Journal of Innovations in Pharmaceuticals and Biological Sciences
www.jipbs.com
JIPBS
ISSN: 2349-2759

Research Article

Biodiversity of Endophytic Fungi in *Withania Somnifera* Leaves of Panchmarhi Biosphere Reserve, Madhya Pradesh

Rajesh Kumar Tenguria[1], Firoz Naem Khan*[2]

[1]Division of Microbiology, Department of Botany, Govt. Motilal Vigyan Mahavidhyalaya, Bhopal 462008, M.P. (INDIA).
[2]Immunology Laboratory, Centre for Scientific Research and Development, People's University, Bhanpur, Bhopal 462037, M.P. (INDIA).

Abstract

Withania somnifera (L.) Dunal possess medicinal properties and can harbour endophytic mycoflora. Many of the pharmaceutical compounds produced by medicinal plants are reportedly produced by their endophytic fungi. Hence, it is important to study medicinal plants for their endophytic mycoflora for biodiversity and to determine their medicinal properties. An attempt has been made to study the biodiversity of endophytic fungi to understand their colonization. A total of 200 segments from plant leaves samples were screened and 49 endophytic fungi isolates belonging to 7 genera with 24.5% colonization frequency was observed. Only Hyphomycetes was recovered and *Alternaria alternata* was present with highest number of isolates followed by *Aspergillus flavus* and *Aspergillus niger.*

Key words: Endophytes, Endophytic fungi, Biodiversity, *Withania somnifera*, *Alternaria alternata*, *Aspergillus niger*, *Aspergilus flavus*, Hyphomycetes, Deuteromycetes, Mycoflora.

***Corresponding Author Firoz Naem Khan,** Immunology Laboratory, Centre for Scientific Research and Development, People's University, Bhanpur, Bhopal 462037, M.P. (INDIA).

1. Introduction

In recent times, focus on plant research has increased all over the world and a large body of evidence has collected to show immense potential of medicinal plants used in various traditional systems and their endophytes. Almost all vascular plant species examined to date were found to anchorage endophytic bacteria and fungi [1][2][3]. Moreover, the colonization of endophytes in marine algae [4][5], mosses and ferns [6][7] had also been detected. Based on fact, endophytes are important components of microbial biodiversity.

De Bary (1866) first defined all organisms that colonize the internal plant tissue as endophyte (Gr. *endon*, within; *phyton*, plant) [8] and endophytic fungi was first identified by Freeman in 1904, and was isolated from *Lolium persicum* (Persian darnel). Since the discoveries of endophytes various definitions had been

provided and widely accepted definition of endophyte is "microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects" and has a mutualistic and symbiotic relationship with their hosts or a suite of microorganisms that grow intra- and/or intercellularly in the tissues of higher plants without causing symptoms on the plants in which they live, and have proven to be rich sources of bioactive natural products [9][10]. Endophytic fungi are poorly investigated and under explored group of microorganisms and received considerable attention after they were found to protect their host against insects, pests and pathogens by secreting bioactive secondary metabolites [11][12][13].

Medicinal plants are known to harbour endophytic fungi that are believed to be associated with the production of pharmaceutical products [3]. *Withania somnifera* is an important tropical medicinal plant which belongs to the family Solanaceae [14] and generally known as Indian Ginseng or Ashwagandha. In traditional ayurvedic systems of medicine it has a wide range of therapeutic properties i.e., anticancerous, anti-oxidant, anti-inflammatory and immunomodulatory [15]. It is believed that medicinal plants and their endophytic flora produce similar pharmaceutical products and the production of pharmacologically active metabolites has been on rise [16]. Endophytes are mostly unexplored group of microorganisms, but a few studies show them as a huge source of medicinal compounds.

The endophytic fungi play important physiological [17] and ecological [18] roles in their host life. The ubiquity of these symbiotic microorganisms is clear, but diversity, host-range, and geographical distributions are unknown [19]**.** Endophytes are now considered as an important component of biodiversity. The distribution of endophytic mycoflora differs with the host. Commonly, numerous of endophyte species can be isolated from a single plant, among them, at least one species shows host specificity. The environmental conditions under which the host is growing also affect the endophyte population [20], and the endophyte profile may be more diversified in tropical areas. Tropical and temperate rainforests are the most biologically diverse terrestrial ecosystems on earth. The most threatened of these spots cover only 1.44% of the land's surface, yet they harbor more than 60% of the world's terrestrial biodiversity [21].

