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DIVERSITY OF ENDOPHYTIC FUNGI IN TRIPHALA TREES OF ACHANAKMAR-AMARKANTAK BIOSPHERE RESERVE CHHATTISGARH

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ABSTRACT

Endophytic fungi are a group which resides within the living tissues of the host plant without producing any apparent disease or disease-like symptoms. Triphala trees such as *T. chebula*, *T. bellerica* and *Emblica officinalis* are important traditional medicinal plants were taken to carry out the studies on endophytic fungi. The forest of central India is basically a tropical semi-evergreen Sal forest. Chhattisgarh state in central India is well recognized for maximum area of forests. The state is also known for maximum varieties of tree species most of which are of pharmaceutical importance. In the present study the above mentioned tree species were screened for the presence of endophytic fungi. The samples were collected from the Achanakmar–Amarkantak Biosphere Reserve in the winter, summer and rainy season. The seasonal occurrence and distribution of endophytic fungi were recorded. In present investigation *Pestalotiopsis* and *Colletotrichum* sp. were found frequently in leaf samples of Bahera, whereas *Alternaria* sp. and *Phyllosticta* sp. were often encountered in Harra tree. Despite the specificity of tree plants, the study reveals importance of the geographical and seasonal factors with respect to the presence or absence of endophytic fungi.

KEYWORDS: **Endophytic fungi, Achanakmar-Amarkantak Biosphere Reserve, seasonal occurrence, Chhattisgarh.**

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INTRODUCTION

Endophytic fungi are a group which resides within the living tissues of the host plant without producing any apparent disease or disease-like symptoms. The term 'endophyte' was introduced by de Bary and was initially applied to any organism found within a plant.[1] Schulz and Boyle described endophytic fungi consisting of three basic ecological groups: the mycorrhizal fungi,[2] the balansiaceous or 'grass endophytes' and the non-balansiaceous taxa. They emphasized that in contrast to balansiaceous endophytes, the non-balansiaceous fungal endophytes are diverse, both phylogenetically and with respect to life-history strategy. The term endophyte has been controversial and mycologists have been cogitating upon its exact meaning for some time.[3] Thus it includes an assemblage of microorganisms with different life history strategies: those that grow saprophyticaly on dead or senescing tissues following an endophytic growth phase[4]; avirulent microorganisms as well as latent pathogens and virulent pathogens in the early stages of infection.[5,6]The ecology of tropical endophyte with reference to host preference, host protection and role in litter degradation and nutrient recycling has scant attention in India. Most work on endophytic fungi has been carried out abroad in temperate regions,[7] but there have been also some investigations of endophytes in tropical ecosystems. In India a limited number of tree species have been studied for the occurrence of endophytic fungi.[8,9,10,11] In this context, the study was initiated to collect information on the endophytic mycobiota associated with naturally growing Triphala plants (*Terminalia chebula*, *T. bellerica* and *Emblica officinalis*) in a 'herbal' state to understand the ecology of fungal endophytes.Triphala (Tri-three + phala-fruit), an Ayurvedic formulation of a mixture of three fruits; Harra (*T. chebula*), Bahera (*T. bellerica*) and Amla (*Emblica officinalis*) also known as 'three Myrobalan', which is a well known free-radical scavenger.[12] References to the use of Triphala can be found in the Sushrut Samhitas and Charak Samhitas which dates back to 1500 BC. Triphala is regarded as an important 'Rasayana' in Ayurvedic medicine. Triphala trees Harra, Bahera and Amla as important traditional medicinal plants have been taken to carry out the studies on endophytic fungi. Although, the de-forestation and planting exotic tree species in a-forestation program put the Triphala plants at the danger of extinction. The geography and climatic condition of Achanakmar-Amarkantak Biosphere Reserve (ABR) is quite favorable for the growth of Triphala trees. ABR of Bilaspur district has been selected for sampling of symptom-less leaf and bark with living tissues, each of which was sampled twice during rainy, winter and summer seasons. Chhattisgarh is one of the largest forests state in India and is basically a hilly state with ranges running from East to West and North to South of which most of the forest cover lying on plateaus of Bastar, Jashpur, Sarguja and Pendra. This floristic province is known as the tribal state which hosts maximum varieties of tree species in central India. The state's forests possess enormous diversity of large canopy tree species. Despite the richness of medicinal tree species in the state and growing awareness of the importance of endophytes, there is least concern on documentation of the mycoflora associated with the tree species of the state's forests. The Sal (*Shorea robusta*) with their associate's tree species viz., *Terminalia tomentosa*, *T. chebula*, *T. bellerica*, *Emblica officinalis*, *Pterocarpus marsupium*, *Anogeissus latifolia*, *Lagerstromia parviflora* and *Boswellia serrata* are found in this region together with some bamboo breaks.Achanakmar-Amarkantak Biosphere Reserve (ABR), a 14th biosphere reserve of India is quite rich in flora and fauna. ABR is one of the threatened 'hot-spots of biodiversity' in the world. This is located in central part of India at the elevation range 383-800m above sea level in Bilaspur district of Chhattisgarh, India. The area of ABR represents the northern tropical deciduous forests. This is known for having a combination of different climatic and edaphic conditions at various altitudes. The vegetational variability particularly of tree species includes *Shorea robusta*, *Anogeissus latifolia*, *Buchanania lanzen*, *Cleistanthus collinus*, *Diospyros melanoxylon*, *Emblica officinalis*, *Pterocarpus marsupium*, *Eugenia jambolana*, *Lagerstroemia parviflora*, *Terminalia bellerica*, *T. chebula*, *T. tomentosa*, *T. arjuna*, *Madhuca indica* and *Butea monosperma*.

