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Isolation and Identification of Endophytic Fungi from Leaves and Stem of Plumbago Auriculata Lam

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Abstract: ***Endophytic fungi live inside the higher plants, apparently without causing any harm to the hosts and its produce the secondary metabolites are potential antimicrobial activity. Plumbago auriculata has been used as medicinal plant. In this study, isolation and identification of the endophytic fungi from P. auriculata collected from in and around Mysuru. About 80 segments (40 segments of each part of the plant) of P. auriculata for the isolation of the endophytic fungi. A total of 13 endophytic fungi was isolated and identified. The leaf segments showed a maximum endophytic fungus than the stem. Among the 13 endophytic fungi, the predominant endophytic fungi isolated belonged to the genera of Trichoderma sp., Aspergillus niger, Pestalotiopsis guepinii, Rhizopus stolonifer, Rhizopus sp., Colletotrichum sp., Fusarium sp. Further studies are required to screen these endophytic fungi for production of novel bioactive compounds.***
Keywords: Endophytic fungi, Plumbago auriculata, Bioactive compound, Stem, Leaves.

I. INTRODUCTION

Endophytes are ubiquitous organisms present in the tissues of the plants, during a part of their life without technically infecting the host [1] and fungi are the dominant endophytes [2]. Majority of the endophytic fungi belong to Ascomycota and colonize the intercellular spaces of the plant without any visual symptoms of their presence [3]. Endophytic fungi are capable of producing a wide range of bioactive compounds.

Through accumulation of secondary metabolites, the endophytic fungi reduce the damage from the pathogens on the hosts [4]. Several of bioactive compounds produced by these fungi have applications in environment, agriculture, food and pharmaceutical industries [5] [6] [7].

Endophytic fungal communities mainly belong to Ascomycota, Basidiomycota and Zygomycota. Identification of the fungal isolates heavily depends on the reproductive structures in classical taxonomy.

Endophytic fungi are the source for natural products with diverse variety of biological activities. The extracts of endophytic fungi have been reported to show antimalarial, antimicrobial and cytotoxic activities on human cell lines [8]. Diverse variety of natural products have recently been identified from endophytic fungi that include substances that have shown promising anti-cancer, antioxidant, anti-viral, immunosuppressing, and other bioactivities. [2] [9].

Plumbago auriculata Lam. is one such plant of great medicinal importance. This plant is commonly known as cape leadwort or cape plumbago, Native to South Africa. It is a weak-stemmed perennial evergreen shrub that grows 6-7' tall and 8-10' wide in its native habitat. it is widespread today in the tropics and subtropics (including the Mediterranean region). In India, it is sprinkled in central India to West Bengal, Maharashtra, Uttar Pradesh, and some parts of South India Karnataka, Telangana etc. Plumbago from Plumbaginaceae family comprises of 10 genera and 280 species. some species of plumbago are *P. zeylanica*, *P. auriculata*, *P. indica* etc. *Plumbago auriculata* endemic to South Africa is a perennial shrub covered with trusses of pale blue flowers, greyish-green leaves and a hairy calyx. The plant contains specialized secretory structures on the leaves and calyces. The minute glands found on the surface of the leaves are reported to be salt. These salt secreting glands assist in the regulation of salt and ion concentrations. [10]

The main objectives of the present thesis include isolation and identification of effective endophytic fungi from leaves, stem, and flower of Plumbago auriculata which further helps to discover their antibacterial and identification of different bioactive compounds from these efficient endophytic fungi.

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II. MATERIALS AND METHOD

A. *Collection of Plant Sample*

The leaf, stem and flowers of *Plumbago auriculata* were collected from in and around Mysuru. The symptomless and apparently healthy plants were collected in pre-sterilized polythene bags and processed within 24 hours of collection.

B. *Chemicals Required*

70% ethanol, 2% sodium hypochlorite, lactophenol cotton blue, water agar media, Potato Dextrose Agar (PDA), distilled water.