It is a difficult task to have an accurate estimate of species diversity for a hyperdiverse group such as the fungi, the diversity of a better studied group, namely the higher plants, is used as a base to predict fungal diversity, principally because different ecological groups of fungi with different resource requirements form temporary or prolonged associations with higher plant [22][23]. It is obvious that the tropics, with their high plant diversity might contain most of the undescribed fungal species [24]. However, there is no study in the sub- tropics that compares the diversity, distribution, and contribution to global fungal variety of different ecological groups associated with plant communities.

Panchmarhi Biosphere Reserve has a well-off microbial diversity due to diverse climatic factors producing favorable conditions for the growth of microbes. In central India it is home to numerous species of mycoflora. The reserve is actually a natural junction of most of the forest representative types prevailing in the State. Perennial streams and dark shady gorges have created micro climatic conditions in the area and provide

hospitable environment for luxuriant growth of several moisture loving species of ferns, orchids, bryophytes, algae and many tiny herbs having immense ecological as well as economic value. The rich plant diversity and the important species are localized in areas may be considered as gene bank of rare species. However, the central Indian sub-tropical hill forests are mainly confined to hill top of the Panchmarhi Plateau, especially in the Panchmarhi Sanctuary [25]. The occurrence of sub-tropical hill forest also makes the area unique. Thus, the present study was conducted to determine the biodiversity of endophytic fungi present in *Withania somnifera* leaves collected from Panchmarhi biosphere reserve, Madhya Pradesh, India.

2. Material and Methods:

Sampling: *Withania somnifera* leaves samples were collected from Panchmarhi Biosphere Reserve (Latitude 22º 11' to 22º 56' N and 77º 47' to 78º 52' E Longitude), Madhya Pradesh, India and wiped with ethanol; stored in sterile bags and kept in ice box for transporting to the laboratory. Isolation of the endophytic fungi was done within 24 hours after sampling.

Isolation of Species: The collected leaves samples were washed thoroughly in running water to remove particles and air dried. Leaf samples were first immersed in 70% ethanol (v/v) for 1 min followed by second immersion in sodium hypochlorite (3.5 % v/v) for 3 min. The samples were rinsed thrice in sterile distilled water and dried on sterile blotters under laminar airflow to ensure complete drying. 200 segments from leaf samples of 5x5 mm size; with and without midrib were excised with the help of a sterile scalpel and the inner tissues were carefully placed on water agar plates [26]. After 5 days of incubation, hyphal tips of the fungi are removed and transferred to Potato Dextrose Agar (PDA) supplemented with streptomycin, chloramphenicol and gentamycin (50 mg/l) each to suppress bacterial growth and cycloheximide was incorporated to inhibit the rapidly growing saprophytic fungi, which can overgrow slow-growing fungi and the plates are monitored for the growth [27]. The efficacy of sterilization was confirmed by pressing the sterilized leaf on to the surface of PDA medium. The absence of growth of any fungi on the medium confirms that the sterilization procedure was effective in removing the exogenous fungi [28][29]. The plates were incubated at 25°C ± 1 with 12 hours light and dark cycles for up to 6 weeks. Periodically the colonies were examined and each colony that emerged was transferred to antibiotic-free Potato Dextrose Agar medium (PDA) for identification. Endophytic isolates were identified on the basis of culture characteristics, morphology of fruiting body and spores. The percent frequency of occurrence was calculated as the number of leaf segments colonized by a specific fungus divided by total number of segments plated x 100 and dominant endophytes were calculated as percentage colony frequency divided by sum of percentage of colony frequency of all endophytes x 100 [30][31][32]. Endophytic Fungi were grown on synthetic media under standardized culture condition. Identification of the isolates recovered was done on the basis of their morphological and cultural characteristics [33].

3. Results

The current study about the biodiversity of endophytic fungi of *Withania somnifera* leaves recovered from Panchmarhi biosphere reserve is first in the central

region of Madhya Pradesh. A total of 200 segments of leaves incubated; 49 isolates were observed which belonged to 07 genera. Amongst the biodiversity of endophytic fungi Hyphomycetes and Sterile mycelium were recovered whereas, Ascomycetes and Coelomycetes were absent. Hyphomycetes showed maximum dominance with (95.92%) possessing *Alternaria alternata* with highest number of recovered isolates (13 nos), colonization frequency (6.5%) and dominance (26.53%) followed by *Aspergillus flavus* with 12 isolates and C.F of 6% and dominance (24.49%) and *Aspergillus niger* showed 10 isolates with 5% colonization frequency and 20.41% dominance. 4 isolates with colonization frequency 2% each of *Cladosporium cladosporioides*, *Fusarium moniliforme* and *Penicillium* sp. were recovered with dominance (8.16%) and Sterile mycelium was 4.08% dominant respectively. The overall colonization frequency of the surface sterilized tissues was 24.5%. it was also observed that older leaves samples produced maximum isolates than the younger leaves samples (Table 1).

