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Diversity of endophytic fungi from the medicinal plant *Mucuna pruriens*

Dharmil N. Vyas[1], Sonal Sharma[2], Anshu Srivastava[2], Neeta Shrivastava[2], Vivek N. Upasani[1*]

[1]Department of Microbiology, M. G. Science Institute, Ahmedabad 380009
[2]B. V. Patel Pharmaceutical Education and Research Development (PERD) Centre, Ahmedabad 380054

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Abstract

Endophytes are microorganisms found within the plants and include fungi, bacteria, algae, insects, etc. However, the most frequently isolated endophytes belong to fungi. The medicinal plant *Mucuna pruriens* is a legume (annual climbing shrub) that is native to tropical countries of Africa and Asia. It is commonly called as Velvet bean, Bengal velvet bean, Florida velvet bean, Cowage, Cowitch, Lucuna bean, Lyon bean, etc. It is found in the wild and is also cultivated for food. It's economically important as a source of the drug L-DOPA, which is used to treat Parkinson disease. However, due to urbanization and over exploitation, it has become scarce, so an attempt has been made to isolate endophytic fungi from this plant and screen for biosynthesis of L-DOPA. The plant samples were collected from eight (8) locations / sites at Ahmedabad, Gandhinagar, Udaipur, Dediyapada, and Baroda in Gujarat and Rajasthan states. The plant material (flowers, seed, stem, roots) was surface disinfected / sterilized and endophytic fungi were isolated on Potato dextrose Agar (PDA) medium. Thirty five (35) different fungal isolates were obtained and identified based on colony and morphological characteristics as well as phylogenetic analysis. Some of the endophytes identified belong to the genera *Cladosporium, Curvularia, Aspergillus,* and *Alternaria.* The isolated strains could be potential candidates for developing sustainable, cost-effective and eco-friendly alternative for bioprospecting of bioactive compounds (L-DOPA, antimicrobials, anticancer drugs, etc.).

Keywords: biodiversity, bioprospecting, endophytic fungi, L-DOPA, medicinal plants, *Mucuna pruriens*.

Introduction:

Endophytic fungi residing in the tissues of the host plant are reported to produce bioactive compounds similar to the native host plant, thus, making them a promising source of novel compounds. Biodiversity studies of many endophytic fungal strains have revealed that they can synthesize phytochemicals characteristic of the native host plant with an unknown mechanism (probably by horizontal gene transfer between endophytic fungi and the host during long term symbiotic relationship) (Taghavi et al., 2005; Heinig et al., 2013). For example, biodiversity study of Himalayan yew tree (*Taxus wallichiana)* for endophytic microorganisms isolation and characterization lead to the discovery of endophytic fungus like *Pestalotiopsis microspora,* which was found to produce taxol (anticancer) (Strobel, 1996). This led to series of many other successful studies related to other plant bioactives such as camptothecin, vincristine, vinblastine,

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phodophytllotoxin, azadirachtin, digoxin, etc. Kjer et al., (2010) has reviewed the methods for isolation of endophytic fungi from marine organisms such as sponges, algae and mangrove plants and their secondary metabolites. Tiwari (2018) has reviewed the role of endophytic fungi that enhanced the growth and yield of *Pongamia pinnata* and *Salvia miltiorrhizia* by production of secondary metabolites including volatile organic compounds, for example *Alterneria sp.* a symbiont of *S. muiltiorrhizia* induced root growth. This makes endophytic fungi a promising source of drugs similar to those produced by its host. *Mucuna pruriens*, commonly known as Cowitch, is a legume well-known for the production of L-DOPA (3,4-dihydroxyphenyl L-alanine) a non-proteinogenic amino acid used in treatment of Parkinson's disease. It is usually found in the wild and also cultivated due to its therapeutic value. However, due to urbanization the plant source has become scarce, so an attempt has been made to explore the endophytic diversity of this plant leading to identification of unique fungal species producing L-DOPA.

In this study, we report the endophytic fungal diversity of *Muccuna* plant samples by the classical cultural method for isolating endophytic fungi. The isolates were further characterized based on colony and morphological characterization. Some of the isolates were also identified based on ITS homology sequencing study. To our knowledge this is the first study reported on endophytic fungi from this medicinal plant.

Materials and methods:

Samples

Plants (*Mucuna pruriens*) were collected in sterile bags from five locations viz. Ahmedabad, Gandhinagar, Baroda, Dediyapada and Udaipur in Gujarat and Rajasthan (Table 1).