C. *Surface Sterilization and Isolation of Endophytic Fungi*

The samples were surface sterilized by the modified method. The samples were washed initially with running tap water for 10 min, then with 2% sodium hypochlorite for 3 - 4 min, followed by 70% ethanol wash for 1 min and then finally rinsed in sterile distilled water three times. The excess moisture was blotted using a sterile filter paper. The surface- sterilized segments were cut in to 1 cm × 1 cm length were placed in petri dishes containing water agar medium supplemented with streptomycin (100 mg/L). The plates were sealed with para film incubating for 7 days and the petri dishes were monitored every day to check the growth of fungal colonies from the sample segments.[11]

D. *Purification, Selection and Preservation of Endophytic Fungi*

Individual hyphal tips that emerged from the edges of each treated plant bits were transferred separately onto fresh PDA medium
Morphological and microscopic identification of endophytic fungi: Fungal identification were based on the morphology of the cultures, the mechanisms of spore production, and characteristics of the spores' Morphological studies were done by plating the fungi on PDA and incubating it for 7 days. The growth appearances were observed both the top and bottom sides of the culture plates. For tentative identification, microscopic slides of each fungal endophyte were prepared. Slides were prepared by tease mount method using lactophenol cotton blue staining and observed under microscope x40.[12]

E. *Statistical Analysis*

Colonization frequency (CF) - To know the endophyte richness, the frequency of fungal endophytes harbored in plant species were calculated by the number of segments colonized endophyte species divided by a total number of segments examined ×100. [13]

$$CF\% = \frac{No.of\ individual\ fungi\ recorded}{no.of\ segments\ analysed} * 100$$

III. RESULTS AND DISCUSSION

A. *Isolation and Identification of Endophytic Fungi*

Total 120 segments were placed, in which 60 segments from leaves and 60 segments from stems. Out of 60 leaf segments, 50 segments showed endophytic fungi and its colonization frequency is 83.3%, similarly on 43 segments from stem out of 60 segments, we found endophytic fungi and whose colonization is frequency is 71.6%. if we consider total number of endophytic fungi isolated segments are 93 and its total colonization frequency is 77.5%. Table1 shows that leaves have predominant number of colonization frequency of endophytic fungi than stem.

Parts of plant	No. of samples	No. of fungi isolated	CF(%)
Leaves	60	50	83.3
Stem	60	43	71.6
TOTAL	120	93	77.5

Table 1. Colonization frequency of endophytic fungi from leaves and stem of *Plumbago auriculata* Lam.

B. Isolation of Endophytic Fungi from the leaves of Plumbago Auriculata

Total 7 endophytic fungi were isolated from 60 segments (30 segments in each replicate) of leaves of *P.auricuata* (Table:2). The fungus *Trichoderma sp.* was found to be the core group fungus with the colonization in leaf samples of 57.5%. The frequency of colonization in leaf samples was varied between 57.5 to 3.3%. The colonization frequency *Trichoderma sp.* was maximum 57.5% followed by *Pestalotiopsis guepinii* (42.5%), *Colletotrichum sp.* (20%). and the frequency of Mycelia sterilia (10%), Unidentified spores *(10%), Fusarium spp. (4%),* Unidentified species (3.3%).

Sl. No.	Isolated fungi	No. of segments	Colonization Frequency (%)
1	Trichoderma spp.	34	57.5
2	Pestalotiopsis guepinii	25	42.5
3	Colletotrichum spp.	12	20
4	Fusarium spp.	4	7
5	Mycelia sterilia	6	10
6	Unidentified species	2	3.3
7	Unidentified spores	6	10

Table 2. Endophytic fungi isolated from leaves of Plumbago auriculata and their colonization frequencies.

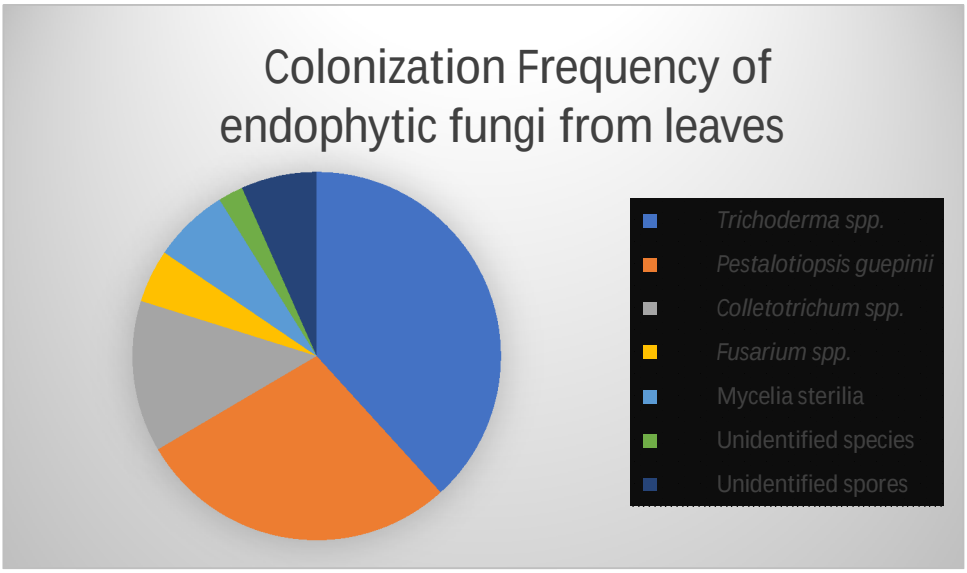

Fig:1 Colonization frequency of different endophytic fungi isolated from leaves of *Plumbago auriculata* Lam.