MATERIAL AND METHODS

Study Site: Achanakmar - Amarkantak Biosphere Reserve (ABR)

The forest of central India is basically a tropical semi-evergreen Sal forest. Tropical forests are regarded as one of the most species diversity rich terrestrial ecosystems. They are distinguished from all other terrestrial ecosystems by a very high diversity in many levels (species, life forms etc.). Tropical dry forests represent the major biome in India covering 46% of the total forest cover.[13] The relic mixed tropical state forests have more tree species and more diverse in comparison to forests of other states. The Achanakmar-Amarkantak Biosphere Reserve has been divided into core and buffer zones. The entire area of the Achanakmar Sanctuary is designated as the core zone of the reserve and the rest of the 3284.36 sq. km. are serving as the buffer zones of this reserve. Out of the total area of the buffer zone, an area of 1224.98 sq. km. falls in the state of Madhya Pradesh and the remaining area of 2059.38 sq. km. falls in the Chhattisgarh state. Processing of samples was done in the laboratory for isolation of endophytic fungi according to the method described by Petrini et al.[14] The leaves were washed thoroughly in the running tap water to eliminate the obligate epiphyllous organisms. These leaves/ bark were cut into the small segments of 0.5 cm with the help of sterile scalpel. The segments were then surface sterilized, leaf segments were sterilized separately. To eliminate epiphytic microorganisms, all the samples were immersed in 70% ethanol for 1–3 min. Then these segments were sterilized with aqueous sodium hypochlorite (NaOCL, 4% available Chlorine) for 3–5 min. and then rinsed in 70% ethanol for nearly 2– 5 sec., followed by a final rinse in sterilized distilled water. Each sample was then dried under aseptic conditions. Segments (a total of 300 at 10 segments per Petri dish) of each sample were placed on growth medium of potato dextrose agar (PDA) amended with streptomycin (100 mg/l). The Parafilm-sealed Petri dishes were then incubated at 27 ± 1^{0}C for 25 days provided with 12-h. light/dark cycle. Endophytic

fungi that grew out from the segments were periodically sub-cultured in other Petri dishes. Those fungi that failed to sporulate were given codes using cultural characteristics such as colony surface, texture and hyphal pigmentation are categorized as 'sterile' forms. The isolated endophytes were maintained in PDA slants in a culture collection. The endophytic fungi were identified according to their macroscopic and microscopic characteristics such as the morphology of fruiting structures, colony and conidial morphology. Standard taxonomic manuals were used to identify the fungal genera.[15,16,17,18]

Analysis of data

The density of colonization (rD%) of a single endophyte taxon in different age groups of leaves was calculated by the method of Fisher and Petrini and was equal to the number of colonized segments divided by the total number of segments observed × 100.[19] The dominant endophytes were calculated as the density of colonization of a given endophyte divided by the sum of the density of colonization of all endophytes ×100.[20] Relative percentage of occurrence of different groups of fungi (viz. coelomycetes, hyphomycetes and ascomycetes) was calculated as density of colonization of one group divided by the total density of colonization of all the groups of endophytes ×100 .[21]

