

COMMUNITIES OF ENDOPHYTIC FUNGI IN MEDICINAL PLANT *WITHANIA SOMNIFERA*

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Abstract

Withania somnifera (L.) Dunal is known to possess medicinal properties. Medicinal plants harbour endophytic mycoflora. Only a few plants have been studied for their endophyte biodiversity and their potential to produce bioactive secondary metabolites. There is a need to understand the biodiversity of endophytic fungi and their potential of producing novel compounds of medicinal importance.

A total of 643 segments (202 leaf, 391 stem, and 50 root samples) from 20 different plants were screened for their endophytic mycoflora. Thirty-three fungal strains of 24 species have been isolated, four belonged to the class Ascomycetes and 20 to class Deuteromycetes. The highest species richness as well as frequency of colonization was in stem; with the exception of *Aspergillus niger*, *A. terreus* and *A. alternata*, all the other fungi were found to be organ-specific. In this study most dominant endophyte was found to be *A. alternata*. Overall colonization frequency was measured as 14.15%.

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 then to determine their medicinal properties. The present work was therefore initiated to study the endophytic fungal population in *Withania somnifera* (L.) Dunal., a commonly used medicinal plant in the subcontinent.

Introduction

Endophytes are microbes which colonize living, internal tissues of plants without causing any harm to their host (Bacon & White, 2000). All vascular plants harbour endophytic organisms (Zhang *et al.*, 2006). These endophytes protect their hosts from infectious agents and adverse conditions by secreting bioactive secondary metabolites (Azevedo *et al.*, 2000; Carroll & Carroll, 1978; Strobel, 2003). The endophytic fungi play important physiological (Malinowaki *et al.*, 2004) and ecological (Tintjer & Rudger, 2006; Malinowski & Belesky, 2006) roles in their host life. The ubiquity of these symbiotic microorganisms is clear, but diversity, host-range, and geographical distributions are unknown (Arnold & Engelbrecht, 2007). Endophytes are now considered as an important component of biodiversity. The distribution of endophytic mycoflora differs with the host.

Medicinal plants are known to harbour endophytic fungi that are believed to be associated with the production of pharmaceutical products (Zhang *et al.*, 2006). Therefore, it is important to explore endophytic mycoflora in the medicinal plants. In the present study, *Withania somnifera*, an important medicinal plant, was investigated for the presence of endophytic community.

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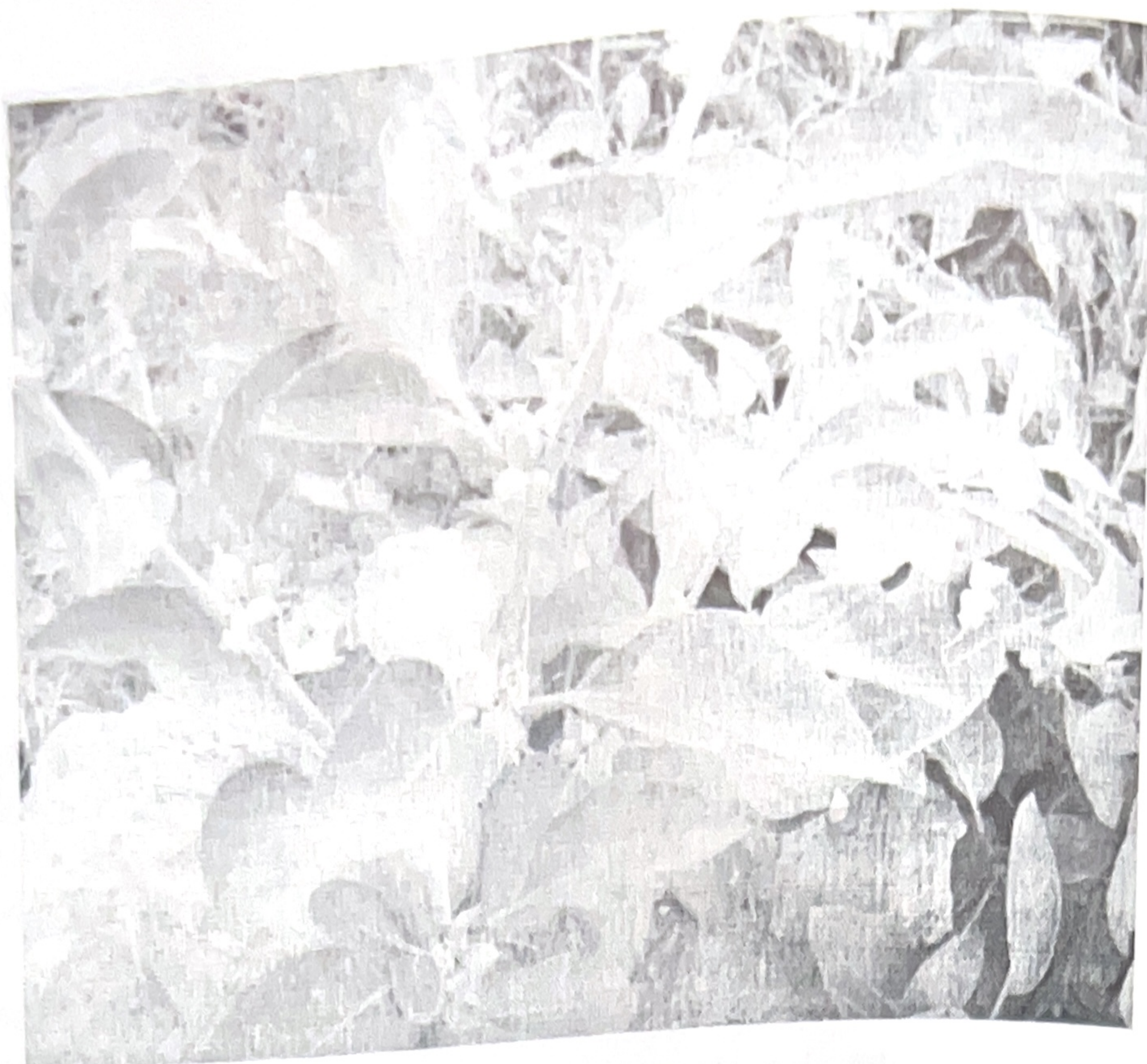