C. Identification of Isolated Endophytic fungi from leaves of Plumbago Auriculata

Identification is based on morphological characteristics of the fungi grown on the culture medium (PDA). The morphology including macroscopic and microscopic characteristics. The macroscopic identification of colony such as color, diameter, colony growth, colony reverse, the observation was done for seven days during the fungal culturing. Microscopic characterization was done by observing shape and size of conidia, and hyphae. Observation of conidia including its arrangements (singular, chain or cluster), cell number (unicellular or multicellular), and conidial measurement. Observation of hyphae was also performed on the presence or absence of septa in hypha, its shape, morphology and modifications of hyphae.

The results obtained were then compared with the literature and monographs. Identified endophytic fungi as follows (Fig:3) *Trichoderma sp.*, *Pestalotiopsis guepinii. Colletotrichum sp. Fusarium sp.*, Unidentified species, Mycelia sterilia and Unidentified spores. And named them as A, B, C, D, E, F, G, and H.

1) Trichoderma Species

a) *Macroscopic Morphology: Trichoderma* species is a rapidly growing mould which matures in 3 to 5 days. Growth begins as fluffy white tufts which then compact and appear woollier. Green tufts may develop within the colony due to the production of conidia. These often appear as concentric rings, typically starting at the edge of the colony. The reverse is typically a light tan to yellow or pale orange.

b) *Microscopic Morphology: Trichoderma* produces septate, hyaline hyphae. Conidiophores are rather short, branching at wide angles (approaching 90^{o}), often giving it a pyramidal appearance. Phialides are flask or ampule shaped (inflated at the base), which again extend from the conidiophore at wide angles. Conidia are round to ellipsoidal and can be smooth or rough walled depending on the species. Single celled conidia (2-3 µm by 2.5 to 5 µm) are often green in colour and accumulate at the tips of the phialides in slimy balls.

2) Pestalotiopsis Guepinii

a) *Macroscopic Morphology:* Colonies on media growing rapidly, covering the whole Petri dish, plane and floccose; mycelium usually white, sometimes off-white to pale brown; reverse pale or in similar colours to the mycelium. Colonies about 10–16 mm diam, of low, white mycelium. Sometimes germination or growth at 58C. Usually no growth at 378C.

b) *Microscopic Morphology:* Conidia produced in flat, black acervuli, borne just beneath the agar surface, opening irregularly at maturity, filled with a dense layer of conidia; conidia fusiform, five celled (four septate), 20–28 6–9 mm, the central 3 cells brown, 15–20 mm long, the apical and basal cells hyaline, the basal one with a single usually unbranched spike-like appendage and the apical one with two or more simple or branched spiky appendages.

3) Colletotrichum Species

a) *Macroscopic Morphology:* Colonies grayish white, with sparse aerial mycelium and small dense felty patches, elsewhere reverse white to grey, conidial masses salmon pink.

b) *Microscopic Morphology:* Some cultures have abundant greyish white aerial mycelium with poor sporulation and no distinct acervuli. Sclerotia absent from both races. Septae sparse. Conidia falcate, fusiform apices obtuse, 15.5-26.5 X 4-5µ. Appressoria sparse, medium brown, clavate or circular, edge entire, 12.5-14.5 X 9.5-2.5 µ.

4) Fusarium sp.

a) *Macroscopic Morphology:* On PDA, growth is rapid, with dense, white to tan or reddish-brown aerial mycelium and sparse, reddish-brown to orange, sporodochia developing as the culture ages. Colonies reach 6.9 cm diameter for 4 days at 24°C.

b) *Microscopic Morphology:* Microconidia are absent. Macroconidia formed from unbranched and branched monophialides are straight to moderately falcate, distinctly septate, thick-walled, with a marked foot cell, mostly 3-6 septate, 31.0 - 58.3 × 4.4 - 5.1 µm. Chlamydospores are sparse and formed slowly from the cells of macroconidia or mycelium.

