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Full Length Research Paper

Comparative analysis of endophytic mycobiota in different tissues of medicinal plants

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Endophytic mycobiota of leaf Lamina, leaf midrib, petiole and stem of five medicinal plants of Solanaceae, viz., *Solanum nigrum* Linn, *Solanum surratense* Burn. F., *Solanum torvum* Sw., *Solanum trilobatum* Linn., and *Withania somnifera* Dunal, were screened for their endophytic fungal assemblages. A total of 1500 isolates were obtained from the 6,000 segments screened. Among the isolates 645 were sterile forms. The remaining isolates were classified into 30 species belonging to 16 genera. The species richness was the greatest in *S. torvum* (21) followed by *W. somnifera* (16), *S. nigrum* (14), *S. surratense* and *S. trilobatum* (13) each. The Endophytic Infection Rates (EIR %) revealed that 30.5% of the tissues were infected by the endophytic fungi in *S. surratense* followed by 29% in *S. torvum,* 23.25% in *S. nigrum,* 22.41% in *S. trilobatum,* and 22.16% in *Withania somnifera*. This is the first report on endophytic fungal populations from these medicinal plants of Solanaceae members.

Key words: Endophytic fungi, colonization frequency, endophytic infection rates, medicinal plants, Solanaceae.

INTRODUCTION

Endophytic fungi are said to be found in almost all plants. These include trees, grasses, algae and other herbaceous plants. Under normal circumstances they live within the host plant without causing any noticeable symptom of disease (Konig, 1999). The endophytic fungi are not considered as sapropytes since they are associated with living tissues, and may in some way contribute to the well being of the plant. Presumably, the plant is thought to provide nutrients to the fungi, while the fungi may produce factors that protect the host plant from attack by animals, insects or microbes (Yang et al., 1999). Investigations related to endophytic micro-organisms isolated from several plants and their tropical hosts have recently increased, due to the increasing importance of fungi in biological control and the ongoing search for pharmacologically active compounds (Azevedo et al., 2000; Breen, 1994; Pereira et al., 1993, 1999; Pinto et al., 2000; Rodrigues, 1994). The delicate equilibrium between the host and the endophytic fungus seems to be controlled in part by chemical factors, for example, herbicidal natural products produced by the fungi versus antifunglal metabolities biosynthesized by the host plant. Meanwhile, there are also documents demonstrating that many antitumor agents, such as taxol could be produced by endophytic fungi (Strobel et al., 1996; Wang et al., 2000). In the present study the endophytic mycobiota of leaf lamina, leaf midrib, petiole and stem of five medicinal plants of Solanaceae, viz., Solanum nigrum Linn., S. surratense Burn. F., S. torvum Sw., S. trilobattum Linn and Withania somnifera Dunal, collected from Botany Field Research and Herbal Science Center, Maduravoyal, University of Madras, Chennai, Tamil Nadu, India, were screened for their endophytic fungal assemblages. This is the first report on endophytic fungal populations from these medicinal plants of Solanaceae members.

MATERIALS AND METHODS

To acquire endophytic fungi the host plants were collected in sterile polythene or paper bags, from their natural habitat. The samples

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were brought to the laboratory and processed within 24 h (Fisher and Petrini, 1987).

Isolation of endophytic fungi

The collected samples were first washed thoroughly in running tap water. The foliage (leaf lamina and leaf midrib) tissues were first surface sterilized in 70% Ethanol for 5 s, then immersed in 4% Sodium hypochlorite for 90 s, rinsed in sterile distilled water and then dried in a sterilized filter paper, (modified method of Fisher et al*.,* 1993). For the petiole and stem, the tissues were first surface sterilized in 75% Ethanol for 60 s, immersed in 4% Sodium hyphochlorite for 180 s, rinsed in 75 % Ethanol for 30 sec, rinsed in sterilized distilled water and dried in sterilized filter paper (modified method of Dobranic et al*.,* 1995). The surface sterilized tissues (viz., leaf lamina, leaf midrib, petiole and stem) were cut into segments of 1cm^2 (from each plant species 1200 segments of 1 cm^2 (300 segments each from leaf lamina, leaf midrib, petiole and stem) were taken, and plated out on PDA medium amended with Streptopenicillin (150 mgL^{-1}) / Ampicillin (150 mgL^{-1}). Ten tissue segments were plated in each Petridish which was sealed with Parafilm™ and incubated in a light chamber at 26 ± 1℃ for a period of 3 to 4 weeks (Bills and Polishook, 1992). The light regime was 12 h bright light followed by 12 h darkness from two Philips "cool white" (6500 k) fluorescent lamps providing energy of 50 $\mu E/m^2$ / s. A Philips 100w comptalax lamp served as the source of incandescent light. The efficacy of the sterilization procedure was ascertained with method of Schultz et al. (1998). The fungi that grow out from the segments were isolated and identified down to the species level. To prevent the rapidly growing fungi from inhibiting the slow growing species the former were removed with the help of sterile scalpel (Bills, 1996). Those fungi that failed to sporulate were categorized as "Sterile forms" based on the culture characteristics, such as colony surface, texture and hyphal pigmentation.

