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Comparative Study of Phylloplane Mycoflora of Non-infected and *Alternaria alternata*-infected Leaves of *Solanum nigrum*

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Abstract

Leaves of *Solanum nigrum* infected by *Alternaria alternata* harboured more fungal species and fungal population cm^{-2} leaf surface than non-infected leaves. A total number of 34 fungal species belonging to various group were isolated from non-infected and infected leaves. Out of these 15 and 25 fungal species were recorded from non-infected and *Alternaria alternata*-infected Leaves respectively. The comparative phylloplane mycoflora of non-infected and infected leaves showed some what distinct group of fungi, *Alternaria alternata*, *Aspergillus niger*, *Cladosporium cladosporioides*, *C.herbarum*, *Fusarium oxysporum* and *Trichoderma lignorum* were represented by a large number of colonies cm^{-2} leaf surface and higher frequency percentage on *Alternaria alternata*-infected leaves.

Keywords: *Solanum nigrum, Alternaria alternate,* Non-infected, Phylloplane, Mycoflora.

Introduction

The leaf surface being nutrient rich and natural habitat is known to provide a complex of niches suitable for a variety of microbes,both pathogenic and non-pathogenic chiefly bacteria and fungi. The presence of fungi on aerial surfaces of plants has long been recognized. Although most emphasis has been placed on the study of fungal pathogen on leaves, stem, and fruits, saprophytic fungi recorded more than a century ago. The earlier work on microfungi in the phyllosphere of wide variety of living plants has been excentlly reviewed by Ruinen (1956), Leben (1965), Sinha (1965), Sharma and Mukerji (1973), Blakeman (1981), Fokkema (1981), Bhattacharya and Purkayastha (1982), Sindhu and Singh (1990), Jain *et al*. (2005-08). Interaction between microorganism on the leaf surfaces have been considered in relation to the influence of saprophytes on the parasite. Thus plant pathologists started comparing the mycoflora present in infected and non-infected leaves to understand the pattern of colonization of mycoflora and search a suitable antagonist if any, to control the pathogen. No work, however,has been done so far to find out phylloplane fungi of non-infected and *Alternaria alternata*-infected leaves of *S.nigrum* and the plant is much of medicinal values,an attempt have been made with a view to study the nature of phylloplane mycoflora in relation to leaf disease caused by *Alternaria alternata*.

Materials and Methods

Comparative study of phylloplane mycoflora of non-infected leaves and leaves infected by *Alternaria alternata* separately was done as follows:

Seeds were sterilized by 0.1% mercuric chloride, washed thoroughly in sterilized distilled water and sown in earthen pots (18×25cm). Only five plants per pot were maintained. Ten pots were used for each treatment. The infected plants were obtained by artificially inoculating the plants with inoculum prepared as follows:

Alternaria alternata infected leaves were collected from the field. The leaf spot lesions were surface sterilized by 0.1% mercuric chloride, repeatedly washed with sterilized distilled water and transferred to Petri dishes containing sterilized potato dextrose agar medium and pure culture was obtained. 10-day old cultures were scraped from the Petri dishes, kept in 150 ml sterilized distilled water and inoclul prepared from the cultures mainly of hyphal fragments and spores.

At the age of 45 days, the plants grown in earthen pots were

inoculated by spraying the inoculums on the foliage until leaves were thoroughly wetted. The plants grown in 10 earthen pots were inoculated. The pots with inoculated plants were covered with polythene bags for 48-72 hr to get good lesions. The control plants were sprayed with sterilized distilled water. Finally infected and non-infected plants were kept in green house.

First collection of *Alternaria alternata* infected leaves was made when spots appeared 15 days after infection. The second collection was made when lesions were matured and third sampling was done when infected tissues were senesced and dead. For isolation of phylloplane mycoflora, four methods, dilution plate, serially washed leaf disc, surface sterilized leaf disc and moist chamber were used .They are described as follows

