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"Fungal Biodiversity of Endophytic Fungi from *Asparagus Racemosus* and Its Molecular Characterization"

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KEYWORDS

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ABSTRACT:

Asparagus racemosus, commonly known as 'Shatavari,' is a medicinally significant plant renowned for its diverse phytochemical composition and extensive therapeutic applications. The shatavari plant is a rich repository for innumerable phytochemicals which are attributed to its vast medicinal properties Keeping in mind the vast application of endophytic metabolites, this study focused on isolating and characterizing fungal endophytes from the root, shoot, and leaf of A. racemosus. A total of 20 fungal endophytes were isolated from the leaf, 5 from the stem, and 6 from the root. These isolates were identified through morphological and molecular characterization, revealing their identity to genera such as Aspergillus, Fusarium, Alternaria, Mucor, and Candida. Among the isolates Alternaria and Mucor species were reported as endophytes of A. racemosus for the first time in this study.

1. Introduction

Endophytes are microorganisms that live in the tissues of healthy plants during specific or all stages of the plant's lifecycle without causing disease or significant morphological alterations to the host. They are recognized as a promising source of novel natural products with diverse biological properties, including antimicrobial, antioxidant, anti-inflammatory, anticancer, and antiviral activities (Guo et al., 2008). Despite the impressive in fungal taxonomy and genome sequencing, the complexity of fungal biosynthetic pathways has left many fungal metabolites unexplored and underutilized. Endophytic microbes affect to plant growth and defense by producing secondary metabolites that protect against pathogens while coexisting with their host plants(Deshmukh et al., 2015). Researchers across the globe showing keen interest on endophytic microbes. Endophytic fungi are having rich repository of bioactive compounds with potential in healthcare and medicine Plants located in biodiversity-rich areas, particularly those with endemic plant species, are more likely to harbor endophytes with novel chemical entities (Deshmukh et al., 2019). Medicinal plants are ideal candidates for endophyte isolation due to their potential to harbor endophytes capable of producing bioactive compounds relevant to human health (Gupta et al., 2018). This approach enhances the likelihood of identifying endophytes that produce bioactive compounds relevant to human health. Endophytic diversity has become an important factor affecting plant health and productivity, which have potential applications in the pharmaceutical, agriculture, and food industries (Strobel*et al* 2003) .*Asparagus racemosus*, commonly known as 'Shatavari,' is an endangered medicinal plant with immense therapeutic value, attributed to its rich phytochemical

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composition (Bopana and Saxena, 2008). Every part of the plant, including tubers, leaves, and fruits, contains pharmaceutical compounds used to treat various ailments, including diabetes, rheumatism, gastric disorders, and headaches (Goyal et al., 2003; Chauhan et al., 2011). The roots of *A. racemosus* are used to treat several biological conditions such as ulcer, cancer, diabetes and obesity. The roots of *A. racemosus* are used as antidepressant, immunomodulatory, anti-inflammatory, anti-urolithiatic, antibacterial, and antidiarrheal (Mandal*et al*., and 2000).The endophytic microorganisms represent a valuable reservoir of bioactive secondary metabolites with tremendous potential in the agrochemical and pharmaceutical industries (Pasrija, *et al* 2022). Endophytic fungi associated with medicinal plants like *A. racemosus* represent a vast, untapped reservoir of bioactive secondary metabolites with potential applications in agrochemical and pharmaceutical industries (Pasrija et al., 2022). Research on endophytic fungi has gained momentum in drug discovery, with an estimated 51% of bioactive metabolites from endophytic fungi possessing unique chemical structures. However, only a small fraction (about 1%) of these fungi has been studied, leaving substantial potential for discovering novel bioactive compounds. Research on endophytic fungi is nowdays focused on overcoming this problem. It is believed that screening for secondary metabolites from endophytic fungi from medicinal plants is a promising way to overcome the dependency on the plants This study focused on the diversity and potential functions of endophytic fungi associated with *A. racemosus*. A combination of morphological and molecular methods, including 18S rRNA sequencing, was employed to identify fungal isolates. Notably, this research reports, for the first time, the occurrence of *Alternaria* and *Mucor* species as endophytes of *A. racemosus*, providing new insights into its endophytic fungal community.