Data analysis

Frequency of Occurrence (%) of an endophyte species was determined using the method of Fisher and Petrini (1987) and was equal to the number of segments colonized by a single endophyte divided by the total number segments observed times 100. Relative Percentage Occurrence of different groups of fungi (viz. Coelomycetes, Hyphomycetes, Ascomycetes and other fungi) was calculated by dividing the number of segments colonized by a group of fungi by the total number of segments colonized by all groups of fungi. Endophytic Infection Rates (EIR%) were calculated by the number of infected segments divided by the total number of segments screened (Petrini and Carroll, 1980).

RESULTS

A synopsis of the Frequency of Occurrence (%) of endophytic mycobiota of leaf lamina, leaf midrib, petiole and stem of five medicinal plants belonging to the family Solanaceae is given in Table 1a and b. Altogether 6,000 segments were screened for the presence of endophytic mycobiota. A total of 1500 isolates were obtained. Among the isolates 645 were strile forms. The remaining isolates were classified into 30 species of endophytic fungi belonging to 16 genera. The overall Frequency of Occurrence (%) of the tissues of the medicinal plants were higher from petiole followed by stem, leaf midrib and leaf lamina regions.

The species richness was higher in *S. torvum* (21) followed by *W. somnifera* (16), *S. nigrum* (14), *S. surratense* and *S. trilobatum* (13) each. Although "Sterile forms" were dominant in almost all the tissues, the following endophytic fungi viz. *Phomopsis vexans,* was dominant in *S. nigrum* and *S. surratense*; *Phoma medicaginis* in *S. torvum*; *Cladosporium cladosporioides* in *Solanum trilobattum; Lasiodiplodia theobromae* in *S. surratense* and *W. somnifera.*

Alternatia alternata, A. niger, A. fumigatus, C. cladosporioides, Curvularia lunata, Nigrospora sphaerica, Colletotrichum gloeosporioides, C. lindemuthianum, P. vexans and *P. medicaginis* were commonly present as endophytes in all the tissue types, viz., leaf lamina, leaf midrib, petiole and stem. The following endophytic fungi are exclusively present in some specific tissues. The fungi such as *Emericella nidulans* and *Phomopsis archerii* were specific to stem of *W. somnifera; Aureobasidium pullulans, Curvularia tuberculata, Penicillium oxalicum* and *Fusarium moniliforme* were specific to leaf midrib of *W. somnifera, S. torvum* and *Solanumm nigrum.*

The Endophytic Infection Rates (EIR%) revealed that 30.5% of the tissues were infected by the endophytic fungi in *S. surratense* followed by 29% in *S. torvum,* 23.25% in *S. nigrum,* 22.41% in *S. trilobatum* and 22.16% in *Withnia somnifera.* Among the tissues it was observed that the endophytic infection rate was maximum in the Petiole tissues followed by stem, leaf midrib and the lamina tissues (Table 2).

The Relative Percentage Occurrence of different groups of endophytic fungi of leaf lamina, leaf midrib, petiole and stem of five medicinal plants was represented in Figure 1. The overall percentage Contribution of Sterile forms (47%) were maximum to the endophytic assemblages followed by Hyphomycetes (27.35%) and Coelomycetes (25.98%).

DISCUSSION

The fungal taxa identified in the present study from the medicinal plants of Solanaceae have been previously recorded as endophytes in various herbaceous and woody tree host plants (Petrini et al., 1982; Bettuci and Saravey, 1993; Fisher et al*.,* 1993, 1995; Rodrigues, 1994; Menendez et al., 1995; Taylor et al., 1999; Lumyong et al., 2000). Frequency of colonization and species diversity has been found to vary with the tissue type in this present study. This is in agreement with the data obtained from several hosts (Carroll et al., 1977; Petrini and Carroll, 1981; Rodrigues, 1994; Bernstein and Carroll, 1977; Brown et al., 1998; Taylor et al., 1999; Umali et al., 1999). Endophytes in tropical plants are thought to benefit the host plant by enhancing absorption of soil nutrients, such as phosporus, by providing

Table 1a. Frequency of Occurrence (%) of endophytic fungi in the Leaf Lamina and Leaf midrib regions of medicinal plants.