Dilution Plate Method

Twenty five non-infected and infected by the pathogen were collected and 100 discs of 6 mm diameter were cut by sterilized cork borer at random from different samples of the leaves. They were placed in 250 ml Borosil conical flasks containing 100 ml of sterilized distilled water. The flasks were hand shaken for 20 minutes to get a homogenous suspension of the fungal propagules. From this suspension, a further dilution (1:10) was made by adding 10 ml aliquot of the suspension with 90 ml of sterilized distilled water. From this dilution 1 ml of the suspension per petridish was added into each of ten sterilized petridishes of 9 cm diameter. Approximately 15 ml of molten, cool, sterilized Czapek's Dox yeast extract agar (CDYA) medium of the following composition was added to the inoculated petridishes: Sucrose, 30 g;$NaNO_3$, 3g; $K_2HPO_4.7H_2O$, 1g;$MgSO_4$, 0.5g; KCl, 0.5g; $FeSO_4.7H_2O$, 0.01 g; agar-agar, 20g; yeast extract 1 g, distilled water, 1 litre; supplemented with rose Bengal (33 mg/litre) and streptomycin (30 µg/ml). The petridishes were inoculated for 7 days at 25±1^{0}C, after which colonies of fungi were identified, counted and recorded. Results are expressed as average number of colonies cm^{-2} leaf surface for each fungus. Quantitative estimation of total fungal population was made by using the following formula:

$$Total\ fungal\ population\ leaf\ surface\ cm^{-2} leaf\ surface = \frac{Average\ no.\ of\ Colonies\ X\ dilution}{Total\ area\ of\ leaf\ discs} X\ 100$$

This method was used to isolate detachable propagules present on leaves.

Serially Washed Leaf Discs Method

This method was followed to isolate actively growing forms present on the leaf surfaces. The leaf discs,used in the Dilution plate method, were transferred to 150 ml sterilized flask containing 50 ml sterilized distilled water and were washed thoroughly. After 15 serial washings, the disc were dried on sterilized blotting papers and 5 such leaf discs were put in 9 cm diameter petridishes containing 15 ml of molten, cool, sterilized CDYA medium. The petridishes were incubated at 25± 1^{0}C for 7 days. Fungi developing from the leaf discs were identified and recorded after every 3 days until no new colonies appeared. Results were expressed for each fungus in terms of its percentage occurrence on total 100 leaf discs examined.

Surface-Sterilized Leaf Discs Method

The leaf discs were surface sterilized with 0.01% mercuric chloride for 60 seconds, washed in three changes of sterilized distilled water, blotted dry and five such leaf discs were placed in each petridish containing molten, cool, sterilized CDYA medium. Results for each fungus expressed as its percentage occurrence on a total of 50 leaf discs examined.

Moist Chamber Method

A pair of Borosil petridishes (9 cm diameter) containing four pieces of blotting papers together with 10 ml of distilled water was autoclaved and designated as moist chambers. Five leaf discs, per petridish, were inoculated into 10 petridish moist chambers. The petridishes were covered with polythene bags to maintain moisture and were incubated at 25± 1^{0}C for 10 days. After incubation, the discs were examined regularly for the presence of fungal species. In some cases, the incubation period was prolonged to ensure appearance of all fungi. Finally the leaf discs were cleared by gently heating in lactophenol, stained in cotton blue, mounted in lactophenol and examined under microscope to get complete picture of fungi. For each fungus, the result were expressed in terms of its percentage occurrence on total of 50 leaf discs examined. The detection of potential fungal colonists present in spore forms or in vegetative state on leaf surface was sought by this method.

Table-1

Mycoflora Isolated from non-infected (N), *Alternaria alternata* - infected (A) Leaves of *Solanum nigrum* by Dilution Plate Method (the number Against Species Represent Number of Colonies cm^{-2} Leaf Surface)

Name of Fungi	Samplings					
	S_1		S_2		S_3	
	N	A	N	A	N	A
Alternaria alternata (Fr.) Keissler	20	28	30	34	28	48
Aspergillus flavus Link ex Fries	35	18	38	21	37	29
A. fumigatus Fresenius	-	-	-	-	6	-
A. lunchuensis Inui	-	-	-	-	8	-
A. nidulans (Eidam) Winter	-	4	8	9	-	-
A. niger Van Tieghem	38	42	32	49	30	69
A. terreus Thom.	-	-	-	8	-	10
Candida albiacns Berkhout	30	18	30	21	36	15
Chaetomium globosum Kunze ex Fries	-	-	9	12	5	20
Cladosporium cladosporioides (Fresen.) de Vries	35	58	39	53	45	64