Fig. 1. *Withania somnifera* (L.) Dunal.

Withania somnifera is an erect, evergreen, tomentose shrub, 30-150 cm in height, with stout and fleshy tap-roots. The leaves are simple, ovate and glabrous; with those in the floral region being smaller and opposite, 5-10 cm long, 2.5 – 07 cm wide, with a broad base and narrow apex. The flowers are inconspicuous, borne in axillaries or umbellate cymes. Orange red berries clothed in persistent calyx, bear many yellow seeds. There are about 600 seeds per gram. The plant flowers and fruits all the year round and is propagated from seeds.

Withania somnifera is an important tropical medicinal plant belongs to the family Solanaceae (Yang *et al.*, 2007). It is known as Indian Ginseng for its wide range of therapeutic uses in ayurvedic and other traditional systems of medicine. More than 91 pharmaceutical products are produced from this plant (Rai *et al.*, 2001). Wide range of activity including anticancer, antistress, anti-inflammatory, antitumor, antibiotic, anticonvulsant, CNS depressant, hepatoprotective, immunomodulatory, insect antifeedant properties are reported (Rasool & Varalakshmi, 2006; Scartezzini & Sparoni, 2000; Agarwal *et al.*, 1999).

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 on rise (Knight *et al.*, 2003). Taxol, a potent anticancer drug, is one such example, produced by *Taxus brevifolia* plant and endophytic fungi, including *Taxomyces andreanae* and several other fungi (Strobel *et al.*, 1996; Strobel, 2003; Gangadevi & Muthumary, 2007).

Endophytes are mostly unexplored group of microorganisms, but a few studies show them as a huge source of medicinal compounds. Dreyfuss & Chapela (1994) estimated that there may be one million species of endophytic fungi alone. There is a lack of information about endophytic diversity in this region. There was an immense need to understand the biodiversity of endophytic fungi in this semiarid region where the climatic conditions remain extreme to high and annual rain-fall is less than 15 mm, most of the time in a year. The present study was conducted to determine the diversity of endophytic mycoflora of *Withania somnifera* in this geographical region.

Sampling: *Withania somnifera* (L.) Dunal was collected from Karachi (latitude 24° 56' 27.88" N and longitude 67° 7' 19.67" E), Pakistan. A sample specimen was deposited in the Karachi University Herbarium (Specimen # CH 67975). The plant was identified by Prof. Dr. Surayya Khatoon (Taxonomist), Department of Botany, University of Karachi, Karachi, Pakistan.

Duration of the study was April 2003–April 2005. Plants with no visible symptoms of disease were carefully selected after physical examination. The plant material was brought to the laboratory in sterile bags and processed within hours after sampling.