5) Mycelia Sterilia

Many fungi do not produce any recognizable sexual/asexual conidia state in culture. Such forms are frequently classified for convenience in the Mycelia sterilia. This group is catchall which may include a few well defined and easily recognizable genera, but more often is a repository for a large number of no descript mycelial isolates.

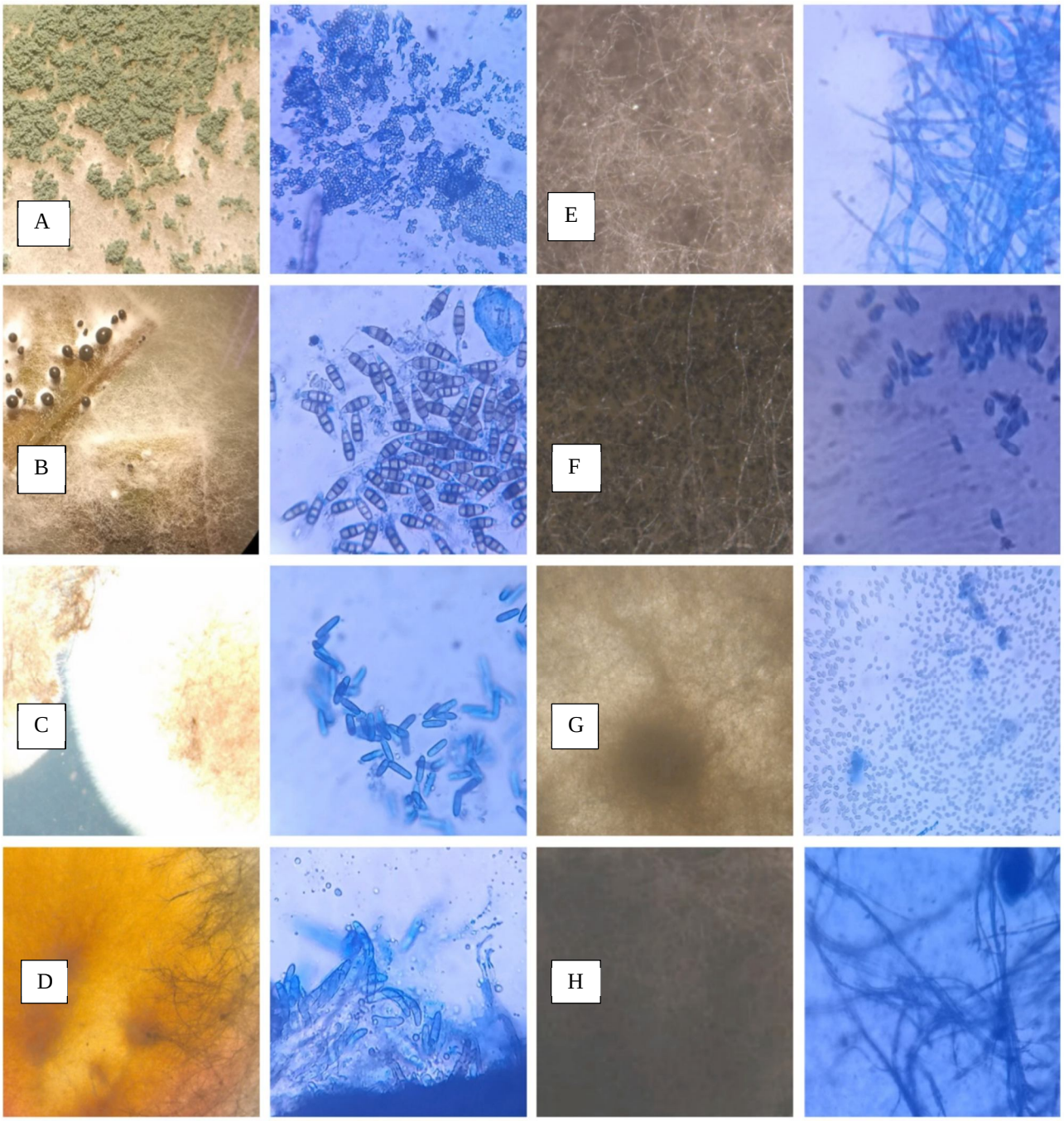

Fig2: Stereomicroscopic view and Microscopic view of the endophytic fungi isolated from leaves of Plumbago auriculata Lam.A-Trichoderma spp.; B- Pestalotiopsis guepinii.; C- Colletotrichum sp.; D- Fusarium sp.; E-Mycelia steralia; F-Unidentified species; G-unidentified spores; H- sterile hyphae.