S/n	Species	Frequency of occurrence (%)*									
		S. nig		*S. sur*		*S. tor*		*S. tri*		*W. som*	
		LL	LM	LL	LM	LL	LM	LL	LM	LL	LM
Hyphomycetes											
01.	*Alternaria alternata*	-	-	1.33	0.70	-	-	-	-	-	
02	*Aureobasidium pullulans*	-	-	-	-	-	-	-	-	-	1.33
03.	*Aspergillus flavus*	-	-					-	1.33		
04.	*A. fumigatus*	-	0.66	2.0	1.0	-	-	-	-	-	-
05.	*A. japonicus*	-	-	-	-	-	2.66	-	2.66	-	-
06.	*A. niger*	-	-	-	-	0.66	0.66	-	1.33	-	-
07.	*Cladosporium cladosporioides*	0.33	0.66	-	-	-	2.66	2.66	3.33	2.33	4.33
08.	*Curvularia lunata*	0.33	0.66	2.70	4.0	-	-	-	-	-	-
09.	*C. tuberculata*	-	-	-	-	-	1.0	-	-	-	-
10.	*Fusarium oxysporum*	-	-	4.0	1.0	-	-	-	-	-	-
11.	*F. moniliforme*	-	0.33	-	-	-	-	-	-	-	-
13.	*Nigrospora sphaerica*	-	-	5.33	4.0	1.0	-	-	-	-	-
14.	*Penicillium. oxalicum*	-	-	-	-	-	-	-	-	-	4.33
15.	*P. purpurogenum*	-	1.33	-	-	-	-	-	-	-	-
Coelomycetes											
16.	*Colletotrichum dematium*	0.66	-	-	-	-	-	-	-	-	-
17.	*C. gloeosporioides*	-	-	0.3	0.70	-	0.66	1.0	3.33	-	-
18.	*C lindemuthianum*	-	-	-	-	0.66	3.33	-	-	-	-
19.	*C. truncatum*	-	-	-	-	-	-	-	-	2.33	5.0
20.	*C. falcatum*	-	-	-	0.70	-	-	-	-	-	-
21.	*Pestalotiopsis funerea*	-	4.33	-	-	-	-	-	-	-	-
22.	*P. vexans*	-	6.0	0.33	4.0	-	-	-	-	-	-
23.	*Phoma medicaginis*	-	-	-	-	-	0.66	0.66	2.0	-	-
24.	*Phyllosticta* sp_1	0.33	-	-	-	-	-	-	-	-	-
25.	*Phyllosticta* sp_2	-	-	1.33	-	-	-	-	-	-	-
26.	*Phyllosticta* sp_3	-	-	-	-	-	-	-	-	1.0	2.0
Sterile Morphospecies											
27.	Sterile form $_{01}$	2.33	9.66	-	-	-	-	-	-	-	-
28.	Sterile form $_{02}$	0.66	5.0	-	-	-	-	-	-	-	-
29.	Sterile form $_{03}$	0.33	6.33	-	-	-	-	-	-	-	-
30.	Sterile form $_{04}$	1.33	1.0	-	-	-	-	-	-	-	-

Table 1a. Contd.

31.	Sterile form $_{05}$	-	-	5.66	4.0	-	-	-	-	-	-
32.	Sterile form $_{06}$	-	-	1.33	1.0	-	-	-	-	-	-
33.	Sterile form $_{07}$	-	-	5.70	4.0	-	-	-	-	-	-
34.	Sterile form $_{08}$	-	-	-	-	5.0	4.33	-	-	-	-
35.	Sterile form $_{09}$	-	-	-	-	0.66	1.0	-	-	-	-
36.	Sterile form $_{10}$	-	-	-	-	3.0	3.0	-	-	-	-
37.	Sterile form $_{11}$	-	-	-	-	-	0.66	-	-	-	-
38.	Sterile form $_{12}$	-	-	-	-	-	-	2.33	6.0	-	-
39.	Sterile form$_{13}$	-	-	-	-	-	-	4.33	6.66	-	-
40.	Sterile form $_{14}$	-	-	-	-	-	-	0.66	-	-	-
41.	Sterile form $_{15}$	-	-	-	-	-	-	0.33	-	-	-
42.	Sterile form $_{16}$	-	-	-	-	-	-	-	-	3.66	6.66
43.	Sterile form $_{17}$	-	-	-	-	-	-	-	-	1.0	1.33
44.	Sterile form $_{18}$	-	-	-	-	-	-	-	-	2.0	2.66
45.	Sterile form $_{19}$	-	-	-	-	-	-	-	-	0.66	1.0
46.	Sterile form $_{20}$	-	-	-	-	-	-	-	-	0.33	2.33
	Total Number of Isolates	19	105	88	75	38	74	36	72	40	82

