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Diversity assessment of endophytic fungi from Azadirachta indica A. Juss. from various regions of Aurangabad, Maharashtra (India)

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Abstract:

The present study was done to assess distribution of endophytic fungi from a medicinal plant *Azadirachta indica* A. Juss. from various regions of Aurangabad. Healthy neem plants were collected from five different areas of Aurangabad i.e. Bidkin region, Khultabad, Osmanpura, Shenrda, Beed bypass. Endophytic fungi were isolated from leaf with midrib, without midrib, petiole, stem. Total 16 fungal isolates were separated. Out of total endophytes isolated most of the genera are from hypomycetes, few from coleomycetes and five are from mycelia sterilla. Colonizing frequency, species richness , Simpson's Diversity indices and Shannon-Wiener indices and evenness were calculated. Maximum endophytes isolated from Loc 4 (30.9%) and minimum endophytes were recovered from Loc 2 (6.1%). The dominant endophytic fungus observed was *Alternaria* sp. Location 4 has lower Simpson's index which indicates it has greater diversity.

Keywords: Endophytes, *Azadirachta indica* A. Juss., Colonizing frequency, Simpson's Diversity, Shannon-Wiener indices

Introduction:

Endophytes are microbes that colonize the living internal tissues of plants without causing any immediate disease symptoms or overt negative effects [4] Fungal entophytes reside within the living tissues of higher plants without producing any apparent symptoms [8,9]. Although they are abundant, the extent of their contribution to fungal biodiversity remains unclear. Endophytic fungi had been previously isolated from leaves, stems and roots of a wide variety of plants in the temperate regions [17,38,51]. Especially in the tropics endophytic fungi are poorly known; therefore the present study of fungal species is probably conservative. Entophytes have a protective role against insect herbivory and many are potential producers of novel antimicrobial secondary metabolites [2].

Until recently, extensive work has been done on endophytic fungi, mostly in the temperate regions of the world [33,39] Although most studies have been conducted on the mere presence and identity of endophytes in the stem or leaf tissues [6,18,40,42], a systematic

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and comparative approach to the specific location in the plant and identification of the endophytes is rarely done especially in tropical trees such as *Azadirachta indica* A. Juss (Meliaceae) [44,49]

"Neem" which is scientifically known as *A. indica* A. Juss (Meliaceae) is one of the most effective medicinal plant in natural therapy and Ayurveda in India. Various dust allergies, fever, skin diseases, rheumatism were treated by all parts of this tree including leaves, bark, and seeds etc. [7]. Various constituents of this plant have already been reported as antibacterial [32],antiretroviral [48] antiarthritic, anti-inflammatory [31], and antiulcer [36].Extracts of neem also exhibited very good results against malaria [13], diabetes [12], and leukemia [35]. However, worldwide, the best-known use of neem is for its insecticidal activities [3,21,25].

It is believed that medicinal plants and their endophytic flora produce similar pharmaceutical products. The use of endophytic fungus for the production of pharmacologically active metabolites has been increased [24]

Several reports in the recent years show that the endophytic fungi from this host produce several bioactive compounds [27,52]. An endophytic fungus, *Phomopsis* sp. isolated from the stems of the neem plant produces some 10-membered lactones, these lactones have very promising activity against plant pathogens *Ophiostomaminus minus* and *Botrytis cinerea* with MIC values 31.25 and 62.50 lg/ml respectively [52]. Again, an endophytic *Geotrichum* sp., isolated from the leaves of the neem tree, has been reported to produce two new chlorinated epimeric1,3-oxazinane derivatives, that have significant activity against the nematodes *Bursaphelenchus xylophilus* and *Panagrellus redivevus* [27]. 'Javanicin' an antibacterial nephthaquinone was isolated and characterized from the endophytic *Chloridium* sp. obtained from root tissues of the *Azadirachta indica* A Juss., this highly functionalized nephthaquinone exhibits strong antibacterial activity against *Pseudomonas* spp., representing pathogens to both humans and plants [27]. Two new solanapyrone analogues were isolated from the fermentation culture of *Nigrospora* sp. YB-141, an endophytic fungus isolated from *Azadirachta indica* A. Juss. The structures of the new compounds were elucidated on the basis of spectroscopic analysis. Most of the compounds exhibited no or only weak antifungal activities [53]. Another isolate 'Drechslera sp. Produces "pestasol" in liquid culture [11]. Thus with these examples it was established that endophytes from neem plant have potential bioactive compounds that need to be characterized [50].