D. Isolation of Endophytic fungi from the stem of Plumbago Auriculata

Total 6 endophytic fungi were isolated from 60 samples of stem of *P.auricuata* (Table:2). The fungus *Aspergillus niger* was found to be the core group fungus with the colonization in leaf samples of 92%. The frequency of colonization in leaf samples was varied between 92 to 3.3%. The colonization frequency *Aspergillus niger* was maximum 92% followed by *Rhizopus stolonifera* (55%), *Rhizopus spp.* (30%), *Mucor spp.* (8.3%) and the frequency of *Unidentified spores (5%) and* Mycelia sterilia (3.3%).

Sl. No.	Isolated fungi	No. of samples	Colonization Frequency (%)
1	*Aspergillus niger*	40	92.5
2	*Rhizopus spp.*	10	30
3	*Rhizopus stolonifera*	25	55
4	*Mucor spp.*	5	8.33
5	Unidentified spores	3	5
6	Mycelia sterilia	2	3.33

Table 3. Endophytic fungi isolated from *Plumbago auriculata*.Lam. stem and their colonization frequencies

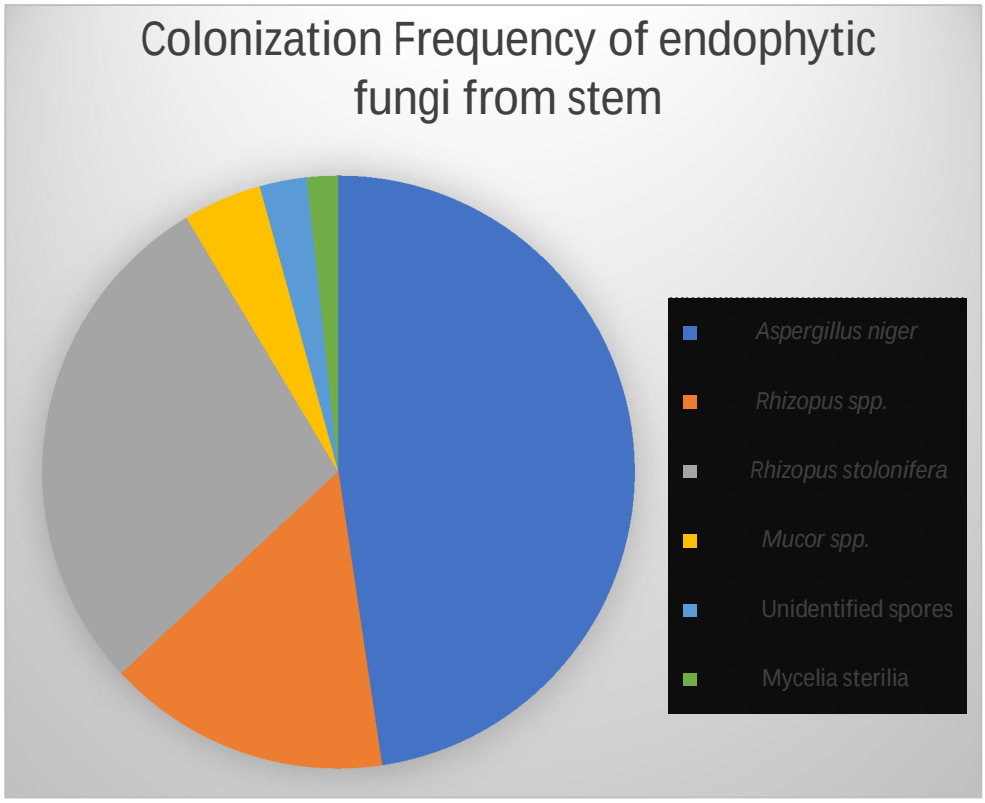

Fig:3 Colonization frequency of different endophytic fungi isolated from stems of *Plumbago auriculata* Lam.

E. Identification of isolated endophytic fungi from stem of Plumbago auriculata

Identification is based on morphological characteristics of the fungi grown on the culture medium (PDA). The morphology including macroscopic and microscopic characteristics. The macroscopic identification of colony such as color, diameter, colony growth, colony reverse, the observation was done for seven days during the fungal culturing.

Microscopic characterization was done by observing shape and size of conidia, and hyphae. Observation of conidia including its arrangements (singular, chain or cluster), cell number (unicellular or multicellular), and conidial measurement. Observation of hyphae was also performed on the presence or absence of septa in hypha, its shape, morphology and modifications of hyphae. The results obtained were then compared with the literature and monographs. Identified endophytic fungi as follows (Fig:5) *Aspergillus niger., Rhizopus sp., Rhizopus stolonifera., Mucor sp.,* Mycelia sterilia. and Unidentified spores and named them as A, B, C, D, D, E, F, G, and H.