RESULTS AND DISCUSSION

Fungal endophytes in tropical trees represent an important and quantifiable component of fungal biodiversity. Highly abundant in a broad assemblage of host species, fungal endophytes appear to be highly diverse in a wide array of tropical angiosperms.[22,23,24] Studying plants from less-studied habitats for their resident endophytes is of immediacy to get a fuller picture of endophyte diversity, and might even lead to identification of novel bioactives. [25]The interest in endophytic fungi is based on several facts: mainly, they represent the main reservoir of biodiversity within the fungal kingdom,[26] including a great variety of taxa from the Eumycota, mostly ascomycetes and their coelomycetous anamorphs. They may also have pharmacological significance, as they represent a rich source of secondary metabolites with biological activity.[27,28,29] The metabolites of plant endophytic fungi could be good potential sources for screening programs of bioactive natural products.[30]Presently, bioprospecting of endophytes is considered important, for which least attempts have been made for characterization of endophytes in reference to useful trees. Since specific rationale for the selection of these plants for isolation of endophytes and their natural products discovery is used, there was a good reason for selection of Triphala trees for the present study. The trees produces a number of highly potent chebulinic acid, tannic acid, gallic acid, ascorbic acid etc. and also it is astringent, purgative, stomachic and laxative. They are useful in asthma, piles and cough.[31]A total of 200 fungal isolates were cultured from the samples of Triphala plants. The survey showed that leaves of Harra, Bahera and Amla plants harbored anamorphic fungi, ascomycetes and the sterile forms as endophytes. Because of structural simplicity, the anamorphic fungi were obtained more often than the complex group ascomycetes. Similarly more number of sporulating endophytes were recovered than that of sterile forms. This has reflected an adaptability of the fungi in response to their spore structure, their dispersal mechanism and mode of penetration in the host. During the isolation and identification of different groups of fungi the dominant endophytic fungi were species of *Pestalotiopsis*, *Colletotrichum* and *Phyllosticta* belongs to the group coelomycetes (Graph 4). Out of 8 genera belonging to the group hyphomycetes, the species of *Alternaria*, *Penicillium* and *Fusarium* were obtained more often than the species of *Cladosporium*, *Monodictys*, *Acremonium* and *Scytalidium*. The group ascomycetes represented only for the genus *Chaetomium* and *Glomerella*. It is of great interest that a variety of fungal species were obtained from Triphala trees. A total of 18 species variants recorded from Harra, whereas Bahera tree represented for 23 species respectively (Table I). The density of colonization (rD%) of endophytic fungal taxa is presented in Graph 1 and 3. Among the mitosporic fungi in Harra, the two coelomycetous species viz., *Dendrophoma* sp., *Pestalotiopsis* sp. showed maximum colonization i.e. 0.6%. The colonization of *Alternaria* sp. belonging to hyphomycetes was also found to be equally fair i.e. 0.6% (Graph 1). In Bahera, *Pestalotiopsis* sp. and *Colletotrichum* sp. showed maximum colonization i.e. 3.6% and 1.3% respectively, followed by *Glomerella* sp. (Graph 3). It explains that these genera are more adapted to Bahera tree. *Colletotrichum* spp. and *Pestalotiopsis* spp. was the most dominant fungi isolated from the leaves of Harra (Graph 2). Numbers of fungal genera were isolated from Harra, probably due to higher density of hairs. Because of smaller in size and sessile conditions of leaves, a very few endophytic fungi were recovered from the Amla plant. The leaves are bipinately compound of which the surface area is quite smaller than that of Harra and Bahera plants, probably might be a reason for the lesser number of endophytic fungi. Moreover most of the endophytic fungi isolated from Amla plant were found out to be non-sporulating group of fungi under the laboratory conditions. In present investigation *Pestalotiopsis* and *Colletotrichum* sp. were found frequently in leaf samples of Bahera, whereas *Alternaria* sp. and *Phyllosticta* sp. were often encountered in Harra tree (Table I). They can gain entry into plant tissues through natural cracks, wounds, lenticels, due to air current or rain water flowing down, or through the agency of insects, beetles, mites and other animals which live and breed in the plants and trees. Ascomycetes and their anamorphic states invariably constitute the endophyte populations of leaves.[32,33] In the present study, among the mitosporic fungi, overall percentages of coelomycetes and hyphomycetes were very high as compared to ascomycetes and other forms (Graph 5). Generally there were higher density of colonization in midrib portion of the leaf than the bark in both Harra and Bahera. Only *Penicillium* sp. was isolated from the bark of Triphala trees. Colonization of fungi was greater in samples from the midrib part of leaf in comparison to those from lamina. Such a variation in distribution of endophytic fungi in leaf is the result of physical properties of the leaf that influence the spore deposition, spore retention and water evaporation. As far as