Surface sterilization and incubation: Isolation of endophytic fungi was done according to the method described by Petrini (1986). The plants samples were rinsed gently in running water to remove dust and debris. Leaf samples were cut into 3–4 x 0.5–1 cm pieces with and without midrib; stems and roots samples were cut into 0.5–1.0 cm long pieces. Each sample was disinfected with 75% ethanol for 1 min followed by immersion in Sodium hypochlorite (NaOCl 1–13% for 3–10 minutes, depending on the type of samples) and then once again in 75% ethanol for 30 seconds. The segments were then rinsed three times in sterile distilled water and the pieces were blotted-dry on sterile blotting paper. The efficiency of surface sterilization procedure was ascertained for every segment of tissues as per method of Schulz *et al.*, (1993). Isolation of fungi from leaves, stems, and roots pieces were made separately. About 5–6 segments were placed on Potato dextrose agar (PDA) supplemented with penicillin G (100 units ml⁻¹) and streptomycin (100 µg ml⁻¹). The dishes were sealed with parafilm and incubated at 27±2°C for 4–6 weeks. Most of the fungal growth was initiated within 10 days of inoculation. The fungi that grew out from the segments were periodically isolated and identified by transferring the hyphal tips to fresh PDA plates without antibiotics.

Colonization Frequency (CF) was calculated as described by Suryanarayanan *et al.*, (2003). Briefly, proper time of incubation was given for CF counting.

$$\text{Colonization frequency of endophyte} = \frac{\text{Number of segments colonized by fungi}}{\text{Total number of segment observed}} \times 100$$

Fungi were grown on specified media under specified culture condition, for identification. The fungi were identified on the basis of their morphological and cultural characteristics (Domsch *et al.*, 1980; Ellis, 1971; Kenneth *et al.*, 1965; Sutton, 1980).

Results

The present study is the first one about the endophytic flora of *Withania somnifera*, a medicinal plant found in this region. A total of 643 segments including 202 leaf, 391 stem and 50 root segments obtained from 20 different plants were screened for the presence of endophytic fungi. Twenty species belonging to 12 genera of fungi were isolated during the present studies. It included 9 fungi from leaves, 20 from stems and 4 from roots (Table 1). All the isolated fungal species were submitted to the Karachi University Culture Collection (KUCC) bank. Among the isolated fungi, four belonged to the class Ascomycetes and 20 to class Deuteromycetes. Most dominant endophyte was *Alternaria alternata* which is not organ-specific. It has been isolated from leaf and stem tissues. It was isolated five times from four different plants at different times (i.e. April, May, and September). Overall colonization frequency was determined 14.15% of surface sterilized tissues.

Table 1. Frequency of Colonization of Endophytic Fungi Isolated from *Withania somnifera*.

S. No.	Fungi	KUCC number	% Frequency of colonization			Plants***
			Leaf	Stem	Root	
	Ascomycota	167	-	16.66	-	
1.	<i>Chaetomium bostrycodes</i>	88	15.47	-	-	1
2.	<i>Eurotium rubrum</i>	365	-	-	10	2
3.	<i>Melanospora fusispora</i>	101	-	21.05	-	1
4.	Unidentified*					1
	Deuteromycota	122	16.66	-	-	
1.	<i>Aspergillus awamori</i>	131	-	10	-	1
2.	<i>Aspergillus auricomus</i>	95	-	10	-	1
3.	<i>Aspergillus flavus</i>	201	-	30	42.85	1
4.	<i>Aspergillus niger</i>	399	-	8.33	-	2
5.	<i>Aspergillus pulvinus</i>	139	-	6.66	66.66	1
6.	<i>Aspergillus terreus</i>	322	-	10	-	2
7.	<i>Aspergillus terreus</i> var. <i>aureus</i>	357	-	5.5	-	1
8.	<i>Aspergillus terricola</i>	275	-	25	-	1
9.	<i>Aspergillus thomii</i>	735	25	5.26	-	1
10.	<i>Alternaria alternata</i>	156	-	20.12	-	5
11.	<i>Cladosporium cladosporioides</i>	142	-	-	16.66	1
12.	<i>Curvularia oryzae</i>	102	-	11	-	1
13.	<i>Drechslera australiensis</i>	326	-	5.26	-	1
14.	<i>Fusarium moniliforme</i>	308	-	25	-	1
15.	<i>Fusarium semitectum</i>	167	-	25	-	1
16.	<i>Myrothecium roridum</i>	107	-	5.26	-	1
17.	<i>Penicillium corylophilum</i>	112	7.14	-	-	1
18.	<i>Penicillium</i> sp.	99	-	5.26	-	1
19.	<i>Phoma</i> sp.	100	7	-	-	1
20.	Unidentified**					1

* This fungus was placed in Ascomycota as it produced ascocarps and asci.