Endophytic organisms have received considerable attention as they are found to protect their host against pest, pathogens and even domestic herbivorous [11]. Only a few plants have been investigated for their endophytic flora and their potential to produce bioactive compounds. Some studies had been conducted about the endophytic biodiversity, taxonomy, reproduction, host ecology and their effects on host and sufficient evidence that endophytic fungi play an important role in host plant physiology[34][35][36][37]. The occurren ce of endophytes seems to be influenced by environment and type of host tissue and is mainly influenced by seasonal variation [38][39].

There is a need to study the biodiversity of endophytic fungi in the tropical region where the climatic conditions remain extreme to high and annual rain-fall is more than 15 mm. Since no information about the endophytic biodiversity in Panchmarhi biosphere reserve is available, the present work was initiated to discover endophytic fungal population in widely used medicinal plant *Withania somnifera*. Our study is quite similar to Suryanarayanan *et al*., (2003) who reported about the endophyte biodiversity in two dry tropical forests of the Nilgiri Biosphere Reserve in India [23].

In the tropics, only a few studies have been carried out on endophytes of tree species [40]. Diverse endophytic population was detected to colonize *Withania somnifera* plant. Previously, 20 species of 12 genera were reported with dominance of *Alternaria alternata* (25%) and *Penicillium* sp. (7.14%) and *Cladosporium sp., Acremonium sp., Paecilomyces sp., Aspergillus sp., Curvularia sp., Penicillium sp.* and mycelia sterilia from leaf sample of *Withania somnifera* [41][42]. In the present study overall colonization frequency was determined 24.5% in surface sterilized tissues whereas, in contrast to Petrini, (1986), Dayle *et al*., (2001) and Khan *et al.,* (2010) colonization frequency was higher but, large number of genera and species are reported as endophytic fungi which live symbiotically with plants [34][41][43]. The isolated fungi in *Withania somnifera* belonged to the class Hyphomycetes and Deuteromycetes and were found to be the most prevalent i.e. *Alternaria alternata, Aspergillus flavus* and *Aspergillus niger* were the most dominant which is similar to the findings of Khan *et al.,* (2010) where Deuteromycetes showed dominance [41].

Table 1. Biodiversity of *Withania somnifera* leaves.

Endophytic fungi	No. of endophytes	Colonization frequency (%)	Dominance (%)	Total (%)
Ascomycetes				
No Isolates	**-**	**-**	**-**	**Absent**
Coelomycetes				
No Isolates	**-**	**-**	**-**	**Absent**
Hyphomycetes				
Alternaria alternata	13	6.5	**26.53**	**95.92**
Aspergillus flavus	12	6	**24.49**	
Aspergillus niger	10	5	**20.41**	
Cladosporium cladosporioides	4	2	8.16	
Fusarium moniliforme	4	2	8.16	
Penicillium sp.	4	2	8.16	
Sterile mycelia	2	1	4.08	**4.08**
Total No. of isolates	**49**	**24.5**		

**Based on 200 segments for frequency analysis.

Attempts had been made to isolate pharmaceutical substances from plants and their endophytic fungi, as endophytes are considered as unexplored source of bioactive natural compounds [44]. Studies had been carried out on endophytic fungi to screen them for antibiotics, anti-tubercular, antiviral and anticancer, antioxidants, insecticidal and immunomodulatory compounds [45]. Currently, the recovered endophytic fungi isolates from *Withania somnifera* is being investigated to obtain the secondary metabolites to facilitate screening against therapeutic targets.

4. Conclusion

In the study the colonization frequency and dominance of hyphomycetes was observed. The ascomycetes and coelomycetes were absent which might be due to the environmental factors prevailing in the region during collection of samples. A lesser diversity was obtained due to the absence of other genus or the antipathy to hyphomycetes.