Surface sterilization, isolation and characterization

The plant tissues (flowers, seeds, stem, and leaves) were surface sterilized with modified method (Shulz et al.,1993). The plant material was cut into an appropriate size, rinsed under running tap water for 10 min, shaken for a few seconds in 70% alcohol, rewashed with sterile distilled water. In the next step, it was treated with $HgCl_2$ (0.1 - 1%w/v) + Teepol 2 drops/100ml (sterile distilled water) for 3-5 min in shaking condition, and then rinsed in sterile distilled water. This was followed by the disinfection with commercial bleach (sodium hypochlorite) 7-15% + Teepol 2 drops/100ml (sterile distilled water) for 10-30 min under shaking condition and rinsed several times in sterile distilled water. The treated plant material was inoculated onto Potato Dextrose Agar (PDA) plates with streptomycin (30µg/ml conc.) and incubated in Plant growth Chamber (Remi model) at 25°C $\pm$ 3°C for isolation of endophytic fungi. The pure fungal cultures were preserved on PDA slants wrapped with parafilm at 2°C to 8°C refrigeration temperature. The following controls were exercised: two (2) PDA plates were taken, one was exposed / kept open at the time of inoculation in the LAF, and the second plate was inoculated with the sterilized distilled water used in the last rinse (one drop) onto PDA plate. All the three (3) controls were incubated to rule out contamination and validate the aseptic procedure.

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Characterization and identification of the isolates

The fungal isolates were identified based on the colony and morphological characteristics as well as phylogenetic analysis (ITS based). Cultural characteristics were noted after 72 hours of growth on PDA agar plates. The growth was mounted on a slide in a drop of lactophenol, teased, covered with cover-slip and microscopic study was carried out using Coslab Phase-contrast microscope with photomicrography attachment as well by bright-field microscopy. The ITS region sequencing of the fungal cultures was done at Chromus Biotech, Bengaluru and Eurofins. The sequences were used for BLAST search to obtain related sequences and further phylogenetic analysis was done by MEGA 6. The ITS sequences (500-538 bp) have been submitted in GenBank with accession numbers (gil137343911 (GL02); gil1373441175 (HebFo1); gil137343482 (Hebs02).

Results and Discussion

Endophytes have been intensively studied over the last several years as prolific sources of new as well as conventional source of bioactive compounds. In fact, an impressive number of natural products have been produced by endophytic fungi (Tiwari, 2018). In addition, some studies have shown endophytes to be good producers of useful enzymes to improve industrial processes. More recently, endophytes have also received attention as biocatalysts in the chemical transformation of natural products and drugs (Borges et al., 2009).

Table 1 shows the location details for collection of the plant material. The plant is seasonal and usually flowers after monsoon. Caution has to be exerted during plant collection (wear gloves) as it causes irritation on contact with skin (Fig. 1).

Table1: *Mucuna pruriens* **plant samples collection sites**

Sr No	Site	GPS co ordinates	Fungi isolated	Plant parts	Site visit
1	Dediyapada, Gujarat	21.6268° N, 73.5859° E	3	Leaves, Flower, shoot	1
2	Baroda, Gujarat	22.3072° N, 73.1812° E	3	Leaves, Flower, shoot	1
3	Udaipur, Rajasthan	24.5854° N, 73.7125° E	6	Leaves, Flower, shoot	1
4	Gandhinagar, Gujarat	23.2156° N, 72.6369° E	6	Leaves, Flower, shoot	1
5	Hebatpur, Ahmedabad, Gujarat	23.0225° N, 72.5714° E	14	Leaves, Flower, shoot	2
6	Jagatpur, Ahmedabad, Gujarat	23.0225° N, 72.5714° E	2	Leaves, Flower, shoot	1
7	South Bopal, Ahmedabad, Gujarat	23.0225° N, 72.5714° E	3	Leaves, Flower, shoot	1

Fig 1: Plant samples of *Mucuna pruriens*, **Hebatpur (Ahmedabad) for flower, pods and leaves.**

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Fig 2: Growth of endophytic fungi isolated from flowers of *Mucuna pruriens* on PDA plate.

Sterilized plant parts were incubated for 48 to 72 hours for growth of endophytic fungi. Fig 2, shows the growth of endophytic fungi from the flower of *Mucuna pruriens*. Two (2) parts have growth, while remaining parts did not show growth; this may be due to harsh surface sterilization method. Forty two (42) endophytic fungal isolates were isolated, characterized and some were identified based on cultural, morphological and ITS analysis. It has been observed that endophytes were easily isolated from flower, shoot, and leaves, but difficult from pods and seeds. Fig 3 shows the cultural characteristics of the isolates that are described in Table 2.