** No perfect stage was seen in this fungus so it was placed in the Deuteromycota.

***Numbers of plants where a particular fungi was isolated

Discussion

Endophytic organisms have received considerable attention as they are found to protect their host against pest, pathogens and even domestic herbivorous (Weber, 1981). Only a few plants have been investigated for their endophytic flora and their potential to produce bioactive compounds. Some studies have been conducted about the endophytic biodiversity, taxonomy, reproduction, host ecology and their effects on host (Faeth *et al.*, 2004; Petrini, 1986; Dayle *et al.*, 2001; Redman *et al.*, 2002; Clay & Schardl, 2002). Currently, endophytes are considered as unexplored source of bioactive natural compounds. They have been found to play a crucial role in the production of beneficial chemical compounds. Taxol, an antifungal and anticancer compound, is one such example. It was found to be a product of endophytic fungi that colonizes on *Taxus brevifolia* and other plants (Strobel *et al.*, 1996; Strobel, 2003; Gangadevi & Muthumary, 2007).

There is a need to study the biodiversity of endophytic fungi in the semiarid regions where the climatic conditions remain extreme to high and annual rain-fall is less than 15 mm. Since no information about the endophytic biodiversity in Pakistan is available, the present work was initiated to find out endophytic fungal population in widely used medicinal plant, *Withania somnifera*.

Diverse endophytic population was detected to colonize this plant. Thirty-three fungal strains of 24 different species were isolated. The endophytic fungal communities found at three different sites were different. Almost all the isolates were recovered from older plant samples than younger ones. The highest species richness, as well as frequency of colonization was found in stems. With the exception of *Aspergillus niger*, *Aspergillus terreus*, and *Alternaria alternata*, all the fungi were found to be organ-specific. However, *Alternaria alternata* was found in both leaf and stem, while *Aspergillus terreus*, and *Aspergillus niger* were isolated from roots and stems. In this study, most dominant endophyte was found to be *Alternaria alternata*. Overall colonization frequency was determined as 14.15% in surface sterilized tissues.

In most of the cases, Ascomycetes, Deuteromycetes and Basidiomycetes are reported as endophytic fungi (Petrini, 1986; Dayle *et al.*, 2001). A large number of genera and species of fungi, belonging to first two classes, are able to live endophytically in plants. In the present study, the isolated fungi belonged to the class Ascomycetes and Deuteromycetes. Fungi of class Deuteromycetes were found to be the most prevalent where 20 out of 24 species were Deuteromycetes. Among the endophytic fungal population, *Alternaria alternata* was the most dominant endophyte in *Withania somnifera*.

Previously, this fungus was reported as a common endophyte to other plant species like *Triticum aestivum*, *Zea mays* (Larran *et al.*, 2002; Fisher *et al.*, 1992; Zhang *et al.*, 2006). *Alternaria alternata* in *Withania somnifera* was not organ-specific. This fungus was isolated from stems and leaves. But frequency of colonization was higher in the leaves (25.57%) compared to stem (5.26%).

There is sufficient evidence that endophytic fungi play an important role in host-plant physiology. They receive nutrition, protection and propagation opportunities from their hosts (Clay & Schardl, 2002; Thrower & Lewis, 1973), while host plants are also benefited from this symbiosis. Endophytes provide protection to their hosts from insects, pests, and herbivore, and help their hosts to adapt in different stress conditions (Knop *et al.*, 2007; Clay, 2005; Clay & Schardl, 2002; Malinowski *et al.*, 2006). However, endophytes also act as opportunistic microorganisms under some conditions (Saikkonen *et al.*, 1998; Faeth *et al.*, 2004).

Attempts are being made to isolate and identify bioactive metabolites from endophytic fungi (Strobel *et al.*, 2004). Studies were also carried out on endophytic fungi to screen them for antibiotics, antiviral and anticancer, antioxidants, insecticidal and immunomodulatory compounds (Tan & Zou, 2001). The endophytic fungi isolated from *W. somnifera* will also be investigated for potential bioactive compounds, in future studies.

In the present study, 24 different fungal species were isolated from *Withania somnifera*. Our results are quite similar to the finding made by Suryanarayanan *et al.*, (2003) who reported about the endophyte biodiversity in two dry tropical forests of the Nilgiri Biosphere Reserve in India. Amongst the 24 plant species, the lowest number of fungal diversity was 10 in *Gmelina arborea* Roxb., and the highest number was 26 in *Shorea roxburghii* G. Don.