2. Material and Methods

2.1 Collection of plants

Healthy and disease-free medicinal plant *Asparagus racemosus* was collected from the Botanical Garden of Karnataka University, Dharwad (15.4589°N, 75.0078°E). The plant was authenticated by the Botanical Survey of India, Southern Regional Centre, Coimbatore, and identified as *Asparagus racemosus* belonging to the family Asparagaceae. The sample was transported to the laboratory under sterile conditions.

2.2 Sterilization of Plant Samples

The root, leaf, and shoot samples of the plant were thoroughly washed under running tap water for 5min to remove surface dirt. They were then surface-sterilized using 70% ethanol, followed by 1% sodium hypochlorite for 3min, and then with 4% Tween-20 for 3min. Finally, the plant parts were rinsed with distilled water to remove any residual sterilizing agents and dried on sterile paper (Rani et al.,2024).

2.2 Isolation of Endophytic Fungi

The sterilized plant parts were dried on sterile filter paper, cut into 1cm pieces using a sterilized blade, and placed on Sabouraud Dextrose Agar (SDA) plates supplemented with 100 mg/mL of Streptomycin to inhibit bacterial growth. The plates were incubated at 25°C for 5–7 days (Lu *et al*. 2012).

2.3 Morphological and microscopic studies

The obtained cultures were stained with Lactophenol Cotton Blue and observed under a light microscope to examine their morphological characteristics. The morphological characteristics including the size and shape of conidia, mycelia septation, and pigmentation, were analyzed for the identification of fungal isolates (Rather *et al*. 2018)

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2.4 **Scanning Electron Microscopy (SEM)**

To identify the mycelium structure (branched/unbranched, septate/aseptate) of the obtained fungal isolates using SEM model SHIMADZU-SS550 electron scanning microscope Then the samples were viewed at high magnification ranging from 10 µm to 100 µm (Nagmani 2005).

2.5 Molecular identification

a) DNA extraction and PCR amplification

Fungal strains subcultured on Potato Dextrose Agar (PDA) were transferred to Potato Dextrose Broth (PDB) medium and incubated in a shaking incubator at 120 rpm for 5–10 days in the dark at 28°C. After incubation, the mycelia were separated by vacuum filtration. Genomic DNA was extracted from the fungal mycelia, and amplification of the 18S rRNA gene using specific primers (18S_18A and 18S_1200R) in a Veriti 96-Well Thermal Cycler (Applied Biosystems). The resulting PCR products were bead-purified and subsequently analyzed through Sanger sequencing to confirm the fungal identity (Kumar *et al*., 2018)

b) **Phylogenetic analysis**

The obtained 18S rRNA sequences were analyzed using the GenBank database using BLAST (NCBI) toidentify similarity to known fungal strains. A phylogenetic tree was constructed todetermine the evolutionary relationships using MEGA X software (Kumar *et al*., 2018), employing the Neighbor-Joining (NJ) method (Saitou and Nei, 1987). Bootstrap analysis with 500 replications was performed to support the reliability of the phylogenetic tree nodes (Felsenstein, 1985).

3. Results and discussion

3.1 Isolation and identification of endophytic fungi

A total of 20 endophytic fungal isolates were obtained from the stems, leaves, and roots of *Asparagus racemosus(Fig.1)*. Of these, 10 isolates were from leaves, 5 from stems, and 5 from roots. Among the 20 isolates, 6 were selected for further characterization based on their distinct morphological features. These selected fungal isolates were identified using both microscopic examination and molecular techniques (Fig.2). The identified endophytic fungal isolates included *Fusarium* sp. and *Mucor* sp. from the root, *Aspergillus* sp. and *Fusarium* sp. from the leaf, and *Alternaria* sp. and *Candida* sp. from the stem(Fig.3) The endophytic fungal isolates characterized by microscopic characters are presented below (Table-1).