1) *Aspergillus niger***:**

a) *Macroscopic Morphology:* Rapidly growing on Potatao-Dextrose Agar starting with a white to yellowish felt-like mat of mycelia, quickly turning black as conidia develop the pigment aspergillin during maturation. Reverse remains white to pale in colour.

b) *Microscopic Morphology:* Septate, hyaline (clear) hyphae. Conidiophores (Stipes) are long (400-3000 µm) with spherical vesicles at the apex measuring 30-75 µm. *Aspergillus niger* is biserate - metulae just about cover the entire surface from which the phialides extend. Conidia are globose, brown to black in colour, measure 3.5-4.5 µm in diameter and have a rough surface.

2) *Rhizopus species:*

a) *Macroscopic Morphology: Rhizopus* is a rapidly growing fungus that can fill a petri dish with fluffy, cotton-candy like growth in under 5 days. Growth is generally whitish in colour which can turn brown with age as a result of the maturation of the sporangiospores within the sporangium**.**

b) *Microscopic Morphology:* Hyphae broad, not or scarcely septate; rhizoids and stolons present; sporangiophores brown, solitary or in tufts on the stolons, diverging from the point at which the rhizoids form; sporangia rather round; apophysis absent or scarcely apparent; sporangiophores ovoid.

3) *Rhizopus stolonifer:*

a) *Macroscopic Morphology:* Colonies covering the whole Petri dish, sometimes low and sparse, with black sporangia only at the margins, sometimes filling the whole Petri dish, reverse pale. Colonies filling the whole Petri dish with floccose white mycelium bearing conspicuous sporangia, at first white, then with maturation rapidly becoming black, reverse uncolored. At 58C, spores barely germinating. At 378C, usually no growth, sometimes colonies up to 15 mm diam, very thin and sparse.

b) *Microscopic Morphology:* Sporangiophores borne in groups of three to five from clusters of rhizoids, stipes unbranched, robust and up to 3 mm long, with brown walls; sporangia 100–350 mm diam, usually spherical; columellae roughly spherical, up to 200 mm diam, in age often collapsing downwards and outwards to produce umbrella shapes, sporangiospores commonly 8-20mm in long axis, pale brownish, with striate walls.

4) *Mucor Species*

a) *Macroscopic Morphology: Mucor* is a rapidly growing fungus which will fill a culture plate in a matter of a few days with a woolly growth resembling cotton candy. New growth is white in colour but turns a greyish-brown with aging. The reverse remains a pale white.

b) *Microscopic Morphology: Mucor* has broad hyphae which are scarcely or non-septate. Sporangiophores are long, may be branched and terminate in a round spore-filled sporangia (50µm-300µm diameter). The sporangia have a thin wall which when mature dissolves (or is disrupted) to release round or somewhat ellipsoidal sporangiospores (4µm-8µm diameter). With the spores scattered, the columella which bore the sporangia is visible, sometimes leaving a collerette at the base of the sporangium.

5) *Mycelia Sterilia*

Many fungi do not produce any recognizable sexual/asexual conidia state in culture. Such forms are frequently classified for convenience in the Mycelia sterilia. This group is catchall which may include a few well defined and easily recognizable genera, but more often is a repository for a large number of no descript mycelial isolates.