colonization of endophytic fungi in relation to host tissue is concerned, mature leaf was found out to harbor more fungi than younger leaves. Various workers have studied distribution patterns of endophytes within plant tissues and in most cases foliar endophytes were examined.[34,35,36] Generally the assemblage of foliar endophytes comprises a relatively consistent growth of fungal genera and species characterized by a few dominant species.[20,37] This study also corroborates well with the same conclusion. The species composition of the endophytic assemblage and frequency of infection varies according to the host species and slight characteristic such as elevation, exposure, associated vegetation, tissue type and tissue age.[38,39,40,34]The number of endophytes that can be recovered from leaf tissue has been shown to increase with age of leaves in several hosts.[4,41,42] Espinosa-Garcia and Langenheim and Stone also reported similar correlation exists between leaf age and endophytic species richness and infections density in many temperate tree species.[43,4] The leaf lamina of mature leaves in Harra and Bahera and its dorsi-ventral position provides a large surface area for the infection of endophytes. Furthermore, the above tree species are semi-evergreen in which leaves persist for several months. Thus increased density of colonization of older leaves is due to their repeated re-infections from the air borne inoculums.[44,45] This is especially relevant in the case of large leaf lamina of mature leaves for endophyte assemblages in Harra and Bahera. In addition, the endophytic assemblage of old leaves were more diverse suggesting that the susceptibility of old leaves to endophyte increase with leaf age. Old leaves contain a higher percentage of cellulose and complex sugar, which act as a good substrate for a large amount of endophytes. Young leaves shown an early incidence of endophytes, therefore, lower numbers of endophytic fungi were isolated from younger leaves. Coley and Barone studied prevalence of antifungal secondary compounds in young leaves and in leaves that develop under high light conditions,[46] has been documented for many tree species in tropical forests. Denslow et al. and Coley and Barone studied well on phenolic compounds which are known to have antifungal effects.[47,46] The smaller size of new-born leaf seems to have little chance of infection of endophytic fungi,[48] therefore, lesser number of fungi was obtained from new-born leaves. In our investigation, *Colletotrichum* species and *Pestalotiopsis* species have been isolated at any age group from the leaves of *T. bellerica*. Thus it seems that above chemical defence mechanism does not influence hyphal growth (*in-vitro*) directly to above fungi and they can be isolated frequently from many other tree species of the forest. Carroll and Carroll and Petrini and Carroll obtained a ubiquitous endophytic genus *Phyllosticta* sp., which features in great amount in the plant species.[49,50] Suryanarayanan et al. had also reported the species of *Phyllosticta* along with *Phomopsis* sp. as dominating endophytes in 20 out of 24 host plants.[9]ABR is one of the tropical unexplored pristine habitats known for maximum variability of medicinal tree plants which may provide a fruitful niche in their foliar and bark tissues for the occurrence of endophytic fungi. It corroborates with the opinion of Arnold that tropics represent a wealth of unexplored endophytic diversity.[51] Bills and Polishook isolated 69 fungal species from the bark of a single *Carpinus caroliniana* tree (Iron wood), suggesting enormous extent of fungal diversity in a single plant.[52] *Pestalotiopsis* sp. is an anamorphic genus obtained in tropical and sub-tropical regions.[53,54] The geographical distribution and occurrence of *Pestalotiopsis* sp. play an important role in the forest ecosystem. It is widespread endophytic fungi found on leaves, bark and their decaying part of wide varieties of plants. Some species of *Pestalotiopsis* produce secondary metabolites with potential use on medical application and control of plant diseases.[55,56] In our investigation number of *Pestalotiopsis* species has been isolated from Harra and Bahera trees (Table I).The fungus *Phyllosticta* was isolated frequently in the rainy season because of its slimy spores that rely at least in part on rain for dispersal.[57] The fungi *Phyllosticta* spp. and *Pestalotiopsis* spp. were not obtained in summer season (Table II and III). The species of *Alternaria* and *Colletotrichum* were found in summer season which shows their mesophilic nature. The fungal species of *Chaetomium* and *Fusarium* that are highly cellulolytic fungi were obtained during rainy season.[58,59,60,61] *Colletotrichum* sp. is also a plant pathogen, infects many plant species reflecting an idea that a pathogen may spend part of its lives in an endophytic stage.[62] Rouhier and Jacquot studied host pathogen relationship to express that both the hosts and the pathogens appear to produce reactive oxygen species, if produced by the plant it may act to limit pathogen colonization and if produced by the pathogen may increase the virulence.[63]During rainy season large surface area of leaves provide a better chance for the fungal propagules in the air to shelter. Rajagopal and Suryanarayanan studied endophytes of Neem (*Azadirachta indica*) leaf for a period of two years and concluded that the colonization frequency of the endophytes increased with rainfall.[11,64] In present study the Triphala trees inhabiting endophytes particularly species of *Pestalotiopsis* showed increased colonization during the rainy season (Table III). The appendages found in the conidia of *Pestalotiopsis* species probably support its attachment to the host surface. The common anamorphic species of *Alternaria*, *Cladosporium* and *Colletotrichum* were exclusively found during the winter season. Arnold and Herre correlated above fungi with their wide range of host preference during moderate temperature and low humid conditions.[65] Arnold and Lutzoni showed that the degree of host specificity is similar in tropical and temperate forests but increases at higher latitudes.[66] Moreover, in present investigation the most frequently recovered endophytic fungi are distinct in terms of their patterns of abundance, diversity and taxonomic composition.

