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ANALYSIS OF FUNGAL DIVERSITY IMPACTS ON *PINUS ROXBURGHII* SEEDS FROM PINE FOREST AND PLANT NURSERIES OF AZAD KASHMIR, PAKISTAN

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Abstract

Pinus tree plays a pivotal role in commercial revenue generation, domestic lives of rural communities and sustaining of climate of Azad Kashmir. Pinus grows in forest as wild species but due to harsh environmental parameters it is also cultivated in nurseries for propagation and plantation. In this research, injurious impacts of mycofloral diversity on seed germination of *Pinus roxburghii* Sarg. in nature (forest) and nurseries were explored from different localities of Azad Kashmir, Pakistan. In the analysis two protocols *viz*., blotter method (BM) and agar plate method (APM) were employed and 11 fungal species of nine genera were isolated. APM was found better (66a±0.32) than BM (60a±0.09). The prevalence of different isolated taxa was as: *Aspergillus niger* (42.75%), *Aspergillus flavus* (24.0%), *Botrytis* sp (14.25%), *Botryosphaeria* sp. (17.75%), *Cladosporium cladosporioides* (32.75%), *Drechslera* sp. (5.75%), *Fusarium* sp. (47.50%), *Penecillium* sp. (7.25%), *Rhizopus stolonifer* (11.50%), *Rhizopus oryzae* (13.0%) and *Mucor* sp.(7.0%). Pathogenicity analysis depicted that *Fusarium* was the most harmful (15.75e±0.54), followed by *Aspergillus flavus* (20.50d±0.32), *Aspergillus niger* (25.75c±0.42) and *Rhizopus sp.* (35.75b±0.12). Different pathogenicity results of analyzed fungal species were found in different areas and it was highest in Muzaffarabad (52.0%), Kotli (45.6%), Samahni (42.4 %) and least in Bhimber (36.0 %). Radical length (mm) of *Pinus roxburghii* was severely affected by *Aspergillus flavus* (46.6a±0.44) in Muzaffarabad, *Rhizopus* sp. (44.1a±0.72) in Samahni, *Fusarium sp.*(42.5a±0.28) in Kotali, *Aspergillus niger* (37.8a±0.44) in Samahni, respectively. The tested species showed that plumule length (mm) of samples was most retarded in Muzaffarbad (37.98%) and least affected in Mirpur (24.58%). The results depict that fungi do cause damage to seed germination and growth of seedlings in nature and nurseries and these findings will be useful for forest and nurseries researchers to produce good quality seeds for growth of pinus trees.

Keywords: Seed Born Fungi; Samahni; *Pinus roxburghii*; Pathogenicity; Forest trees.

Introduction

Pinus roxburghii Sarg. (Pinaceae), commonly known as chir pine, is a tall tree with enormous economic and medicinal potential. *P. roxburghii* is key species in forests of Azad Kashmir and it controls microclimate of area and thus controls other growth and propagation other flora in sub-canopy. It is also a medicinal plant used as disinfectant, colds, influenza, tuberculosis, bronchitis, diaphoretic, diuretic, rubefacient, refresher and vermifuge (Langenheim, 2003; Puri *et al.,* 2011; Mittal *et al*., 1993). Pines are source of many valuable goods, having timber, pulpwood, firewood, resin and edible seeds which are used as food phytonyms in indigenous therapeutics (Ishtiaq, *et al.,* 2012). It is also used as charcoal, stain, herbicide, gum and commercial wood (Ahmed *et al*., 2009; Martín *et al.,* 2008).

Many microorganisms infect seed and plant parts of pinus in various climates impeding its growth. One of those is fungi which produces mycotoxin in infected seeds of pinus that may cause a irreparable loss to human kidney (Mohanan *et al*., 2005; Domsch *et al.,* 1980). *Aspergillus flavus* produces aflatoxins in seeds of pinus which are noxious and produce liver cancer (Bilgrami & Ghaffar, 1993).