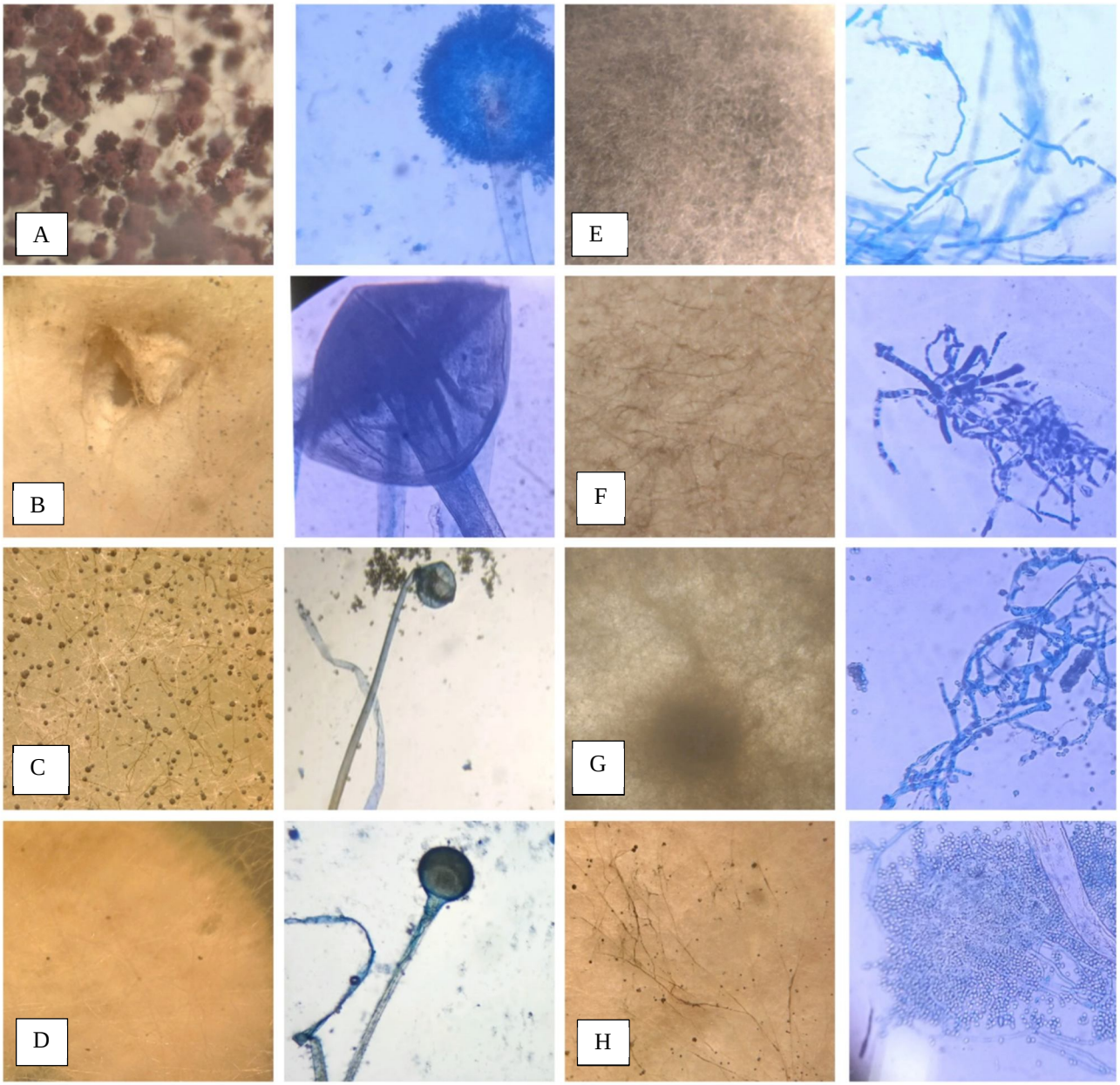

Fig:4 Stereomicroscopic view and Microscopic view of the endophytic fungi isolated from stem of Plumbago auriculata. Lam. A- Aspergillus niger. B- Rhizopus spp. C- Rhizopus stolonifera. D- Mucor spp. E,F,G- Mycelia sterilia. H-Unidentified spores.

This study was carried to isolate and identify endophytic fungi from different parts of *Plumbago auriculata*. In the study, a total of 13 fungal colonies were isolated from 120 segments. Genera of endophytic fungi identified as *Trichoderma spp, Pestalotiopsis guepinii, Colletotrichum sp., Fusarium sp., Aspergillus niger, Rhizopus spp., Rhizopus stolonifera,* and *Mucor sp.* This reported *Trichoderma sp.,* (55%) *and Aspergillus niger* (92%) as dominant endophytic fungi from the leaves and stems of *P.auriculata.* Where in leaves *Trichoderma sp.* forms 57.5%, *Pestalotiopsis guepinii* forms 42.5%, *Colletotrichum spp* forms 20% *Fusarium sp.* forms 7%. Mycelia sterilia was about 10%, Unidentified species 3.3% and unidenfied spores 10%. Where in stem *total 6 endophytic fungi were isolated from 60 samples of stem of P.auricuata* . The colonization frequency *Aspergillus niger 92%* followed by *Rhizopus stolonifera* (55%), *Rhizopus spp.* (30%), *Mucor sp.* (8.3%), Unidentified spores (5%) and Mycelia sterilia (3.3%). No species of endophytic fungi found common in both parts of plant *P. auriculata.* Leaves have predominant number of colonization frequency of endophytic fungi than stem as leaves have CF% of 83.3 where stem is 71.6% and total colonization frequency is 77.