C. herbarum (Pers.) Link ex Gray	18	38	23	32	-	-
Curvularia lunata (Wlker) Boedijn	10	15	15	17	17	19
C. pallescens Beodijn	-	-	-	-	6	-
Drechslera australiensis (Bugnicourt) Subram. and Jain ex Ellis	12	15	14	19	19	22
D. hawaiiensis (Bugnicourt) Subram. and Jain	-	-	-	-	7	-
Doratomyces sp.	-	-	-	7	-	6
Epicoccum purpurascens Ehrenb. ex Schlecht.	22	10	30	10	18	5
Fusarium oxysporum Schlechtendahl	14	36	35	42	26	37
Graphium sp. Corda	-	-	-	5	-	-
Humicola brevis (Gilman and Abbott) Gilman	-	-	6	10	7	11
Melanospora sp. Cord (Frontispiece)	-	-	-	-	3	-
Mucor racemosus Fresenius	-	4	-	-	3	3
Myrothecium indicum Rama Rao	-	-	-	-	27	28
Nigrospora sphaerica (Sacc.) Mason	8	7	9	7	8	6
Penicillium sp. Link	-	-	-	-	4	4
Pestalotia sp. de not	-	-	3	5	-	-
Phoma humicola Gilman and Abbott.	-	-	3	6	-	6
Rhizopus arrhizus Fischer	7	16	13	15	11	16
Sclerorium sp. Tode	-	3	-	5	-	5
Staphylotrichum sp.	-	-	-	-	-	3
Sterile hyphae (brown)	-	-	-	-	-	3
Thielaviopsis sp. Went	-	-	-	6	-	6
Torula herbarum (Pers.) Link ex Gray	-	-	-	-	9	13
Trichoderma lignorum (Tode) Harz.	14	37	18	33	14	30
Total fungal species	**13**	**16**	**18**	**23**	**23**	**25**
Total fungal population (Average number of colonies of fungi cm^{-2} leaf surface)	**263**	**349**	**355**	**426**	**374**	**478**

Aspergillus flavus, *Candida albicans* and *Epicoccum purpurascens* and *Nigrospora sphaerica* were found with more number of colonies cm^{-2} leaf surface on non-infected leaves than infected leaves, while *Alternaria alternata, Aspergillus niger*, Cladosporium *cladosporioides, C. herbarum, Fusarium oxysporum Humicola* brevis and *Trichoderma lignorum* were represented by more number of colonies cm^{-2} leaf surface on infected leaves than non-infected leaves.

Aspergillus terreus, Doratomyces sp., *Graphium* sp., *Sclerotium* sp. *Staphylotrichum* sp. and *Thielaviopsis* sp. were confined to *Alternaria*-infected leaves. It was observed that *Alternaria*-infected leaves harboured more fungal population (number of colonies of fungi cm^{-2} leaf surface) than non-infected leaves in the dilution plate method.

Table -2

Mycoflora isolated from non-infected (N), *Alternaria alternata* - infected (A) leaves of *Solanum nigrum* by Serial washed leaf discs (SW) and Surface-sterilized leaf discs (SS) methods (frequency percentage of occurrence)