CONCLUSION

The challenges remains to recover and identify those elusive and rarely cultured taxon with broad and narrower host ranges and to elucidate the ecological roles of these little-known symbionts in a wide forest area of Chhattisgarh. ABR, rich in tree diversity represents a wealth of endophytes, which may encounter the chance of new species of fungi. The

chances of isolating novel metabolites from endophytes is also very high. In this context, the abundance and potential of Triphala trees are worth for further study on endophytes.

Table I
List of endophytic fungi isolated from leaves of Harra and Bahera.

Endophytic fungi	Harra	Bahera
Acremonium sp.	-	+
Alternaria alternata	+	-
Alternaria sp.1	-	+
Alternaria sp.2	+	-
Chaetomium sp.	+	-
Cladosporium elatum	-	+
Colletotrichum sp.1	+	-
Colletotrichum sp.2	-	+
Colletotrichum sp.3	-	+
Colletotrichum sp.4	-	+
Colletotrichum sp.5	-	+
Curvularia sp.	+	-
Dendrophoma sp.1	+	-
Dendrophoma sp.2	+	-
Fusarium sp.	+	-
Glomerella sp.1	-	+
Glomerella sp.2	-	+
Monodictys fluctuata	+	-
Penicillium sp.	-	+
Pestalotiopsis sp.1	+	-
Pestalotiopsis sp.2	-	+
Pestalotiopsis sp.3	-	+
Pestalotiopsis sp.4	-	+
Pestalotiopsis sp.5	-	+
Pestalotiopsis sp.6	-	+
Pestalotiopsis sp.7	+	-
Pestalotiopsis sp.8	-	+
Pestalotiopsis sp.9	-	+
Pestalotiopsis sp.10	-	+
Pestalotiopsis sp.11	-	+
Pestalotiopsis sp.12	-	+
Pestalotiopsis sp.13	-	+
Phomopsis sp.	+	-
Phyllosticta sp.1	+	-
Phyllosticta sp.2	-	+
Scytalidium thermophilum	+	-
Unidentified sp. HL 2	+	-
Unidentified sp. HL 3	+	-
Unidentified sp. HR 11	+	-

+ indicates positive result and
- indicates negative result.

Table II
Seasonal occurrence of endophytic fungi in Harra.