Fig: 1 *Asparagus racemosus* leaf, root and stem sample

Fig.2 Isolation of fungal endophytes

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Table 1: **Total number of identified endophytes from different parts of *A. racemosus*.**

Sl. no	Plant part	No. of isolates	Code	Probable identity of microorganism
1	Leaf	01	ARL1, ARL2	*Aspergillus*sp.*Fusarium* sp.
2	Stem	02	ARS	*Alternaria* sp.
3	Root	03	ARR, PCB PCW	*Fusarium* sp.,*Mucor* sp.,*Candida* sp.

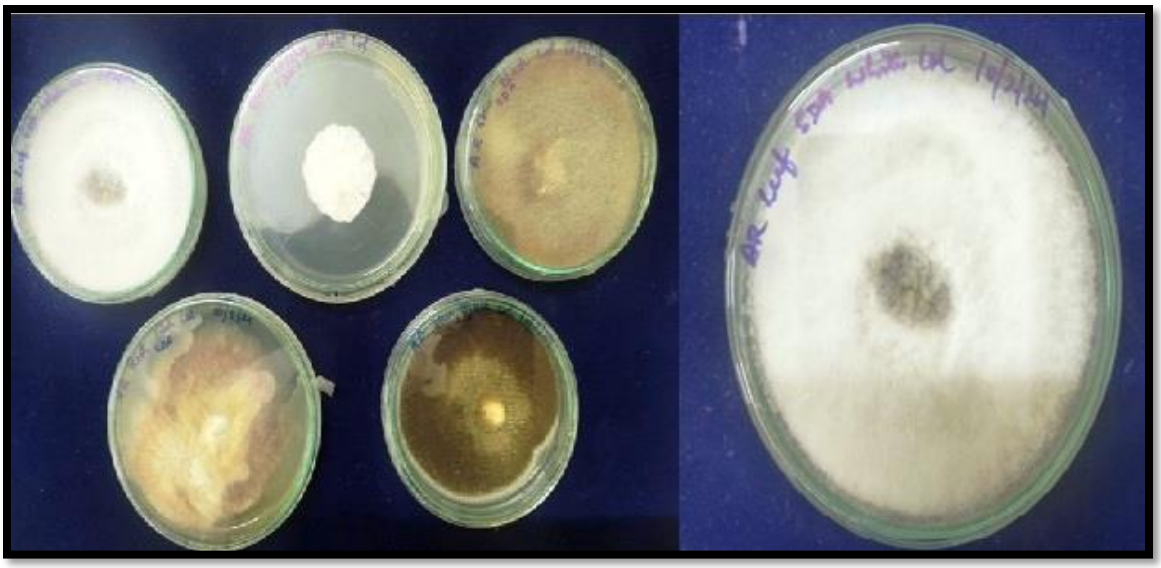

Fig 3: Different Fungal isolates isolated from *Asperagus racemosus*

3.2 Molecular identification of endophytes

The previously identified endophytes were further confirmed through molecular identification involving DNA extraction and amplification. PCR amplification of the ITS region produced amplicons approximately ~1300 bp in size. These amplicons were subjected to bidirectional sequencing using the same set of primers for precise identification (Table-2).

3.3 Phylogenetic analysis

Based on the BLAST search, the most similar sequences were taxonomically identified. The ITS sequence of isolate ARR was identified as *Fusariumoxysporum* sp. dianthi, showing 98.03% similarity with *Fusariumoxysporum* (CUS220317_R18 18S region). Similarly, the ITS sequence of isolate AR S was identified as *Alternaria* sp., AR L1 as *Aspergillus* sp., AR L2 as *Fusarium* sp. F16, PCB as *Mucorcircinelloides*, and PCW as *Candida* sp. The strains showed the following similarities: AR S (98.47%) with *Alternaria* sp., AR L1 (94.23%) with *Aspergillus* sp., AR L2 (93.91%) with *Fusarium* sp., PCB (99.54%) with *Mucorcircinelloides*, and PCW (93.75%) with *Candida* sp (fig.4a-e).