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Fig 3: Cultural characteristics of the fungal isolates obtained from Gandhinagar and South Bopal, Ahmedabad plant samples on PDA plate.

Table 2: Cultural characteristics of few endophytic fungal isolates on PDA medium.

Sr no	Sample site	Material part	Growth and Morphological characteristics	Identification based on ITS*
1	Gandhinagar	Leaf (GL02)	Colonies are olive-green to olive-brown and appear velvety or powdery. Microscopic study shows mycelia growth with long chain of conidia	*Cladospoium cladpsporides*
2	Hebatpur, Ahmedabad	Flower (HebF01)	Shiny velvety-black, fluffy growth on the plate, hyphae producing brown, geniculate conidiophores. The poroconidia are curved slightly to distinctly, transversely septate, with an expanded third cell from the pore end of the conidium	*Cochliobolus* sp. (*Curvularia lunata)*
3	Hebatpur, Ahmedabad	Stem (HebS01)	Blackish powdery growth on plate, microscopic study shows typical foot cell, conidiophores structures.	*Aspergillus multicolor*

* See the phylogenetic trees Figure 5.

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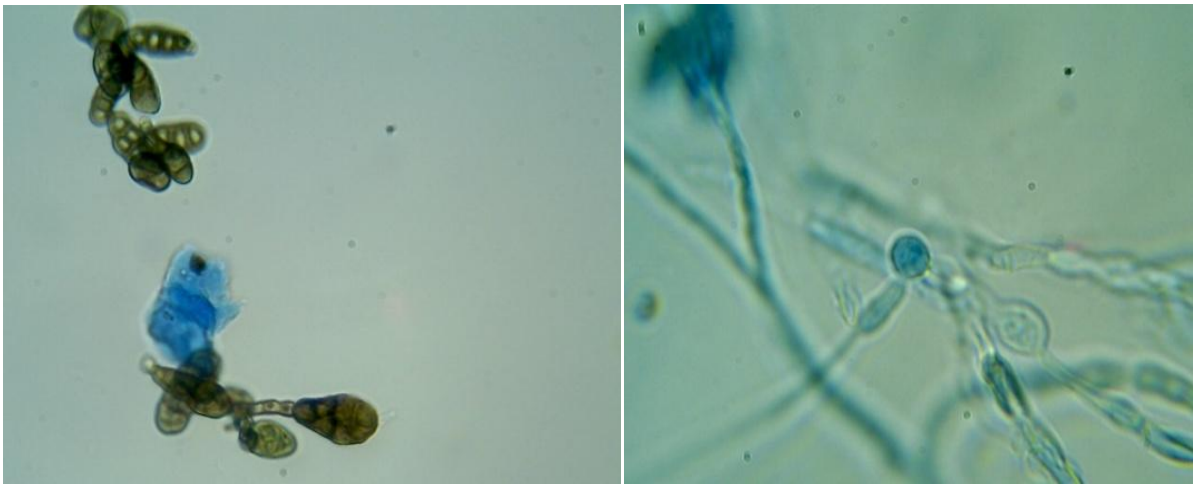
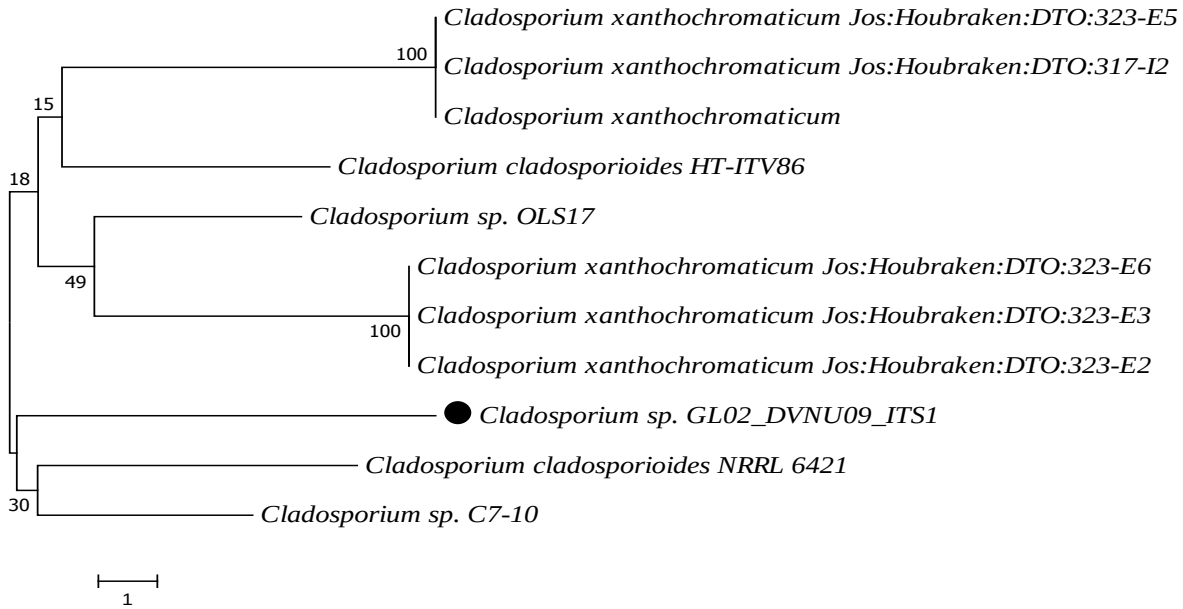
Fig 4: Endophytic fungi isolated from *Mucuna* plants shoot and flowers, morphological study shows