S. nig - Solanum nigrum; S. sur - S. surratense; S. tor - S. torvum; S. tri - Solanum trilobatum; W. som - Withania somnifera LL - Leaf Lamia; LM - Leaf Midrib. * Based on 300 segments screened

protection from insect attack and by possibly inhibiting the development of plant pathogens (Thomson et al., 1986; Lactch, 1993; Stone et al*.,* 2000). There was a little evidence of tissue specificity exhibited by the endophytes isolated in the present study. Differences in endophyte assemblages in the different tissue type (leaf tissues versus petiole and stem) would reflect the tissue preferences of individual dominant taxa. In the present study, *Penicillium oxalicum. P. citrinum* and *P. purpurogenum* were recorded as endophytes in leaf midrib of *W. somnifera;* leaf midrib of *S. nigrum,* stem of *S. torvum* and petiole and stem of *S. nigrum. Penicilium* spp*.* have been commonly recorded as endophytes from leaves and roots of various hosts, viz. *Alnus glutinosa* (L.) Gaetn, *Picea abies* (L.) Karst., *Picea marina* (Micc) and *Sorbus* spp. (Cappellano et al., 1989; Summerbell, 1989; Valla et al., 1989; Holdenrieder and Sieber, 1992).

In the present study, *A. alternata* and *Aureobasidium pullulans* were recorded as endophytes in *S. surratense* and *W. somnifera.* These endophytic fungi were previously considered as ubiquitous genera, (Vujanovic and Brisson, 2002; Kliejunas and Kuz, 1997). *P. vexans, Phoma medicaginis, C. cladosporioides* and *Lasiodiplodia theobromae,* the dominant endophyte in the present study has been routinely isolated as endophytes of leaves from wheat, soyabean and other plants (Siber, 1985; Petrini, 1986; Larren et al., 2002), and from maize (Wellacher, 1991). The results obtained in the present investigation are in almost perfect agreement with reports on endophytes from other hosts in which generally a large number species can be isolated from a given host, but only a few species are present in significant amounts (dominant species) (Petrini et al., 1992). In the present study the endophytic fungi were more concentrated in the petiole, than stem, leaf midrib and the leaf lamina regions, the possible reason could be the nutrient accumulations is higher in petiole when compare to other tissues, whereas in a few tropical hosts the endophytes are more concentrated in the leaf midrib region than in the leaf lamina region (Rodrigues and Samuels, 1990; Brown et al., 1998).

In the present study large numbers of sterile forms were obtained as endophytes.

As suggested by Carroll (1991) simply listing fungal strains as unidentified or sterile forms is not sufficient. Manipulations of growth media and laboratory environment to induce sporulation and

Table 1b. Frequency of Occurrence (%) of endophytic fungi in petiole and stem tissues of medicinal plants.