Attempts have also been made to isolate pharmaceutical substances from plants and their endophytic fungi, as endophytes are considered to be rich source of novel compounds (Strobel *et al.*, 2004).

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References

- Agarwal, R., S. Diwanay, P. Patki and B. Patwardhan. 1999. Studies on immunomodulatory activity of *Withania somnifera* (Ashwagandha) extracts in experimental immune inflammation. *J. Ethnopharmacol.* 67: 27-35.
- Arnold, A.E. and B.M.J. Engelbrecht. 2007. Fungal endophytes nearly double minimum leaf conductance in seedlings of a neotropical tree species. *Journal of Tropical Ecology*, 23: 369-372.
- Azevedo, J.L., J.O.P. eireira and W.L. Araújo. 2000. Endophytic microorganisms: a review on insect control and recent advances on tropical plants. *Electronic Journal of Biotechnology*, 3(1): 40-65.
- Bacon, C.W. and J.F. White. 2000. *An overview of endophytic microbes: Endophytism Definition, Microbial Endophytes*, Marcel Dekker, New York, pp. 3-5.
- Carroll, G.C. and F.E. Carroll. 1978. Studies on the incidence of coniferous needle endophytes in the Pacific Northwest. *Canadian Journal of Botany*, 56: 3032-3043.
- Clay, K. and C. Schardl. 2002. Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. *Am. Nat.*, 160: S99-S127.
- Clay, K., C. Fuqua, C. Lively and M.J. Wade. 2005. Microbial community ecology of tick-borne human pathogens. In: *Disease Ecology: Community Structure and Pathogen Dynamics*, (Ed.): S.K. Collinge and C. Ray. Oxford University Press, Oxford, pp. 41-57.
- Dayle, E.S., N.O. Polans, D.S. Paul and R.D. Melvin. 2001. Angiosperm DNA contamination by endophytic fungi: Detection and methods of avoidance. *Plant Molecular Biology Report*, 19: 249-260.
- Domsch, K.H., W. Gamas and T.H. Anderson. 1980. *Compendium of Soil Fungi*, Academic press, New York. Vol. 1: pp. 168-169, 540, 559-560.
- Dreyfuss, M.M. and I.H. Chapela. 1994. Potential of fungi in the discovery of novel, low-molecular weight pharmaceuticals. In: *The discovery of natural products with therapeutic potential*, (Ed.): V.P. Gullo. Butter-worth-Heinemann, London, United Kingdom. pp. 49-80.
- Ellis, M.B. 1971. *Dematiaceous Hypomycetes*. Commonwealth Mycology Institute, Kew, Surrey, England. pp. 319, 413-414, 465-466, 555-556.
- Faeth, S.H., M.L. Helander and K.J.T. Saikkonen. 2004. Asexual Neotyphodium endophytes in a native grass reduce competitive abilities. *Ecology Letters*, 7: 304-313.
- Fisher, P.J., O. Petrini and H.M. Lappin-Scott. 1992. The distribution of some fungal and bacterial endophytes in maize (*Zea mays* L.). *New Phytopathology*, 122(2): 299-305.
- Gangadevi, V. and J. Muthumary. 2007. Preliminary studies on cytotoxic effect of fungal taxol on cancer cell lines. *African Journal of Biotechnology*, 6(12):1382-86.
- Kenneth, B.R., I.F. Dorothy and K.C.A. Peter. 1965. *The genus Aspergillus*. The Williams & Wilkins Company, Baltimore. pp. 277-278; 408-411.
- Knight, V., J.J. Sanglier, D. DiTullio, S. Braccili, P. Bonner, J. Waters, D. Hughes and L. Zhang. 2003. Diversifying microbial natural products for drug discovery. *Appl. Microbial. Biotechnol.*, 62: 446-458.
- Knop, M., S. Pacyna, N. Voloshchuk, S. Kjant, C. Müllenborn, U. Steiner, M. Kirchmair, H.W. Scherer and M. Schulz. 2007. *Zea mays*: Benzoxalnone Detoxification under sulfur deficiency conditions- A complex allelopathic alliance including endophytic *Fusarium verticillioides*. *Journal of Chemical Ecology*, 33(2): 225-237.
- Larran, S., C. Rollán, H.B. Ángeles, H.E. Alippi and M.I. Urrutia. 2002. Endophytic fungi in healthy soybean leaves. *Invest. Agr. Prod. Prot. Veg.*, 17(1): 173-178.