Azad Kashmir lies between longitude 73-75° and latitude 33- 36° with an area of 5734 sq miles (12397 Km^2) (Ishtiaq *et al.*, 2007). Research area has spatial longitudinal and latitudinal paradigms with dynamic topography. The climate is inconstant between lower and higher altitude and fluctuating temperature and humidity (Nazar *et al*., 2013) which make it as appropriate climate for growth of pinus species (Muhammad *et al*., 2012; Rawat *et al*., 2006). Pinus is dominant species of many forests regions of Azad Kashmir and it is playing an important economic boost in revenue of state. Pinus trees are also controlling macro and micro climate of the area and thus subsequently affecting other plant taxa of zone.

Pinus regenerate through seed germination. Its seeds grow naturally in wild the growth and regeneration is but hampered due to different factors such as fire, grass cutting, grazing, construction, volcanos, road infrastructure and Sweden agriculture practice (Ishtiaq *et al*., 2012). The number of mature trees is also decreasing day by day due to anthropogenic effects and other climatic fluctuations.

Due to dynamic and fluctuating environment, seed production by forest trees is so inconstant that for some species, there may be no yearly production of seeds, and/or production may be very less or of deprived value (Edwards, 1981; Runion, & Kelley, 1991). Secondly, alike all other seeds, forest tree seeds also show response to diverse biotic and abiotic aspects that can affect the regular growing procedure. So, alternative method is to grow Pinus seeds commercially in nurseries and do a-forestation, re-forestation and off-forestation in different appropriate climate of various areas of Azad Kashmir for propagation of *Pinus roxburghii*. One of the greatest impenetrable difficulties is providing pure and healthy seeds for coniferous seedlings stock in nursery beds. There are complaints of low seed germination and less survival of seedlings, both in nature and nurseries by different stakeholders and beneficiaries of pinus forest. This plethora is of multifarious in nature but major role is

of pathogenic fungi associated with seeds which cause seed infections in conifers (Sahai & Mehrotra, 1982; Sutherland *et al*., 1987), as well as pre- and post-emergence damping-off of sprouts (Kirti, *et al.,* 2004). Fungi do seed infestation through parental plant or through stigma/style during flowering periods (Sesan & Taut, 1998; Marsh & Payne, 1984). Another prominent cause of seed contamination is that they are shed at high humidity content affecting seed viability and making it susceptible for fungi and it makes them liable to contamination once fall on the ground, or in packing bottles if processed for sale to nurseries (Shuaib *et al.,* 2013; Sutherland, 1995).

Pinus roxburghii is very important plant for indigenous, rural communities of Azad Kashmir being part and parcel of their life and livelihood, and sustainable ecosystems of the area. There is a dire need to collect and process wild seeds to make them pure and contamination free for food and medicine purpose. Healthy seed germplasm is prerequisite for flourishing of plantlets in nurseries so that healthy seedlings and sprouting's may be provided and planted in forest and rural pasture areas. This is first attempt to study impact of fungi on *Pinus roxburghii* seeds, its germination in pine forest and in different plant nurseries of Azad Kashmir. The purpose of research was: (a) to explore and prepare checklist of mycoflora associated with pine seeds in forest and commercial seed bags of plant nursery, (b) to determine incidence and pathogenicity impact of mycoflora on pinus seeds and seedlings, (c) to determine impact of fungi on different parts of seedlings and (d) to determine impact of ecoclimatic factors on prevalence and pathogenicity from different areas of research.

Materials and Methods

Sample collection: Seed samples of *Pinus roxburghii* Sarg. were collected from different forest nurseries and localities (Bhimber, Mirpur, Kotli, Samahni and Muzaffarabad) of Azad Jammu and Kashmir. 50 seeds were collected in triplicates, placed in polythene bags, labeled and brought in Plant Pathology Laboratory, Department of Botany Mirpur University of Science & Technology (MUST) Bhimber Campus, Bhimber Azad Jammu and Kashmir, Pakistan.

Mycological assessment: Mycoflora associated with seeds were detected by standard methods as described by ISTA (International Seed Testing Association) and cited by Hussain *et al.* (2013). The existence and type of fungi were determined according to their development on seeds which had been incubated on substrates such as water soaked standard blotter named as blotter method (BM) and agar plate method (APM).