Though these studies are done further systematic studies are required about distribution of these endophytes. Obviously a collection of such endophytes then serve as a library to start more characterization studies. Very few fragmentary reports are done on detailed distribution of endophytes of neem in the present area. Studies are required how the locations are affecting on the population of endophytes. This work is a beginning to understand the distribution of endophytes separating isolates and to work on their abilities to synthesize metabolically important compounds.

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Materials and Methods

Collection of Samples.

Five different locations were selected for sampling and were denoted as location 1, Zalta corner (Locl); location 2, the Shendra MIDC (Loc2); location 3, Osmanpura (Loc3) , location 4 , Bidkin area (Loc 4) and location 5, Khultabad (Loc 5) Leaves, stem were collected from individual plants at each location. Samples were labeled and collected, and each was assigned a code .All samples were immediately brought to the laboratory in sterile bags, and the tissues were screened for endophytic fungi

Screening, Identification of Endophytes.

All the samples were washed properly in running tap water for half an hour before processing. The samples were cut into small pieces. Leaves with midrib, leaves without midribs, petiole and stem samples were cut into 1.0 x 1.0 cm pieces. To eliminate epiphytic microorganisms, all the samples were initially surface treated [34]. The samples were immersed in 0.1 % mercuric chloride for two minutes followed by 70% ethanol for 1-3 min and then sterilized with distilled water for 3-5 min. Each sample was then dried under aseptic conditions. Segments of each sample were placed on potato dextrose agar (PDA) . The Parafilm -sealed petri dishes were then incubated for 72 hrs. The endophytic fungi were identified according to their macroscopic and microscopic characteristics such as the morphology of fruiting structures and spore morphology. Standard taxonomic manuals were used to identify the fungal genera [1,5]. All isolated and identified endophytic fungi were assigned specific code and subcultured and cultures were kept in deep freeze.

Analysis of Data.

The relative frequency (percent CF) of colonization of endophytic species was calculated as the number of segments colonized by a single endophyte divided by the total number of segments observed x 100 [19].

This is expressed as %CF = (N_{col}/ N_t) x 100; where N_{col} the number of segments colonized by each fungus, and N_t = the total number of segments. The dominant endophytes were calculated as the percentage colony frequency of a given endophyte divided by the sum of the percentage of colony frequencies of all endophytes x 100 [26]. Utilizing the data of percentage colony frequency in leaves with midrib, leaves without midribs, petiole and stem of different locations, Simpson's Diversity indices and Shannon-Wiener indices were calculated [20].

Results and Discussion:

Endophytic fungi from leaf, leaf with midrib, petiole and stem were isolated, identified and evaluated for their existence.Plants were collected from five different localities. A total 101 isolates belonging to 16 fungal taxa were obtained from 120 segments observed. Out of total endophytes isolated most of the genera are from hypomycetes.Very few from coleomycetes and five are from mycelia sterilla.

Maximum endophytes isolated from Loc 4 (30.9%) followed by Loc1 (29.8%) and minimum endophytes were recovered from Loc 2 (6.1%). Among 101 isolates, 29 isolates (6 from leaf with midrib, 7 from leaf, 8 from stem and 8 from petiole) were separated from Loc 1, 6 isolates (3 from leaf with midrib, 2 from leaf, 1 from stem and nothing from petiole) recovered from Loc 2 , While Loc 3 shows 17 isolates (4 from leaf with midrib, 10 from leaf, 2 from stem and 1 from petiole), Loc 4 exhibited 30 isolates (9 from leaf with midrib, 7 from leaf, 13 from stem and 1from petiole) and Loc 5 recovered 15 isolates (4 from leaf with midrib, 2 from leaf, 5 from stem and 4 from petiole) (Table 1).

The percent colonization at tissue samples by endophytic fungi at location 4 was higher than percent colonization of other locations. Significant fluctuation was observed with respect to occurrence of fungi and various tissue samples. Generally stem samples from loc 4 (54.1) and Loc 1 (33.33) exhibited maximum diversity from other stem samples. Leaf samples from Loc 3 harbored higher endophytic % frequency as compared to other leaf samples and their location. Samples of leaf with midrib harbor more diversity at Loc 2, Loc 4, Loc 5. Petiole samples in all locations showed lower % frequency as compared to the other (Table 3).