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Sl no	Plant part	Isolate Code	No of base pairs	Probable identity of the fungal endophytes	Percentage of similarity
1	Root	ARR	1300bp	LT841236.1*Fusarium oxysporum* f. sp. dianthi	98%
2	Stem	ARS	1209bp	KJ489375.1 *Alternaria* sp. GE 18S	98%
3	leaf	ARL1	1152bp	MF503668.1 *Aspergillus sp. isolate* wfb.R	94%
4	leaf	ARL2	1154 bp	KF562839.1 *Fusarium*sp. F16	95%
5	Root	PCB	1149 bp	HQ845293.1 *Mucorcircinelloides* strain NRRL 5437	99%
6	Root	PCW	1155bp	AY242199.1 *Candida sp.* BG01-7-23-017A-1-2	96 %

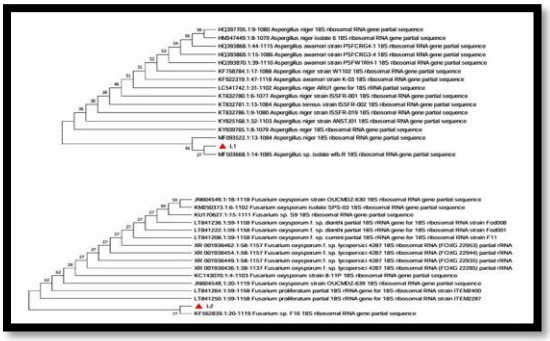

Fig.4.a) **Phylogenetic tree of the fungal isolate ARL1 b) Phylogentic tree of the fungal isolate ARL2**

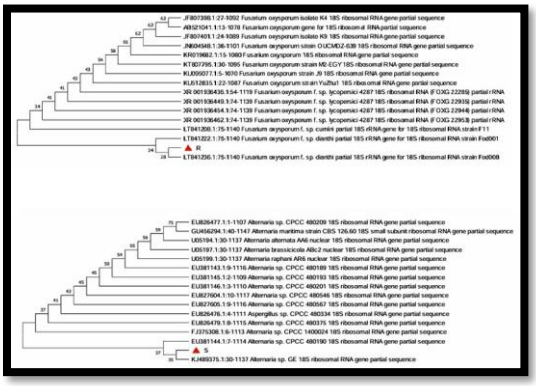

Fig.4c Phylogenetic tree of the fungal isolate ARR and ARS

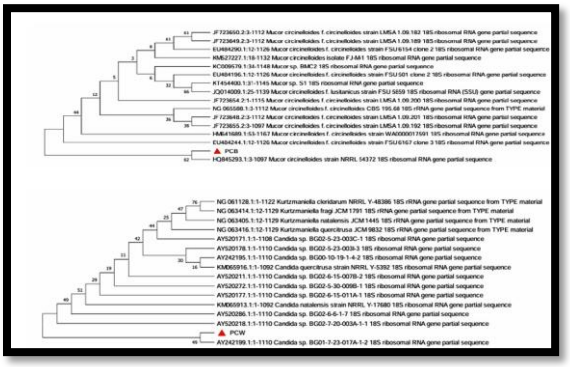

Fig 4d: Phylogenetic tree of the fungal isolate PCB e. Phylogenetic tree of the fungal isolate PCW

3.4 Scanning Electron Microscopy (SEM) technique

Scanning Electron Microscopy (SEM) images of the isolated endophytes revealed the structural details of the mycelium. The SEM visuals showcasing the mycelial morphology of the fungal isolates are showed below. The SEM image of fungal isolate ARL1 reveals smooth, elongated hyphae with bead-like conidia arranged in a string-like pattern. A broom-like configuration of spores radiating from hyphal nodes highlights its reproductive and dispersal