Blotter method: Sample seeds were sterilized in 1% $Na(OCl)_2$ solution for 10 minutes and washed in double distilled (d.dist) water for 5-7 min and dried properly (Elmer *et al*., 2001). Ten to fifteen seeds of each sample were placed on three layer water soaked filter papers in 9 cm diameter Petri dishes. The plates were incubated at 22±2^{o}C for 12 hour of alternating cycles of day/night under fluorescent light (Mustafa, 2009). Seeds were examined under stereoscopic microscope for fungi identification and population of mycoflora was recorded by Colony counter technique (CCT) as suggested by previous researchers (Tanveer *et al.,* 2010; Ishtiaq, 2013). Identification of Fungi was conducted by comparing with permanent fungi slides prepared and preserved in previous research in our lab and relevant hard and soft literature using internet facility (Habib *et al*., 2007; Barnett & Hunter, 1998).

Agar plate method: The preparation of medium was done according to recipe of Hussain *et al.* (2011) with some minute modifications as: mixing 20g agar, 20g dextrose and 20g potato starch in 1000 mL distilled water, medium was purified at 15 psi for 20 minutes. Nearly 15ml of purified refined medium was transferred in each Petri dish under aseptic circumstances. Seeds from each sample were surface sterilized in 1% $Na(OCl)_2$ for one minute and then given three washings in sterilized distilled water, were plated 5-seeds per 9cm diameter Petri dish. Incubation conditions were the same as for the blotter test.

Percent incidence of fungi: Fungal percent incidence (FPI) values were calculated to determine the most susceptibility of different seeds of different zones of Azad Jammu and Kashmir to various fungal infections recorded as per available methods (Mustafa, 2009; Hussain *et al.,* 2013).

$$\text{Fungal percent incidence} = \frac{\text{No. of infected seeds}}{\text{Total no. of seeds were examined}} \times 100$$

Fungi were subcultured directly on Petri plates in potato dextrose agar (PDA) growth medium for further studies. The fungus culture was refined by hyphal tip culture practice for better identification purpose (Hussain *et al.,* 2013).

Pathogenicity impact measurement: Isolated fungi were tested for their pathogenicity impacts on the seeds of *P. roxburghii* by following procedure.

Preparation of inoculum (fungal suspension): The culture of pathogenic fungi isolated were grown by cultivating them on Potato dextrose agar (PDA) as medium in Petri plates (9cm) at 25±2^{o}C for 15 days in an incubator with 12 hour of alternate light and dark arrangement. The colonies were agitated along with agar and creased to form a paste and diluted in 200 mL of sterilized distilled water to make a fungal suspension for inoculation of seeds (Mustafa, 2009).

Determination of pathogenic fungi by their effect on seedling vigour: The effect of pathogenic fungi on seeds germination and seedling vigor was assessed by obtaining a culture filtrate. Suspensions of different fungi were filtered through Whatsman filter paper No.4. Seeds were soaked in each culture filtrate separately. Ten seeds were placed in each Petri plate at 25±2^{o}C. The experiment was run in four replications with Randomized Completely Block Design (RCBD) arrangements (Mustafa, 2009; Hussain *et al.,* 2013).

Seed to seedling transmission: Transmission experiments were conducted by using a water-agar method. Seed were surface sterilized with 1% $Na(OCl)_2$ for one min followed by washing three times with sterilized water and then dried by blotting on sterile filter paper. For each fungus, seeds were then transferred to a 250-mL glass beaker comprising homogenized mycelial suspension, the inoculum preparation as described above.

Statistical analysis: The data obtained was formulated in tabular form and analyzed by applying analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) at $p< 0.05$ level by using SPSS and MVSP softwares (Steel *et al*., 1996; Steel & Torrie, 1980).

Results

This work analyses the mycoflora associated with the seeds of *P. roxburghii* collected from 5 different ecological zones of Azad Kashmir. In this analysis 9 fungal species were isolated from seeds of pinus and their infection rate in different ecological areas was determined. Eleven fungal species belonging to 9 genera were isolated from seed samples: *Aspergillus niger, Aspergillus flavus, Botrytis* sp, *Botryosphaeria* sp, *Cladosporium cladosporioides, Drechslera* sp., *Fusarium* sp., *Penecillium* sp., *Rhizopus stolonifer, Rhizopus oryzae* and *Mucor* sp. (Table 1; Fig. 4). The highest frequency was associated with *Fusarium* sp. with leading infection rate of (47.50%) and *Aspergillus niger* showed (42.75%) while *Aspergillus flavus* infection was third (24.0%). The lowest infection was seen in *Drechslera* sp. which was (5.75%). The analysis demonstrated that phytogeographical variation does pose biotic and abiotic impacts on quality and quantity of mycoflora. This investigation depicted that highest infection was found in Bhimber (54.0%), and other were as Samahni (48.0%), Muzaffarabad (45.0%) and Kotli (39.25%), respectively (Table 1).