- Malinowski, D.P. and D.P. Belesky. 2006. Ecological importance of *Neotyphodium* spp. Grass endophytes in agroecosystems. *Grassland Science*, 52(1): 23-28.
- Malinowski, D.P., H. Zuo, D.P. Belesky and G.A. Alloush. 2004. Evidence for copper binding by extracellular root exudates of tall fescue but not perennial ryegrass infected with *Neotyphodium* spp., Endophytes. *Plant and Soil*, 267: 1-12.
- Petrini, O. 1986. Taxonomy of endophytic fungi of aerial plant tissues. In: *Microbiology of the phyllosphere*. (Ed.): N.J. Fokkema, J. Van Den Heuvel. Cambridge University Press, Cambridge. pp. 175-187.
- Rai, M., D. Acharya, A. Sing and A. Varma. 2001. Positive growth responses of the medicinal plants *Spilanthes calva* and *Withania somnifera* to inoculation by *Piriformospora indica* in a field trial. *Mycorrhiza*, 11: 123-128.
- Rasool, M. and P. Varalakshmi. 2006. Immunomodulatory role of *Withania somnifera* root powder on experimental induced inflammation: An *In vivo* and *In vitro* study. *Vascular Pharmacology*, 44: 406-410.
- Redman, R.S., K.G. Sheehan, R.J. Rodriguez and J.M. Henson. 2002. Thermotolerance generated by plant/ fungal symbiosis. *Science*, 298: 181.
- Saikkonen, K., S.H. Faeth, M. Helander and T.J. Sullivan. 1998. Fungal endophytes: a continuum of interactions with host plants. *Annual Review of Ecology and Systematics*, 29: 319-343.
- Scartezzini, P. and E. Speroni. 2000. Review on some plants of Indian traditional medicinal with antioxidant activity. *Journal of Ethnopharmacology*, 71(1-2): 23-43.
- Schulz, B., S. Guske, U. Dammann and C. Boyle. 1998. Endophyte-host interactions. II. Defining symbiosis of the endophyte- host interaction. *Symbiosis*, 25: 213-227.
- Schulz, B., S. Wanke, S. Draeger and H.J. Aust. 1993. Endophytes from herbaceous plants and shrubs: effectiveness of surface sterilization methods. *Mycol. Res.*, 97:1447-1450.
- Strobel, G., B. Daisy, U. Castillo and J.J. Harper. 2004. Natural product from endophytic microorganisms. *Nat. Prod.*, 67: 257-268.
- Strobel, G.A. 2003. Endophytes as source of bioactive products. *Microbiol. Infect.*, 5: 535-544.
- Strobel, G.A., H.M. Hess, E. Frod, R.S. Sidhu and X. Yang. 1996. Taxol from fungal endophytes and issue of biodiversity. *Journal of industrial microbiology and biotechnology*, 17(5-6): 417-423.
- Suryanarayanan, T.S., G. Venkatesan and T.S. Murali. 2003. Endophytic fungal communities in leaves of tropical forest trees: Diversity and distribution patterns. *Current Science*, 85(4):489 - 492.
- Sutton, B.C. 1980. *The Coelomycetes, Fungi Imperfecti with Pycnidia, Aceruli and Stromata*. Robert Mac Lechose and Co. Ltd., University of Glasgow. England. pp. 82, 382, 385.
- Tan, R.X. and W.X. Zou. 2001. Endophytes: a rich source of functional metabolites. *Nat. Prod. Rep.*, 18: 448-459.
- Thrower, L.B and D.H. Lewis. 1973. Uptake of sugars by *Epichloë typhina* (Pers. Ex Fr.) Tul. in culture and from its host, *Agrostis stolonifera* L. *New Phytologist*, 72: 501-508.
- Tintjer, T. and A.J. Rudger. 2006. Grass-herbivore interaction altered by strains of a native endophyte. *New Phytologist*, 170: 513-521.
- Weber, J. 1981. A natural control of Dutch elm disease. *Nature*, London, 292: 449-451.
- Yang, H., G. Shi and Q.P. Dou. 2007. The tumor proteasome is a primary target for the natural anticancer compound Withaferin A isolated from Indian Winter Cherry. *Molecular Pharmacology*, 71: 426-437.
- Zhang, H.W., Y.C. Song and R.X. Tan. 2006. Biology and chemistry of endophytes. *Nat. Pro. Rep.*, 23: 753-771.

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