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strategy(fig.5.a,b). The SEM image of fungal isolate ARS reveals conidia arranged in branched structures connected to the stomatal layer. the hyphae exhibit distinct cup-like bilobed projections on their surface, highlighting unique morphological features(Fig5.c). The SEM image of fungal isolate PCB reveals a distinct pinhead-like structure of conidia. These conidia are small, rounded, and prominently positioned at the tips of the hyphae, resembling tiny pins(Fig5.e). The SEM image of fungal isolate PCW reveals cup-like projections intertwined with a network of hyphae. Germ tubes from some germinated conidia are seen gradually elongating into hyphae, spreading extensively across the leaf surface (Fig.5f).

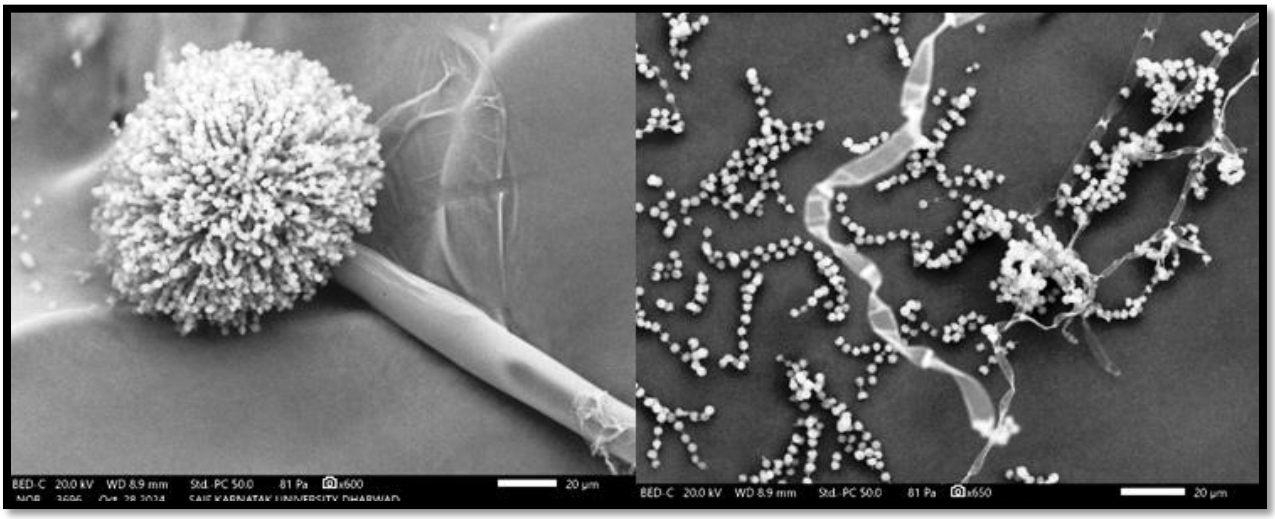

Fig.5a SEM image of fungal isolate ARL1 shows bead-like conidia on hyphae and a broom-like spore arrangement.