Another study done by Varma [49] according to their research leaf samples harboring higher colonizing percentage, but in our study we recorded in some locations leaf samples as well as stem samples are showing higher percentage of colonizing percentage.

Alternaria sp. was observed as the dominant endophytes fungus in total screened samples. Though *Colletotrichum truncatum* was showing lower dominance though it is isolated from all the locations. *Alternaria* sp., *Colletotricum truncatum* and *Aspergillus* sp. are showing higher colonizing frequency. *Trichoderma viridae* was showing lowest colonizing percentage in contrast of some studies where *T. viridae* was observed to be dominant fungal sp. in *A. indica* (Table 2) [30].

Similar type of results are also recorded by Tenguria and Khan [47] when they isolated endophytic fungi from *Azadiracta* leaves from Panchmarhi reservoir. They also recorded *Alternaria sp.*, *Fusarium* Sp. In their work dominant fungi recorded were *Trichoderma*, *Pestalotiopsia* and *Penicillium* sp. But as per our study *Alternaria* and *Colletotrichum* is the dominant sp. *Penicillium* sp. showed lower CF% and dominance.

In one of the previous studies *Fusarium* sp. and some sterile fungi had been recorded in the leaves of A. indica and effect of endophytic assemblage and colonization by leaf tissue type, site and seasonality was shown [37] Acccording to study done by Mahesh *et.al.* [30]

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they reported *Trichoderma, Penicillium* and *Pestalotiopsis* as a dominant species from neem bark collected from Mysore region.

According to studies done by Verma *et.al.* [50] *Alternaria* sp. , *Acromonium* and *Cladosporium* and *Aspergillus* sp. were dominant. They also recorded *Pestalotiopsis, Trichoderma, Curvularia* and *Penicillum* sp. But we able to report *Alternaria sp.* and *Trichoderma sp.*

Table No.1 Occurrence and identification of endophytic fungi from leaf with midrib and leaf without midrib, stem and petiole samples of *Azadirachta indica* growing at five different locations

Name	Beed bypass* Loc1				Shendra* Loc2				Osmanpura* Loc3				Bidkin* Loc4				Khultabad* Loc5			
	LW	L	S	P	LW	L	S	P	LW	L	S	P	LW	L	S	P	LW	L	S	P
A.flavus	-	-	-	-	-	-	-	-	-	-	2	-	1	1	-	-	-	-	-	-
A. sp. 1	-	-	-	-	-	-	-	-	1	2	-	-	-	-	-	-	-	-	-	-
A.sp. 2	3	-	1	-	-	-	-	-	1	4	-	-	1	-	1	-	-	-	1	-
Alternaria sp	2	5	3	4	-	-	-	-	-	-	-	-	-	-	-	-	3	-	1	2
Colletotrichum truncatum	-	-	-	-	-	-	1	-	1	4	-	1	-	-	5	-	-	1	-	-
Trichoderma viridae	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-
Cladosporium sp	-	-	-	-	-	-	-	-	-	-	-	-	2	3	3	-	-	-	-	
Penicillum sp1	-	-	-	-	-	-	-	-	-	-	-	-	3	1	-	-	-	-	-	-
Penicillum sp2	-	-	-	-	-	-	-	-	-	-	-	-	1	1	3	1	-	-	-	-
Nigrospora sp.	-	-	-	-	-	-	-	-	-	-	-	-	1	1	-	-	-	-	-	1
Fusarium sp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1		1	1		1
A4S2	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sterile mycelium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	2
Linoet gold(C5L1)	-	-	-	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A5l1 (brown	1	2	3	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A5l4(white my)	-	-	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* Zalta corner (Locl); t Shendra MIDC (Loc2); Osmanpura (Loc3) , Bidkin area (Loc 4) , Khultabad (Loc 5)

Similar diversity studies were done by Suwannarach *et.al.* [46] on the endophytes isolates from *Cinnamomum bejolghota* . They calculated various indices and recorded that

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Colletotrichum gloeosporioides, *Phomopsis* sp. and *Guignardia mangiferae* and xylariaceous forms were dominant regardless of the site, season and tissues.