IV. CONCLUSION

Medicinal plants are good source for isolation of endophytic fungi that colonize the tissue without causing apparent symptoms. Endophytic organisms have received considerable attention as they are found to protect their hosts against pests, pathogens and even domestic herbivores. In this study, a total of 8 endophytic fungi were isolated from the *Plumbago auriculata*, *P. auriculata* a well-known medicinal plant contains various chemical compounds. Isolation of endophytic fungi from this plant produces novel bioactive compounds.

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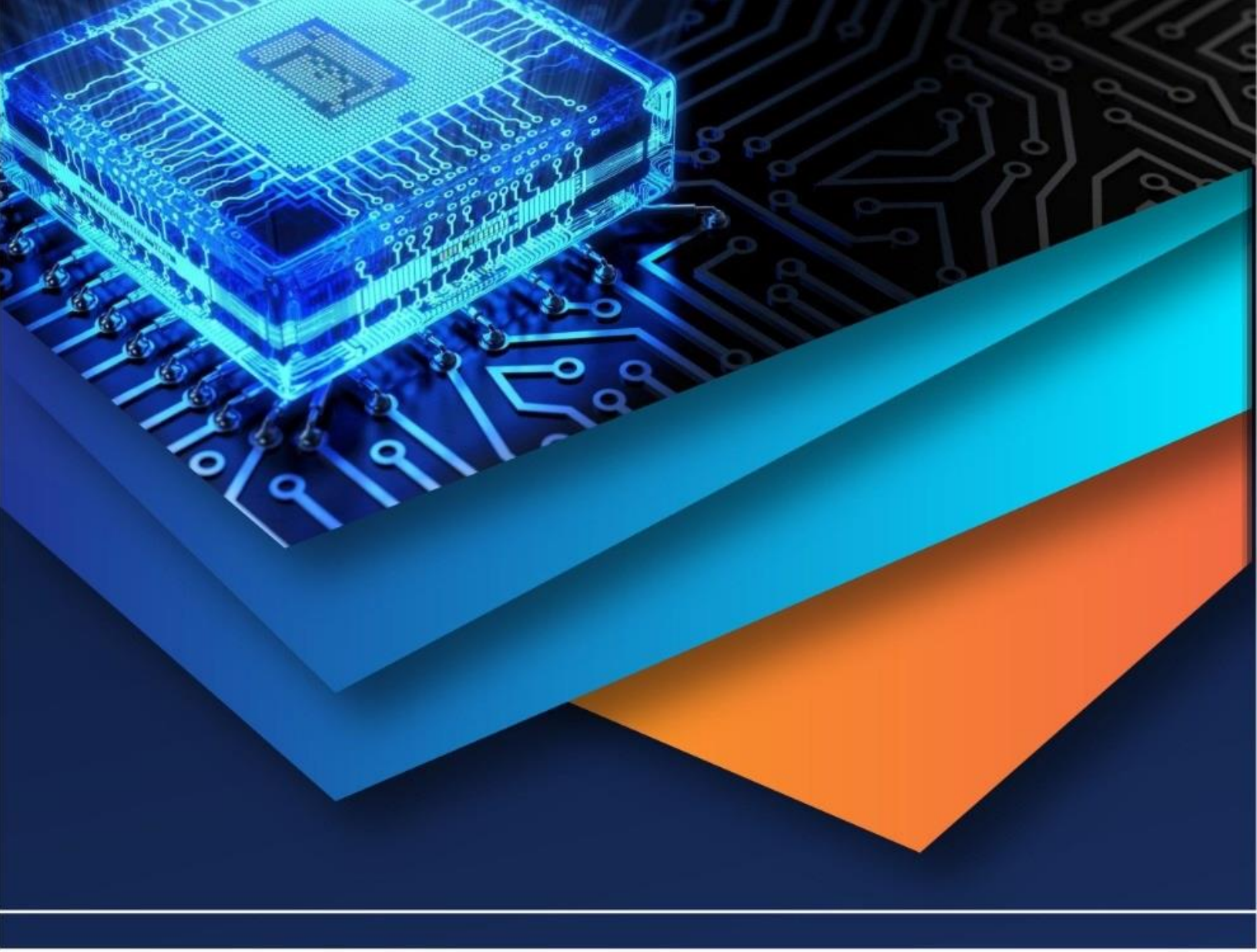

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