a) *Alterneria,* and **b)** *Cladosporium*, **respectively.**



Screening of these isolates may reveal their capacity to produce economical important compounds. It was interesting to note that a few isolates were EPS producing, some having antifungal or antibacterial activity (unpublished results). Thus, they have high potential for drugs and bioactive intermediate. *Mucuna pruriens* is a rich source of L-DOPA, our hypotheses is that the isolates may be producing L-DOPA or intermediates for its biosynthesis.

Fig 5. Phylogenetic analysis using MEGA6 for identification of the endophytic isolates by Neighbour-Joining tree based on ITS region sequences.



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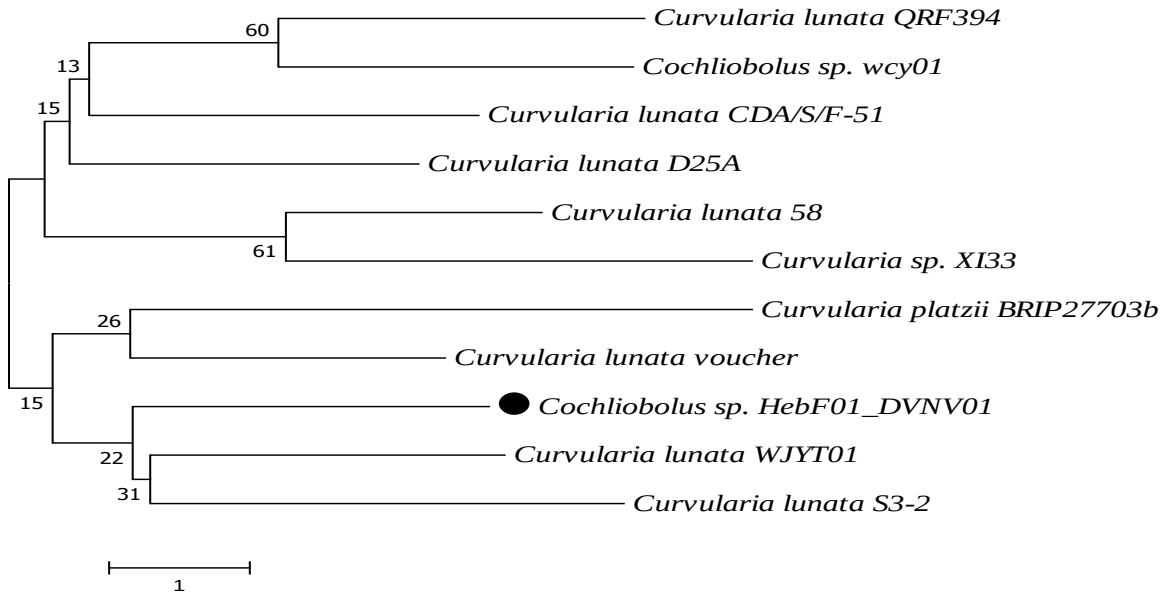