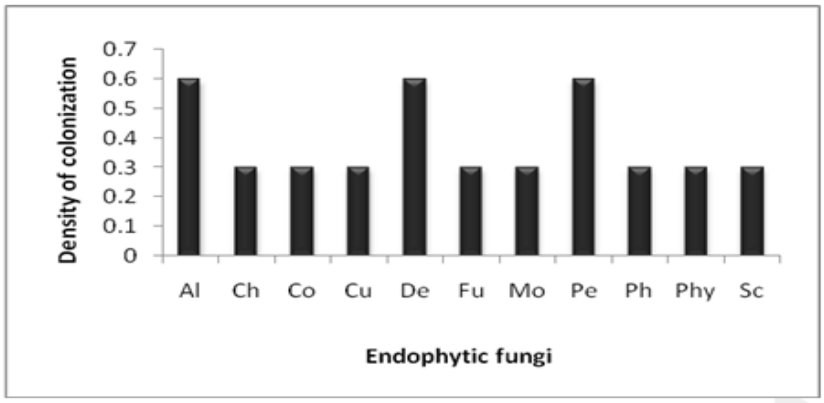
Endophytic fungi	Summer	Rainy	Winter
Alternaria alternata	-	+	-
Alternaria sp.2	+	-	+
Chaetomium sp.	-	+	-
Colletotrichum sp.1	-	+	-
Curvularia sp.	-	+	-
Dendrophoma sp.1	-	-	+
Dendrophoma sp.2	-	-	+
Fusarium sp.	-	+	-
Monodictys fluctuata	-	-	+
Pestalotiopsis sp.1	-	+	-
Pestalotiopsis sp.7	-	-	+
Phomopsis sp.	-	-	+
Phyllosticta sp.1	-	+	-
Scytalidium thermophilum	-	-	+
Unidentified sp. HL 2	-	+	-
Unidentified sp. HL 3	-	-	+
Unidentified sp. HR 11	-	+	-

+ indicates positive result and
- indicates negative result.

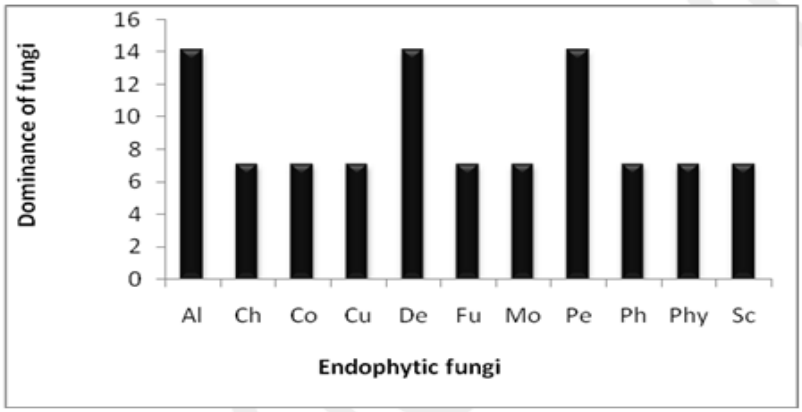
Table III
Seasonal occurrence of endophytic fungi in Bahera.

Endophytic fungi	Summer	Rainy	Winter
Acremonium sp.	-	-	+
Alternaria sp.1	-	-	+
Cladosporium elatum	-	-	+
Colletotrichum sp.2	-	-	+
Colletotrichum sp.3	-	+	-
Colletotrichum sp.4	-	+	-
Colletotrichum sp.5	+	-	-
Glomerella sp.1	-	-	+
Glomerella sp.2	-	-	+
Penicillium sp.	-	-	+
Pestalotiopsis sp.2	-	+	-
Pestalotiopsis sp.3	-	+	-
Pestalotiopsis sp.4	-	+	-
Pestalotiopsis sp.5	-	-	+
Pestalotiopsis sp.6	-	-	+
Pestalotiopsis sp.8	-	-	+
Pestalotiopsis sp.9	-	-	+
Pestalotiopsis sp.10	-	-	+
Pestalotiopsis sp.11	-	-	+
Pestalotiopsis sp.12	-	-	+
Pestalotiopsis sp.13	-	-	+
Phyllosticta sp.2	+	-	-