Name of Fungi	Samplings											
	S_1				S_2				S_3			
	N		A		N		A		N		A	
	SW	SS	SW	SS	SW	SS	SW	SS	SW	SS	SW	SS
Alternaria alternata (Fr.) Keissler	20	14	38	19	30	18	50	24	33	28	50	48
*Aspergillus fla*vus Link ex Fries	20	-	10	-	26	10	28	12	36	20	22	11
A. niger Van Tieghem	14	-	37	20	29	20	60	43	32	29	72	42
Candida albicans Berkhout	36	-	28	-	48	-	28	-	46	30	34	22
Chaetomium globosum Kunze ex Fries	-	-	-	-	10	-	14	-	12	10	20	12
Cladosporium cladosporioides (Fresen.) de Vries	43	23	56	28	49	23	62	24	35	20	69	48
Curvularia lunata (Walker) Boedijn	12	2	15	6	17	7	20	12	18	8	25	12
Drechslera australiensis (Bugnicourt) Subram, and Jain ex Ellis	8	-	13	-	10	6	14	12	12	8	28	18
Fusarium oxysporum Schlechtendahl	13	7	38	13	18	12	32	19	15	11	37	19
Mucor racemosus Fresenius	-	-	-	-	-	-	-	-	13	10	15	10
Myrothecium indicum Rama Rao	-	-	-	-	-	-	-	-	10	10	19	16
Nigrospora sphaerica (Sacc.) Mason	-	-	-	-	5	3	10	4	8	3	14	6
Phoma humicola Gilman and Abbott.	-	-	-	-	4	-	10	-	6	2	10	4
Rhizopus arrhizus Fischer	-	-	12	-	10	16	-	-	12	-	20	-
Sterile hyphae (brown)	-	-	-	-	-	2	-	-	-	-	4	-
Trichoderma lignorum (Tode) Harz.	8	-	22	10	11	4	28	13	14	10	27	13
Total fungal species	9	4	10	6	13	11	12	9	15	14	16	14
Total fungal population (Average number of colonies of fungi cm^{-2} leaf surface)	174	46	269	96	267	121	356	163	302	199	466	281

Table-2 revealed that more fungal species were isolated from infected leaves than non-infected leaves. The frequency percentage of occurrence of *Alternaria alternata, Aspergillus niger, Cladosporium cladosporioides, Fusarium oxysporum* and *Trichoderma lignorum* was observed more on infected leaves than non-infected leaves. Relatively less fungal species were recorded by surface-sterilized leaf discs method.

Table - 3

Mycoflora isolated from non-infected (N) and *Alternaria*-infected (A) leaves of *Solanum nigrum* by moist chamber method (frequency percentage of occurrence).

Name of Fungi	Samplings					
	S_1		S_2		S_3	
	N	A	N	A	N	A
Alternaria alternata (Fr.) Keissler	60	100	76	100	100	100
Cephalosporium curtipes Saccardo	-	-	5	3	6	8
Chaetomium globosum Kunze ex Fries	-	-	8	12	14	16
Cladosporium cladosporioides (Fresen.) de Vries	56	68	62	76	70	84
C. herbarum (Pers.) Link ex Gray	12	18	18	24	-	-
Colletotrichum sp. Corda	-	-	11	13	16	20
Curvularia lunata (Walker) Boedijn	7	15	28	27	32	40
C. pallescens Boedijn	-	-	-	-	-	8
Drechslera australiensis (Bugnicourt) Subram. and Jain ex Ellis	5	8	18	20	15	23
Epicoccum purpurascens Ehrenb. ex Schlecht.	34	11	26	15	-	-
Fusarium oxysporum Schlechtendahl	12	25	23	36	38	52
Graphium sp. Corda	-	-	-	-	-	8
Humicola brevis (Gilman and Abbott) Gilman	-	-	-	15	15	21
Melanospora sp. Corda (Frontispiece)	-	-	-	-	7	-
Memnoniella echinulata (Riv.) Galloway	-	-	-	18	-	22
Myrothecium indicum Rama Rao	-	-	-	-	15	24
Nigrospora sphaerica (Sacc.) Mason	3	5	7	12	13	3
Pestalotia sp. de not.	-	-	13	19	-	-
Phoma humicola Gilman and Abbott.	7	13	6	18	16	24
Stachybotrys atra Corda	-	-	13	18	20	27
Torula herbarum (Pers.) Link ex Gray	-	-	-	15	7	13
Trichoderma lignorum (Tode) Harz.	-	12	15	26	18	25
Trichothecium roseum Link	-	-	13	-	15	-
Total fungal species	9	10	16	18	17	18
Total fungal population (Average number of colonies of fungi cm^{-2} leaf surface)	196	275	342	467	417	518

It can be seen from Table 3 that :

1. *Cephalosporium curtipes, Colletotrichum* sp., *Memnoniella echinata*, *Stachybotrys atra* and *Trichothecium roseum* were recorded only by moist chamber method.
2. *Alternaria alternata, Cladosporium cladosporioides, Fusarium oxysporum* and *Trichoderma lignorum* were represented by more frequency percentage of occurrence on infected leaves than non-infected leaves.

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