S/n	Species	Frequency of Occurrence (%)*									
		S. nig		*S. sur*		*S. tor*		*S. tri*		*W. som*	
		Petiole	Stem	Petiole	Stem	Petiole	Stem	Petiole	Stem	Petiole	Stem
Ascomycetes											
01.	*Emericella nidulans*	-	-	-	-	-	0.66	-	-	-	-
Hyphomycetes											
02.	*Alternaria alternata*	-	-	1.33	1.0	-	-	-	-	-	-
03.	*Aspergillus flavus*	-	4.0	-	-	2.33	-	-	-	-	0.33
04.	*A. fumigatus*	1.0	2.66	-	-	0.66	1.33	1.0	-	-	-
05.	*A. japonicus*	-	-	-	-	-	1.33	-	-	-	-
06.	*A. niger*	-	-	-	-	5.33	4.0	3.0	2.66	-	0.33
07.	*Cladosporium cladosporioides*	5.0	3.33	-	-	1.33	-	1.66	3.0	4.0	2.66
08.	*Curvularia lunata*	1.0	-	1.0	0.70	-	-	-	-	2.66	-
09.	*Fusarium oxysporum*	-	-	1.33	-	-	-	-	-	-	-
10.	*F. moniliforme*	0.33	0.33	-	-	-	-	-	-	-	-
11.	*Myrothecium roridum*	-	-	-	-	-	2.66	-	-	-	-
12.	*Nigrospora sphaerica*	-	-	2.70	0.70	2.66	-	2.66	-	-	-
13.	*Penicillium citrinum*	-	-	-	-	-	0.66	-	-	-	-
14.	*P. purpurogenum*	1.33	1.33	-	-	-	-	-	-	-	-
Coelomycetes											
15.	*Colletotrichum dematium*	7.66	-	-	-	-	-	-	-	-	-
16.	*C. gloeosporioides*	-	-	2.70	4.0	-	-	1.66	3.0	-	-
17.	*C lindemuthianum*	-	-	-	-	6.33	3.0	-	-	-	-
18.	*C. truncatum*	-	-	-	-	-	-	-	-	-	1.66
19.	*C. falcatum*	-	-	1.0	5.70	-	-	-	-	-	-
20.	*Lasiodiplodia theobromae*	-	-	5.70	4.0	-	1.66	-	-	-	10.66
21.	*Pestalotiopsis funerea*	0.66	1.66	-	-	-	-	-	-	-	-
22.	*Phomopsis archeri*	-	-	-	-	-	-	-	-	-	0.66
23.	*P. vexans*	0.66	2.33	6.70	10.0	-	-	-	-	-	-
24.	*Phoma medicaginis*	-	-	-	-	13.0	8.0	1.0	-	-	-
25.	*Phyllosticta* sp_1	0.66	-	-	-	1.33	5.66	-	-	-	-
26.	*Phyllosticta* sp_2	-	-	1.0	7.0	-	-	-	-	-	-
27.	*Phyllosticta* sp_3	-	-	-	-	-	-	2.0	1.33	-	-
Sterile morphospecies											
28.	Sterile form $_{01}$	9.33	2.0	-	-	-	-	-	-	-	-
29.	Sterile form $_{02}$	5.66	5.33	-	-	-	-	-	-	-	-
30.	Sterile form $_{03}$	1.0	4.66	-	-	-	-	-	-	-	-
31.	Sterile form $_{04}$	-	-	2.33	1.67	-	-	-	-	-	-
32.	Sterile form $_{05}$	-	-	3.33	2.33	-	-	-	-	-	-
33.	Sterile form $_{06}$	-	-	4.33	3.70	-	-	-	-	-	-
34.	Sterile form $_{07}$	-	-	-	-	2.0	5.0	-	-	-	-
35.	Sterile form $_{08}$	-	-	-	-	3.0	2.0	-	-	-	-

Table 1b. Contd.

36.	Sterile form $_{09}$	-	-	-	-	1.33	1.33	-	-	-	-
37.	Sterile form $_{10}$	-	-	-	-	-		5.0	4.0	-	-
38.	Sterile form $_{11}$	-	-	-	-	-	-	-	5.0	-	-
39.	Sterile form $_{12}$	-	-	-	-	-	-	5.33	3.33	-	-
40.	Sterile form $_{13}$	-	-	-	-	-	-	2.0	1.33	-	-
41.	Sterile form $_{14}$	-	-	-	-	-	-	-	-	3.33	0.66
42.	Sterile form $_{15}$	-	-	-	-	-	-	-	-	13.33	-
43.	Sterile form $_{16}$	-	-	-	-	-	-	-	-	-	1.33
44.	Sterile form $_{17}$									4.0	1.0
	Total Number of Isolates	103	52	100	103	127	107	78	71	58	86

S. nig - Solanum nigrum; S. sur - S. surratense; S. tor - S. torvum; S. tri - Solanum trilobatum; W. som - Withania somnifera. ***** Based on 300 segments screened.

Table 2. Endophytic infection rates (EIR %) in the leaf lamina, leaf midrib, petiole and stem tissues of medicinal plants.

S/n	Hosts	No of segments infected by the endophytic fungi				Endophytic Infection Rates (EIR %)			
		Leaf Lamina	Leaf Midrib	Petiole	Stem	Leaf Lamina	Leaf Midrib	Petiole	Stem
01.	*Solanum nigrum* Linn.	19	105	103	52	6.33	35.00	34.33	17.33
02.	*S. surattense* Burn.F.	88	75	100	103	29.33	25.00	33.33	34.33
03	*S. torvum* Sw.	38	74	127	107	13.00	25.66	42.33	36.00
04.	*S. trilobatum* Linn.	36	72	78	71	12.00	24.00	26.00	24.00
05.	*Withania somnifera* Dunal	40	82	58	86	13.33	27.33	19.33	29.00

the techniques like extraction and comparison of both nuclear and mitochondria DNA by means of RFLPS (Manicom et al., 1987; Taylor, 1986*)* should be carried out. Such work is usually challenging one, which is a must in future. The mechanism of interaction of the endophytic fungi with the host plants is not clear. Investigations on these lines should be studied in future.

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