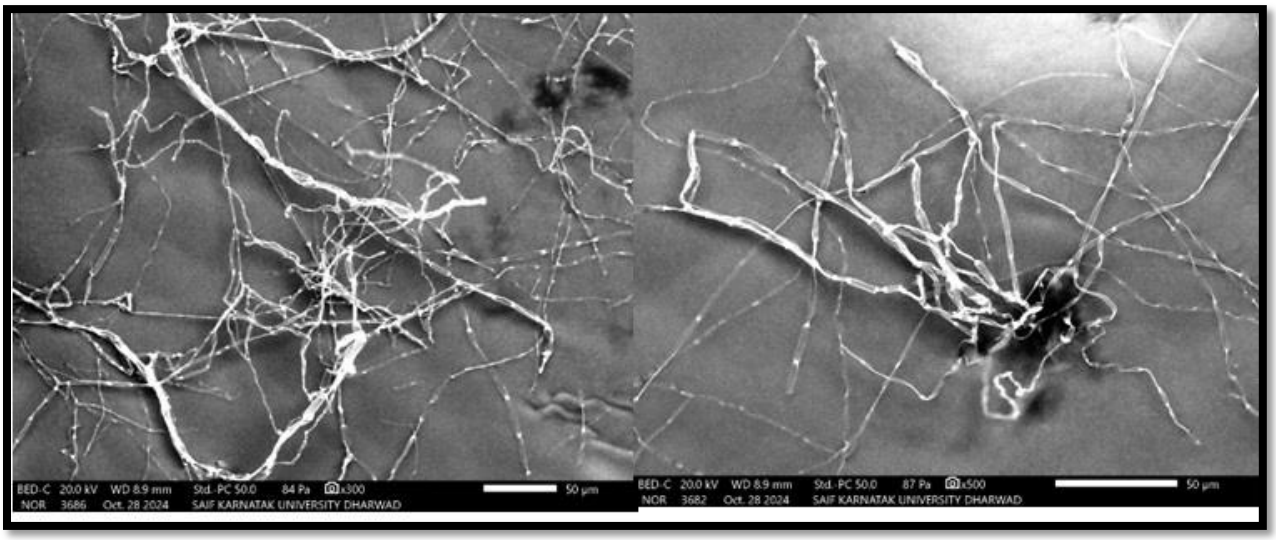

Fig.5b **SEM image of the fungal isolate ARL2 (The threads like Hyphae)**

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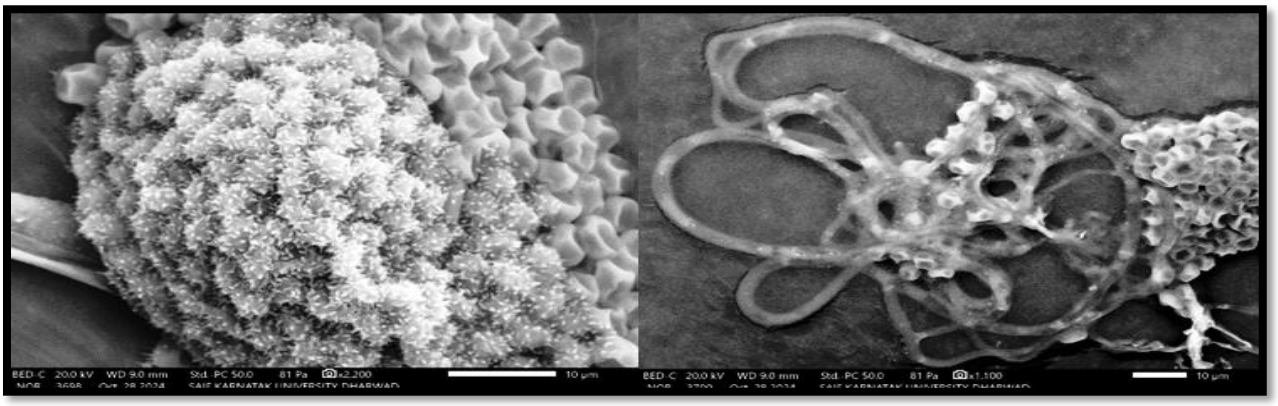

Fig.5c SEM image of fungal isolate ARS shows branched conidia on the stomatal layer and cup-like bilobed structures on hyphae.