Acknowledgment

The authors are thankful to Shri S. N. Vijaywargia and Ms. Megha Vijaywargia, Director, People's University, Bhopal for granting financial assistance to carry out the present research work.

References

1. Arnold E, Maynard Z, Gilbert GS, Coley PD, Kursar TA: Are tropical fungal endophytes hyperdiverse? Ecology Letters 2000; 3: 267-74.
2. Sturz AV, Christie BR, Nowak J. Bacterial endophytes: potential role in developing sustainable systems of crop production. Critical Review of Plant Sciences 2000; 19: 1-30.
3. Zhang HW, Song YC, Tan RX. Biology and chemistry of endophytes. Natural Product Reports 2006; 23: 753-71.

4. Smith CS, Chand T, Harris RF, Andrews JH. Colonization of a submersed aquatic plant, Eurasian water mildfoil (*Myriophyllum spicatum*), by fungi under controlled conditions. Applied Environmental Microbiology 1989; 55(9): 2326-32.
5. Stanley SJ. Observations on the seasonal occurrence of marine endophytic and parasitic fungi. Canadian Journal of Botany 1992; 70: 2089-96.
6. Petrini O, Fisher PJ, Petrini LE. Fungal endophyte of bracken (*Pteridium aquilinum*), with some reflections on their use in biological control. Sydowia 1992; 44: 282-93.
7. Raviraja NS, Sridhar KR, Bärlocher F. Endophytic aquatic hyphomycetes of roots of plantation crops and ferns from India. Sydowia 1996; 48: 152-60.
8. De Bary, A. Morphologie und Physiologie der Pilze, Flechten, und Myxomyceten. 1866; Vol. II. Hofmeister's Handbook of Physiological Botany. Leipzig, Germany.
9. Bills G, Dombrowski A, Pelaez F, Polishook J, An Z. Recent and future discoveries of pharmacologically active metabolites from tropical fungi. 2002; p. 165-94. In R. Watling, J. C. Frankland, A. M. Ainsworth, S. Issac, and C. H. Robinson. (ed.), Tropical Mycology: Micromycetes, 2. CABI Publishing, New York, N.Y.
10. Li R, Tian S, Liu X, Chen L, Guo K, Che Y. Pestalotheols A-D, bioactive metabolites from the plant endophytic fungus *Pestalotiopsis theae*. Journal of Natural Product 2008; 71(4): 664-68.
11. Weber J. A natural control of Dutch elm disease. Nature, London, 1981; 292: 449-51.
12. Azevedo, J.L.; Pereira, J.O. and Araújo, W.L. Endophytic microorganisms: a review on insect control and recent advances on tropical plants. Electronic Journal of Biotechnology 2000; 3(1): 40-65.
13. Strobel GA. Endophytes as source of bioactive products. Clinical Microbiology and Infection 2003; 5: 535-44.
14. Yang H, Shi G, Dou QP. The tumor proteasome is a primary target for the natural anticancer compound Withaferin A isolated from Indian Winter Cherry. Molecular Pharmacology 2007; 71: 426-37.
15. Rasool M, Varalakshmi P. Immunomodulatory role of *Withania somnifera* root powder on experimental induced inflammation: An *In vivo* and *In vitro* study. Vascular Pharmacology 2006; 44: 406-410.
16. Knight V, Sanglier JJ, DiTullio D, Braccili S, Bonner P, Waters J, Hughes D, Zhang L. Diversifying microbial natural products for drug discovery. Applied Microbial Biotechnology 2003; 62: 446-58.
17. Malinowski DP, Zuo H, Belesky DP, Alloush GA. Evidence for copper binding by extracellular root exudates of tall fescue but not perennial ryegradd infected with *Neotyphodium* spp., Endophytes. Plant Soil 2004; 267: 1-12.
18. Malinowski DP, Belesky DP. Ecological importance of *Neotyphodium* spp. Grass endophytes in agroecosystems. Grassland Science 2006; 52(1): 23-28.
19. Arnold AE, Engelbrecht BMJ: Fungal endophytes nearly double minimum leaf conductance in seedlings of a neotropical tree species. Journal of Tropical Ecology 2007; 23: 369-72.
20. Hata K, Futai K, Tsuda M. Seasonal and needle age-dependent changes of the endophytic mycobiota in *Pinus thunbergii* and *Pinus densiflora* needles. Canadian Journal of Botany 1998; 76: 245-50.
21. Mittermeier RA, Meyers N, Gil PR, Mittermeier CG. Hotspots: Earth's biologically richest and most endangered ecoregions. Toppan Printing Co., Tokyo, Japan 1999.
22. Hawksworth DL. The magnitude of fungal diversity: the 1.5 million species estimate revisited. Mycology Research 2001; 105: 1422-31.
23. Suryanarayanan TS, Venkatesan G, Murali TS. Endophytic fungal communities in leaves of tropical forest trees: Diversity and distribution patterns. Current Science 2003; 85(4): 489-92.
24. Hyde KD, Soytong K. The fungal endophyte dilemma. Fungal Diversity 2008; 33: 163-73.