Despite significant variations in the specific recovery of the endophytic community from plat tissue in each location, the inter-site comparison is significant. For instance Location 4 has lower Simpson's index which indicates it has greater diversity while location 1 has higher Simpson's index reflects lower diversity. Shannon Wiener Index is also higher for loc 4 indicating maximum number of fungal isolates. When evenness were compared Loc 4 though having more isolates all are evenly distributed (Table 4).

Loc 4 (Bidkin region) is said to be harboring more no. of endophytic isolates when compared to other locations, this region is comparatively less disturbed. Loc 5 is also having considerable diversity which is a tourism sight hence remain to be less polluted. Most of the area is conserved and entry is restricted.

When we observed endophytic diversity at loc 2 and loc 1 which has less diversity, less Shannon wiener index. These areas are industrial may tend to limit the rate of endophytic colonization.

Table No.2 Colonizing frequency and dominance of fungi isolated from *Azadirachta indica*

Name	Total isolates	CF	Dominance of Fungi
A.flavus	4	3.33	3.96
A. sp. 1	3	2.50	2.97
A.sp. 2	12	10.00	11.88
Alternaria sp.	20	16.67	19.80
Colletotrichum truncatum	13	10.83	12.87
Trichoderma viridae	1	0.83	0.99
Cladosporium sp.	9	7.50	8.91
Penicillum sp1	4	3.33	3.96
Penicillum sp2	6	5.00	5.94
Nigrospora sp.	3	2.50	2.97
Fusarium sp.	4	3.33	3.96
A4S2	2	1.67	1.98
Sterile mycelium	5	4.17	4.95
C5L1	3	2.50	2.97
A5l1	9	7.50	8.91
A5l4	3	2.50	2.97

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Various researches have studies distribution patterns of endophytes within plant tissues and in most cases, foliar endophytes were examined [28,38,51]. The species composition of the endophytic assemblage and frequency of infection varied according to host species, site characteristics such as elevation, exposure, associated vegetation, tissue type [15,16], and tissue age [14,38]. On the other hand, for large woody perennials, growth stage and position in the canopy may also affect the distribution of endophytes [22]. Generally, the assemblage of foliar endophytes for a given host comprises a relatively consistent group of fungal genera and species, characterized by a few dominant species, and this study also corroborates this conclusion [11]. Similar findings were obtained in case of Sequoia sempervirens [41].

Table No. 3 Dominant endophytes, their species richness and percentage colonization of *A. indica* at each location

Localities	Sampling location	Total isolates	frequency % CF	Dominance of fungi	Species richness
Loc1	AZLW	6	25.00	6.19	6
	AZL	7	29.17	7.22	7
	AZS	8	33.33	8.25	7
	AZP	8	33.33	8.25	7
Loc2	AZLW	3	12.50	3.09	3
	AZL	10	41.67	10.31	10
	AZS	1	4.17	1.03	1
	AZP	0	0.00	0.00	0
Loc3	AZLW	4	16.67	4.12	4
	AZL	10	41.67	10.31	10
	AZS	2	8.33	2.06	2
	AZP	1	4.17	1.03	1
Loc4	AZLW	9	37.50	9.28	9
	AZL	7	29.17	7.22	7
	AZS	13	54.17	13.40	13
	AZP	1	4.17	1.03	1
Loc5	AZLW	4	16.67	4.12	4
	AZL	2	8.33	2.06	2
	AZS	5	20.83	5.15	5
	AZP	4	16.67	4.12	4

Table 4 Different diversity indices for each location

Indices	Loc1	Loc2	Loc3	Loc4	Loc5
Simpson's diversity index	0.31	0.26	0.21	0.14	0.18

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Shannon wiener index	0.53	0.44	0.63	7.22	5.43
Evenness	0.88	0.92	0.90	7.99	6.42

Nearly all endophytes studied, so far, produce bioactive compounds [45] Bioprospecting, and sometimes, these com pounds have an enormous pharmaceutical, agricultural, and industrial potential. *Alternaria* is one of the dominant fungi in the present study, known for presence of various bioactive compounds [29]. Another fungal form *Cladosporium* sp. was also studied for its insecticidal property [43]

Isolation and identification of the endophytic fungi from *Azadiracta* was done in the present study. Along with isolation of endophytic fungi, their distribution with the help of indices was studied. This work focuses on occurrence of endophytic fungi is somewhat related with the environmental condition. Isolated strains can be further used to screen presence of various metabolites. May be these fungal strains can be potential source of different bioactive compounds.

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