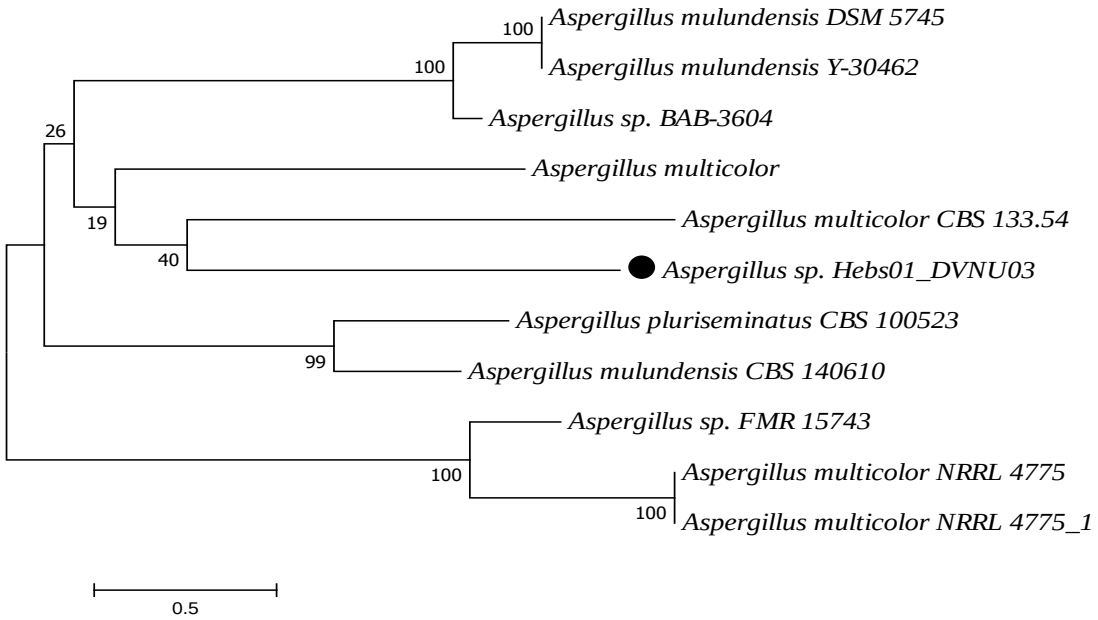

We have observed that there is a difference in the diversity of endophytic fungi isolated from plant samples collected from Rajasthan and Gujarat. Study shows different diversity with change of location as also reported by Nalini et al. (2014), *Aspergillus* and *Curvularia* were frequently isolated from samples collected from Rajasthan, whereas in Gujarat the isolated belonged to the genera *Cladosporium, Alternaria* and *Fusarium.* Similarly, the diversity of endophytes in samples collected from Ahmedabad was different from those collected from Udaipur. Phylogenetic

analysis based on ITS sequence homology using MEGA6 the isolates were identified as members of the genera *Cladosporium, Alternaria, Aspergilus, Curvularia, Chaetomium, Acremonium spp*., etc. (Fig. 5). Su et al. (2010) has reported that the species diversity of endophytic fungi was higher in roots than in leaves of *Stipa grandis* plants collected from the Inner Mongolian steppes (China). The phylogenetic diversity of endophytic fungi in the plant *Pinus halepenesis* (Botella and Diez, 2011) has shown the dominance of *Dothideomycetes* class. Sun and Guo (2012) have reviewed the various traditional as well as molecular methods used in identifying endophytic fungi for studying their diversity. Krishnaveni et al., (2009) have reported the isolation of fungi (non-endophytic) *Acremonium rutilum, Aspergillus niger, Cladosporium herbarum, Scyttalidium* spp., *Fusarium equiseti, Fusarium moniliforme and Odeocephalum* spp. from various plant materials rich in phenolic substances from Gulbarga region. They have reported for the first time the ability of *A. rutilum* to able to transform L-tyrosine to L-DOPA having tyrosinase activity. Under submerged conditions using Potato dextrose broth this isolate produced 0.89 mg/ml L-DOPA and had 1095 U/mg tyrosinase activity. Similarly Ali and Haq (2010) showed the production of 3,4-dihydroxy L-phenylanaine by *Aspergillus niger* strain isolated from bread waste. Future enzymatic studies on our endophytic fungal isolates may also identify potential candidates with tyrosinase activity.

Conclusion:

Endophytic fungi are ubiquitous and have been isolated from diverse plant species including sponges and algae. They are endosymbionts and produce various secondary metabolites that affect the growth of plants as well as have therapeutic value. Some endophytes have also been reported to produce the drug that is indigenous to the plant species. We report here for the first time the isolation, characterization and identification of endophytic fungi from the medicinal plant *Mucuna pruriens* for screening for L-DOPA synthesis. Some of the isolates were identified as species of the genus *Aspergillus* (isolate Hebs02), *Cladospoium* (isolate GL02) and *Cochliobolus / Curvularia* (isolate HebF01). Further work on screening for L-DOPA and bioactive compounds has been undertaken.

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*corresponding author: **vnu_halophiles@yahoo.com**