FREQUENCY OF MYCOFLORA ASSOCIATED WITH SHISHAM (*Dalbergia sissoo*) DECLINE IN DISTRICT FAISALABAD, PAKISTAN

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Abstract

Shisham (*Dalbergia sissoo* Roxb.) is a very famous agroforestry tree but in previous years Shisham dieback disease has caused high mortality in standing Shisham trees. Objective of this study was to investigate fungal pathogens that cause decline in Shisham trees. Contaminated samples including roots, bark from neck portion, stem, branches, leaves and infected soils were collected for the isolation of associated fungal pathogens. Isolations were made on Potato Dextrose Agar (PDA) and on Czapek dox agar media. Identification of isolated fungi was done microscopically. The most frequently isolated fungus from Shisham tree was *Botryodiplodia theobromae* from roots, stem, bark, and twigs. R. solani was more frequent with leaves than B. theobromae. *Fusarium oxysporum* from the stem was the second most frequent fungus after *B. theobromae*. However, *F. oxysporum* was less frequently isolated from the root parts of Shisham. The study revealed that *B. theobromae* was the most isolated fungus and can be a major cause of shisham decline.

Introduction

Forests of Pakistan are facing number of biotic and abiotic constraints; decline is major which is defined as an interaction of exchangeable, specially abiotic and biotic factors to create a slow death of trees (Manion, 1991). During last decades, enormous losses have been recorded due to decline. This syndrome has engaged the attention of forest pathologists, but the information available today has not yet become sufficient to solve this problem. However, studies on tree decline may provide foundations for developing management strategies to combat this syndrome in agro-forest plantations (Manion and Lachance, 1992).

Decline in forest trees has been reported almost in all forest growing areas. Decline is characterized by the presence of symptoms such as reduced growth, shortened internodes, root necrosis, premature fall, yellowing and loss of foliage, dieback of twigs and branches. Generally it begins from the upper crown and increased the progressive vigor and health loss of tree (Manion, 1991). Mortality varying from 8.94 to 20.80% has been reported in Shisham due to dieback in plantations of Eastern Utter Pradesh (Varanasi, India) (Ahmad *et al.,* 2013).

Khan (2002) reported that fungal pathogens are major cause of Shisham decline in Pakistan - these include *F. solani, F. moniliforme, F. equiseti, F. oxysporum, F. semitectum, Rhizoctonia solani, Alternaria alternate, Curvularia lunata, A*spergillus *niger* and few species of *penicillium* species. However, there is still no concrete solution for this syndrome. The present study was therefore designed with the objective "isolation and identification of fungal pathogens associated with declining trees in district Faisalabad".

Materials and Methods

Survey and sample collection: A survey of Faisalabad district was conducted to note the disease incidence in Shisham trees. For survey purpose, Faisalabad district was divided into two separate zones: 1) urban areas and 2) cultivated lands. Then, 50 infected trees of *Dalbergia sissoo* were randomly selected from both zones at different locations in Faisalabad District and then separate samples for stem, twigs, roots, leaves and bark were collected from each tree. Cutters and knives used in sampling were sterilized with 2.5% sodium hypochlorite. Tree roots were dug up to 30-45 cm, whereas aerial parts were harvested depending upon the size of tree and portion of affected by the disease.

Preservation, isolation and identification of associated mycoflora: Samples were taken in sterilized polythene bags, labeled and stored in a refrigerator at 4^{o}C until processed. Stored samples were cut into small pieces and treated with 1% Mercuric chloride solution and then with distilled water. After this, samples were

inoculated in laminar air flow cabinet on media with the help of foreceps. Isolations were made on potato dextrose agar (PDA) and Czapek dox agar media. The ingredients and their quantity for PDA were:

Distilled water	1 Litre
Potato	200 g
Glucose	20 g
Agar	20 g

The sliced and unpealed potatoes were boiled in distilled water for 30 minutes and filtered through cheese cloth saving effluent. After that, glucose and agar were added in potato dextrose filtrate, made the volume of conical flask 1 Litre by adding distilled sterilized water and autoclaved for 15 minutes at 121^{o}C.
Czapek dox broth is a semisynthetic medium used for general cultivation of fungi. The ingredients and their quantities for this medium were:

Sucrose	30 g/L
Sodium nitrate	3 g /L
Dipotassium phosphate	1g /L
Magnesium sulphate	0.5g/L
Potassium chloride	0.5g/L
Ferrous sulphate	0.01g/L
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Final pH	7 ± 0.2

All the ingredients were mixed in 1000 mL distilled water and heated to dissolve the medium completely. The medium was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

The Samples from different locations were processed individually. The fungi isolated were recognized with the help of keys (Neergaard, 1979). Organisms were maintained on PDA media for more studies. The fungi grown from contaminated tissues were recognized on the basis of colony characteristics and conidial morphology wing standard keys (Neergaard, 1979; Hawksworth *et al.,* 1995; Gill *et al.,* 2001). The frequency of fungal species was calculated by following formula:

$$\text{Frequency of isolated fungi} = \frac{\text{Samples yielding fungus}}{\text{Total number of samples}} \times 100$$

Characterization of associated mycoflora: Collected and isolated fungi were identified on the basis of colony color, texture and shape as well as conidial morphology. After that, pure cultures were made of these identified fungi for microscopical characterization. Isolated fungi were transferred to the new petri-plates of PDA and Czapek dox agar media to prepare their pure cultures. Identified fungal pathogens were characterized for their maximum growth on optimized medium conditions i.e. pH and temperature.

Results and Discussion

Results of the survey indicated that, in cultivated lands, disease incidence in Shisham trees was 18.5%, 11.9% and 29.6% at Jhang Road, Sargodha Road and Satiana Road, respectively. However, in urban areas, disease incidence was 14.5, 24.8, 29.1 and 27.5% at Jinnah Garden, Gatwala Forest Park, Punjab Forestry Research Institute and University of Agriculture, respectively (Table 1). Findings of this study are well in accordance with Bajwa *et al.* (2003), Bajwa and Javaid (2007) and Pathan *et al.* (2007) who have reported that Dieback disease incidence in *Dalbergia sissoo* varied from 10 to 40% at various locations of Punjab province, Pakistan.

Table 1. Disease incidence in *Dalbergia sissoo* in urban areas and cultivated lands.

Urban areas		Cultivated lands	
Sites	Disease incidence (%)	Sites	Disease incidence (%)
Jinnah Garden	14.5	Jhang Road	18.5
Gatwala Forest Park	24.8	Sargodha Road	11.9
PFRI*	29.1	Satiana Road	29.6
UAF*	27.5		

PFRI: Punjab Forestry Research Institute; UAF: University of Agriculture, Faisalabad

The analysis of variance (ANOVA) of data on frequency of fungal species recorded from bark, stem, roots, twigs and leaves of Shisham trees growing on road sides of Jhung road, Sargodha road, Satyana road and PFRI, Jinnah Garden, Getwala park and University of Agriculture, Faisalabad is presented in Table 2. The ANOVA indicated highly significant difference among the frequencies of fungi isolated from different plant parts collected from different locations of Faisalabad.

The relative frequencies (RFs) of fungi recorded from different plant parts were statistically different from each other (Table 3). Eight fungal species were associated with stem and bark of which three species were relatively predominantly frequent in occurrence e.g., *B. theobromae, F. oxysporum* and *R. solani*. There were nine fungal species which associated with root, twigs and leaves. *B. theobromae* was predominant with stem, roots, twigs and leaves. The fungi viz. *F. solani, A. alternata* and *B. theobromae* were equally frequent with leaves with relative frequency ranging from 12.26-13.21% relative frequency. C. lunata, G. lucidum, A.flavus and *P. digitatum* were present with low frequency – RF: 1.48-5.99. *C. lunata, Ganoderma lucidum, A. flavus* and *penicillium digitatum* occurred with substantially lower values of relative frequency as compared to that of *B. thoebromae, F. oxysporum, F.solani* and *A.alternata. C. lunata* was, however, recorded more frequently from roots than other parts. *Ganoderma, Aspergillus* and *penicillium* were recorded below 10% relative frequency (Table 3).

Table 2. ANOVA on the basis of frequency of fungi associated with Shisham decline.