25. Environmental Planning and Coordination Organization (EPCO) Biosphere Reserve Information Services (BRIS), 2002; 2(2), ed. Panchmarhi Biosphere Reserve.
26. Tenguria RK, Khan FN. Distribution of endophytic fungi in leaves of *Azadirachta indica* A. Juss. (Neem) of Panchmarhi Biosphere Reserve. Current Botany 2011; 2(2): 27-29.
27. Tenguria RK, Khan FN, Quereshi S. Endophytes-Mines of pharmacological therapeutics. World Journal of Science and Technology 2011; 1: 127-49.
28. Strobel GA. Microbial gifts from rain forests. Canadian Journal of Plant Pathology 2002; 24:14-20.
29. Schulz B, Boyle C. The endophytic continuum: Review. Mycology Research 2005; 109(6): 661-98.
30. Fisher PJ, Petrini O. Tissue specificity by fungi endophytic in *Ulex europaeus*. Sydowia 1987; 40: 46-50.
31. Kumaresan V, Suryanarayanan TS. Endophytes assemblages in young mature and senescent leaves of *Rhizophora apiculata*: evidence for the role of endophytes in mangrove litter degradation. Fungal Diversity 2002; 9: 81-91.
32. Suryanarayanan TS, Murali T, Venkatesan G. Occurrence and distribution of fungal endophytes in tropical forests across a rainfall gradient. Canadian Journal of Botany 2002; 80: 818-26.
33. Domsch KH, Gamas W, Anderson TH. Compendium of Soil Fungi, Vol. **I**. Academic Press, New York. 1980: p. 205.
34. Petrini O. Taxonomy of endophytic fungi of aerial plant tissues. In: *Microbiology of the phylosphere*. (Ed.): N.J. Fokkenna, J. Van Den Heuvel. Cambridge University Press, Cambridge. 1986. pp. 175-187.
35. Redman RS. Sheehan KG. Rodriguez RJ. Henson JM. Thermotolerance generated by plant/ fungal symbiosis. Science 2002; 298: 1581-82.
36. Clay K, Schardl C. Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. American Nature 2002; 160: S99-S127.
37. Faeth SH, Helander ML, Saikkonen KJT. Asexual Neotyphodium endophytes in a native grass reduce competitive abilities. Ecology Letters 2004; 7: 304-13.
38. Rodrigues KF. The foliar fungal endophytes of the Amazonian palm *Euterpe oleracea*. Mycologia, 1994; 86: 376-85.
39. Halmschlager E, Butin H, Donaubauer E. Endophytische Pilze in Bl ttern und Zweigen von *Quercus petraea*. Europian Journal of Pathology 1993; 23: 51-63.
40. Fröhlich J, Hyde KD. Biodiversity of palm fungi in the tropics: are global fungal diversity estimatesrealistic? Biodiversity and Conservation 1999; 8: 977-1004.
41. Khan R, Shahzad S, Choudhary MI, Khan SA, Ahmad A. Communities of endophytic fungi in medicinal plant *Withania somnifera.* Pakistan Journal of Botany 2010; 42(2): 1281-87.
42. Madki MA, Manzoor AS, Powar PV, Patil KS. Isolation and biological activity of endophytic fungi from *Withania somnifera*. International Journal of Pharmaceutical Sciences 2010; 2(3): 848-58. ISSN 0975-4725.
43. Dayle ES, Polans NO, Paul DS, Melvin RD. Angiosperm DNA contamination by endophytic fungi: Detection and methods of avoidance. Plant Molecular Biology Report, 2001; 19: 249-60.
44. Strobel G, Daisy B, Castillo U, Harper JJ. Natural product from endophytic microorganisms. Natural Products 2004; 67: 257-68.
45. Tan RX, Zou WX. Endophytes: a rich source of functional metabolites. Natural Products Report 2001; 18: 448-59.