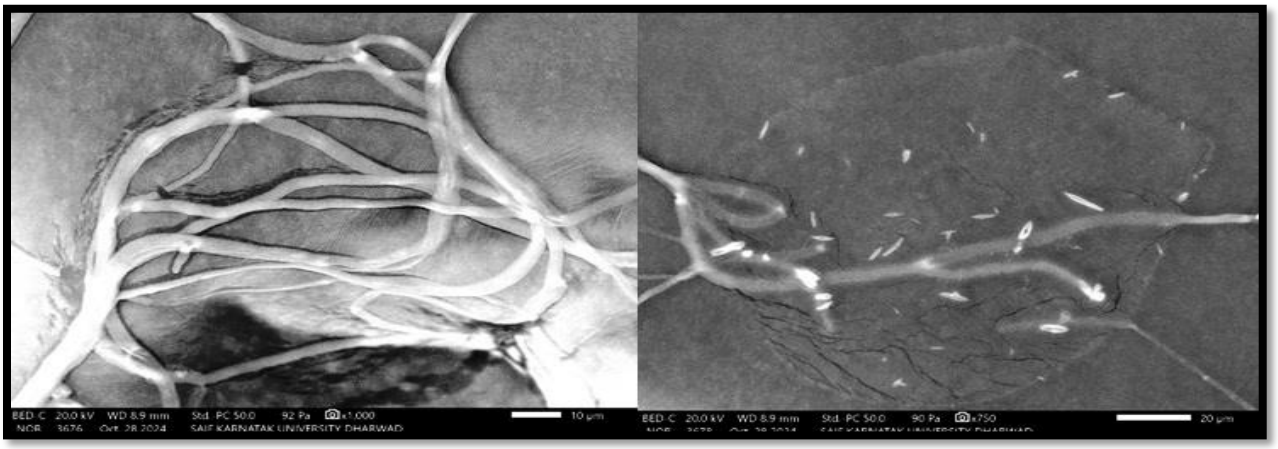

Fig.5d **SEM image of the fungal isolate ARR (Conidial spores intertwined with hyaphae)**

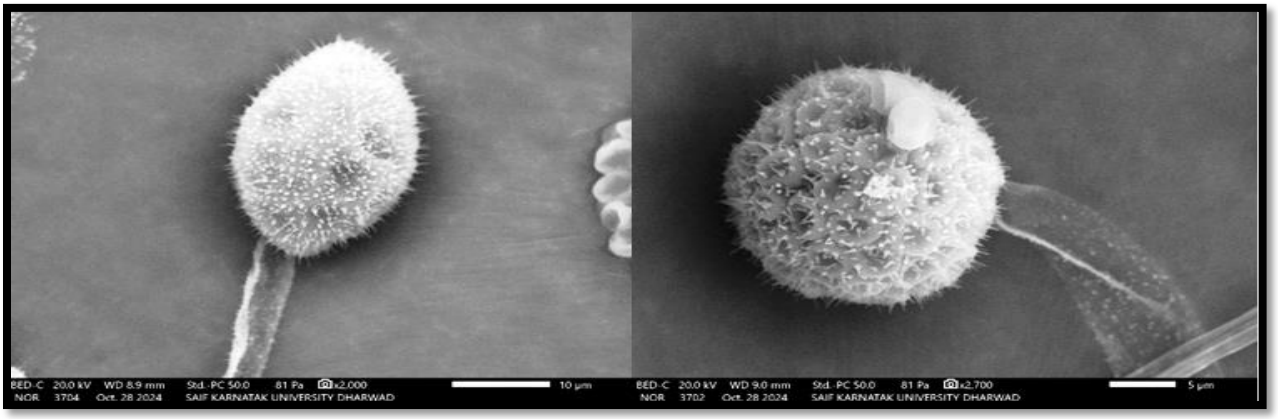

Fig.5e **SEM image of the fungal isolate PCB (Pin head like structure of conidia)**