Source of variation	Degrees of freedom	Mean squares				
		Stem	Roots	Twigs	Leaves	Bark
Treatment	8	3.36193**	1.07798**	0.94547**	1.70943**	2.28090**
Error	18	0.00512	0.00307	0.00170	0.00440	0.00543
Total	26					

** = Highly significant ($p < 0.01$)

The fungi in different parts of shisham were recorded in following order of their relative predominance:

Stem: *B. theobromae > F. oxysporum > R. Solani ≈ A. Alternata*

Bark: *B. theobromae > F. oxysporum > A. alternata ≈ R. Solani*

Root*: B. Theobromae > F. Oxysporum > R. solani ≈ A. alternata = C. lunata*

Twigs: *B. theobromae > F. oxysporum > R. solani = A. alternata > F. Solani*

Leaves*: R. Solani > F. Oxysporum > F. solani ≈ A. alternata = B. theobromae*

It is obvious from the data *B. theobromae, F. oxysporum, F. solani* and *R. solani* were most frequently associated with Shisham die back inn district Faisalabad.

Shisham is inflicted with two severely damaging diseases viz., wilt and dieback, in Pakistan in the recent years and incidence is also reported in the Tarai tract of Nepal, believed to be its home. In wilt disease effects produced on trees are more or less of the same type as those produced by drought or frost. The disease was first observed by Bakhshi (1954) both in natural forests and plantations in UP, India. The disease is systemic that is the entire tree shows symptoms of the attack. In the early stage, an affected tree is characterized by drooping of leaves and branches, due to loss of turgor. The leaflets turn yellow, dry up and eventually drop off rendering the branches bare. The entire tree becomes thin in contrast to the adjoining dense green trees. Death of the affected tree is rapid and occurs within 1–6 months after the crown shows the symptoms of wilt. *F. solani* is suggested to be the cause of this disease (Bakhshi, 1954; Manandhar and Shrestha, 2000; Bajwa *et al.*, 2003; Ahmad *et al.*, 2013). The pathogen is mostly restricted to roots. The fungal hyphae and jelly like substances plug the vessels resulting in wilt symptoms (Bakhshi and Singh, 1959). In our study, during the isolation of pathogens from samples collected from University of Agriculture Faisalabad and satayana road, *F.solani, B. theobromae* and *F. oxysporum* were found to be the maximum. *F. solani* and *A. alternata* were rather more abundantly isolated from the Shisham trees on Satayana road where incidence of disease was (2-100%). This is in accordance with the previous findings.

Table 3. Average relative frequency (%) of fungi isolated from declined Shisham.

Fungi associated	Stem	Roots	Twigs	Leaves	Bark
Fusarium oxysporum	23.78b	14.98b	17.38b	20.19b	18.54b
Fusarium solani	6.69e	8.99e	10.47d	13.21d	10.94 d
Alternaria alternata	13.17d	10.83d	13.93c	12.90e	12.77c
Botryodiplodia theobromae	30.17a	25.24 a	20.94a	12.26c	29.48a
Rhizoctonia solani	16.58c	11.81c	13.93c	25.48a	10.94d
Curvularia lunata	3.30f	10.83d	8.69e	5.29f	7.60e
Ganoderma lucidum	4.50ef	7.15f	4.29g	4.86g	7.60e
Aspergillus flavus	1.69g	5.31g	4.29h	1.48i	2.93f
Penicillium digitatum	0.00 h	3.58h	6.07f	4.33h	0.00 g

Dieback takes place in successive stages and is characterized by progressive death of shoots and roots starting at the tip. There are controversial reports regarding the causal agent of dieback. The fungus *Phellinus gilvus* has also been isolated from the roots of dying back Shisham trees (Bakhshi, 1974). Another fungus *G. lucidum* is considered to be the primary cause of this disease. This pathogen is root inhabiting and infects the roots through intact as well as injured surfaces. Lateral spread of the disease in plantations is through root contact (Sharma *et al*., 2000). According to Gill *et al.* (2001), the primary cause of Shisham dieback is *Phytophthora cinamomi*. One research group in Punjab University Lahore has isolated *F. oxysporum* and *P. cinamomi* from dying back Shisham trees. The causal pathogens of the mortality were identified as *F. solani* and *G. lucidum* which were favoured by high moisture content and heavy soil in the root zone (Barnett and Hunter, 1972; Ahmad *et al.*, 2013).

Conclusion

It was found that *B. theobromae* in combination with *F. solani* and *F. oxysporum* were the most destructive mycoflora responsible for Dieback disease in *Dalbergia sissoo*.

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