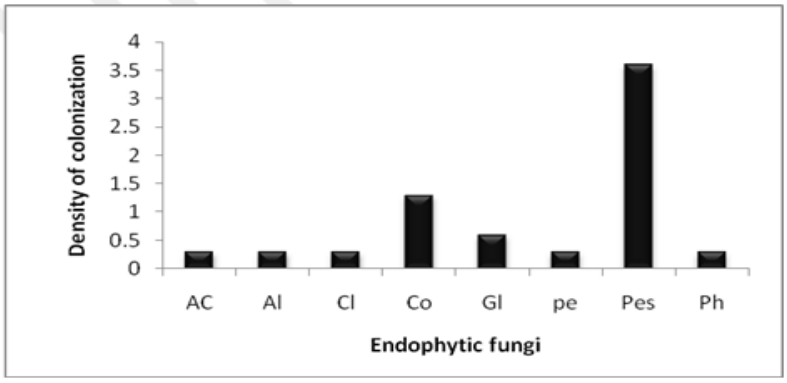
+ indicates positive result and
- indicates negative result.



Graph 1
Density of colonization of endophytic fungi in Harra. The sequence of the endophytes is: Al- Alternaria sp., Ch- Chaetomium sp., Co- Colletotrichum sp., Cu- Curvularia sp., De- Dendrophoma sp., Fu- Fusarium sp., Mo- Monodictys sp., Pe- Pestalotiopsis sp., Ph- Phomopsis sp., Phy- Phyllosticta sp., Sc- Scytalidium sp.

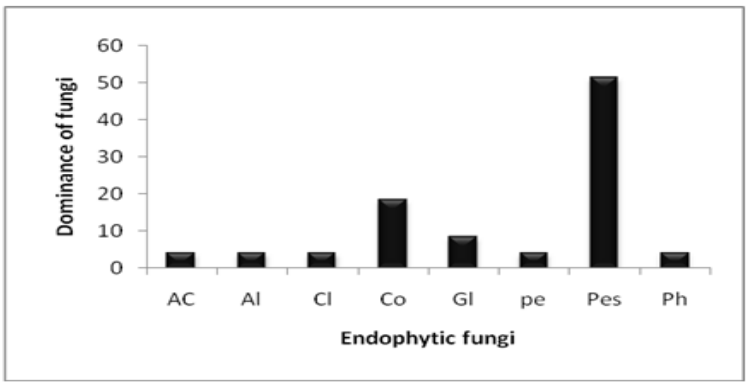


Graph 2
Dominance of endophytic fungi in Harra. The sequence of the endophytes is: Al- Alternaria sp., Ch- Chaetomium sp., Co- Colletotrichum sp., Cu- Curvularia sp., De- Dendrophoma sp., Fu- Fusarium sp., Mo- Monodictys sp., Pe- Pestalotiopsis sp., Ph- Phomopsis sp., Phy- Phyllosticta sp., Sc- Scytalidium sp.



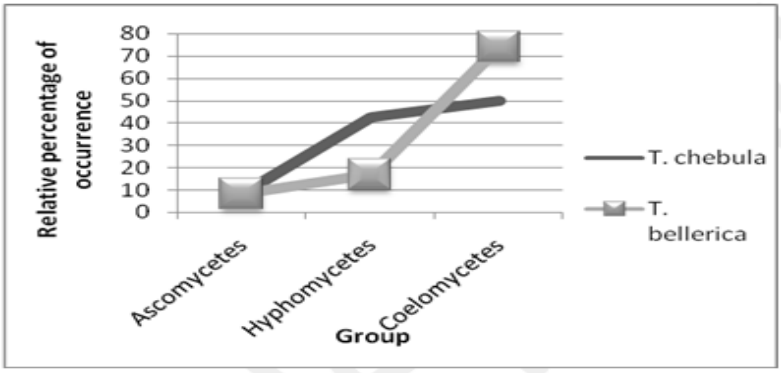
Graph 3

Density of colonization of endophytic fungi in Bahera. The sequence of the endophytes is: Ac- Acremonium sp., Al- Alternaria sp., Cladosporium sp., Co- Colletotrichum sp., Gl- Glomerella sp., Pe- Penicillium sp., Pes- Pestalotiopsis sp., Ph- Phyllosticta sp.



Graph 4

Dominance of endophytic fungi in Bahera. The sequence of the endophytes is: Ac- Acremonium sp., Al- Alternaria sp., Cl- Cladosporium sp., Co- Colletotrichum sp., Gl- Glomerella sp., Pe- Penicillium sp., Pes- Pestalotiopsis sp., Ph- Phyllosticta sp.



Graph 5

Relative percentage of occurrence of different groups of fungi in Harra and Bahera.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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