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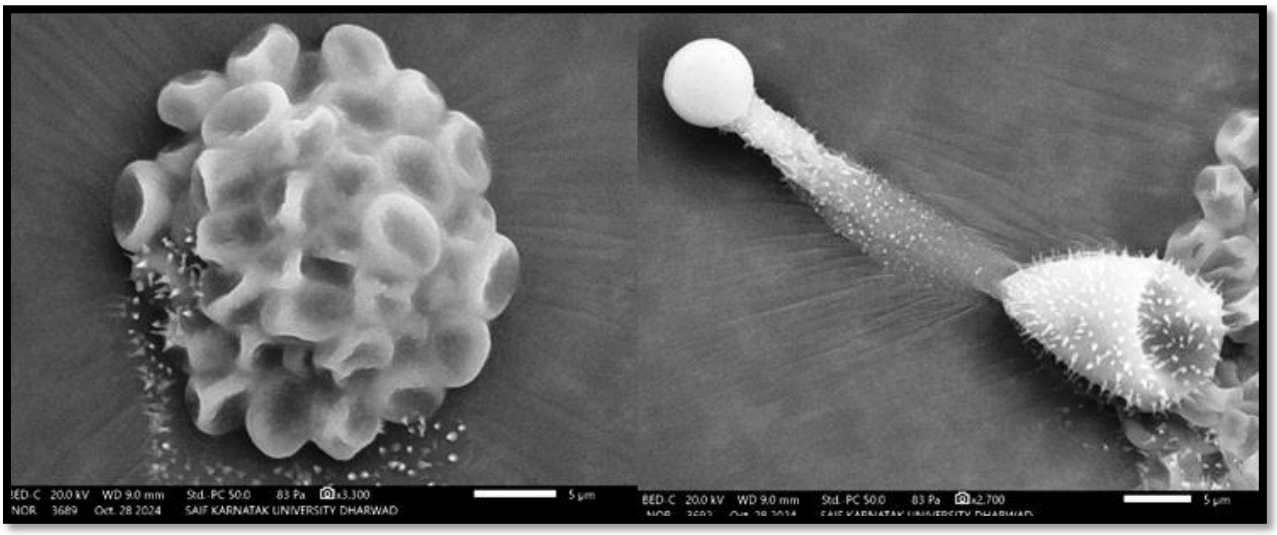

Fig.5f. SEM image of fungal isolate PCW shows cup-like projections with intertwined hyphae and elongating germ tubes spreading across the leaf surface.

Discussion

Endophytes inhabit the healthy plant tissues and have in a mutualistic symbiosis with the plant it produce a bioactive compounds that support the plant. Numerous studies have indicated that these fungi have an impressive array of biotechnological potential, such as enzyme production, bio control agents, and plant growth promoting agents, bioremediation, biodegradation, biotransformation, biosynthesis and nutrient cycling. The actual number and diversity of endophytic fungi are probably enormous and remain unknown. Endophytic fungal communities have different distributions in different tissues of a single tree species. Generally, the colonization rate of endophytic fungi is significantly higher in the stems than in the leaves. Furthermore, studies on the endophytic fungi of plants are necessary to provide fundamental information for the assessment of global fungal diversity and distribution, as well as for the discovery of new species. In our study the more number of endophytic fungi were isolated from root. Endophytic Fungal communities within leaf and root tissues are significantly different. Asparagus (*Asparagus officinalis* L) is an economically important crop, rich in nutrients, and is also conducive to solving ecological and environmental problems from our study we have isolated 20 fungal endophytes, among the 20 isolates 6 prominent fungal species were characterized by morphological and molecular methods. The endophytic fungi isolated from root are designated as PCW, PCB and L1 showed similarity with *Candida*, *Mucor* and *Fusarium* species respectively. The Endophytic fungi isolated from leaf are showing similarity with L1 (*Aspergillus)* and L2 (*Fusarium).* The fungal isolated from shoot designated as S showed a striking similarity with *Alternaria* species. Endophytic fungi are a noble and consistent source of unique metabolites.

Conclusion

Asparagus racemosus a medicinal plant is a rich repository for diverse phytochemicals. In our study the endophytic fungi were isolated from different parts such as Leaf, Stem and Root. The isolated endophytic fungi belong to diverse groups we have isolated more endophytic fungi from root samples. The diversity of the endophytic fungi was studied by morphological and molecular methods. The present study gives as the immense insight in to

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the diversity of endophytic fungi isolated from medicinal plants and helps us to explore more about the endophytic secondary metabolites secreted by them.

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