Two protocols *viz*. blotter method (BM) and agar plate method (APM) were employed to isolate and identify various fungi species associated with pinus seeds collected from wild (forest) and nurseries of selected areas (Table 2). There was considerable variation found for both protocols in different sampled areas. APM method showed better results for isolation of fungi than the other. In blotter method Bhimber showed highest incidence (66.0%) and Muzaffarabad showed least incidence (36.0%). In agar plate method all localities showed maximum incidence (Table 2; Fig. 1).

The impact of different seed borne fungi causes rot of seeds, lessen seed germination and retardation in growth of different parts of seedlings. There is a decrease in germination rate (15.75%) of seeds and maximum rotted seeds (84.25%) observed in case of *Fusarium* sp., due to heavy colonization as compared to control (65.50%) (Table 3). Maximum number of seeds (35.25%) resisted the effect of fungi and produced maximum number of healthy seedlings (29.25%) observed in *Rhizopus* sp. as compared to *Fusarium* sp. (Table 3).

To determine seed vigour of *P. roxburghii* tree; four pathogenic fungi species namely *Aspergillus flavus, Aspergillus niger*, *Fusarium* sp. and *Rhizopus* sp. were used and it was determined that *Fusarium* sp. was the most harmful for seeds and *Rhizopus* sp. was least injurious for the samples (Table 3).

The seed germination (% age) was determined using four fungal pathogenic species as test case and correlation of seed germination with phytogeographical variation was also determined. The pathogenicity test showed pathogenic effects on seed germination on samples of different localities. With respect of eco-climatic perceptions efficiency of different fungi imposed variable effects on seed germination and seed growth. *Aspergillus flavus* produced toxicity impact on seed growth of pinus (35.20%) and it was most prevalent (52 %) in Muzaffarabad zone (Table 4).

The effect of fungal taxa on seedling growth and its various parts was determined. The length of radical was checked and it was found that pathogenic fungi do cause hectic impacts on its length. The ecological variation alters pathogenicity of different species and its correlation is determined in this research as described in Table 5 and Fig. 2. The effect of test fungi on seedling parts pronounced that *Fusarium* sp. was the most severe pathogen which hampers radical length leading its growth (28.7%) and it was followed by *Aspergillus niger* with toxicity potential (32.4%) on pinus seedlings (Table 5).

Table 1. Mycoflora associated with seeds of *Pinus roxburghii* and its infection values (%) in different areas of Azad Kashmir.

Fungal species	Sampling localities					Total infection
	Bhimber	Samahni	Mirpur	Kotli	Muzaffarabad	
Aspergillus niger	12.5	10.25	5.25	6.0	8.75	**42.75**
A. flavus	6.25	5.0	4.0	3.25	5.5	**25.0**
Botrytis sp.	-	4.5	3.25	3.5	3.0	**14.25**
Botryosphaeria sp.	2.5	3.25	4.25	5.25	2.5	**17.75**
Cladosporium cladosporioides	7.5	8.25	5.75	4.5	6.75	**32.75**
Drechslera sp.	3.25	-	2.5	-	-	**5.75**
Fusarium sp.	10.5	8.25	7.75	10.25	10.75	**47.5**
Penicillium sp.	3.25	2.25	-	-	1.75	**7.25**
Rhizopus oryzae	3.25	2.5	1.75	2.75	1.5	**11.75**
Rhizopus stolonifer	2.5	3.75	-	3.75	2.75	**12.75**
Mucor sp.	2.75	-	2.5	-	1.75	**7.0**
Total infection	**54.0**	**48.0**	**37.0**	**39.25**	**45.0**	

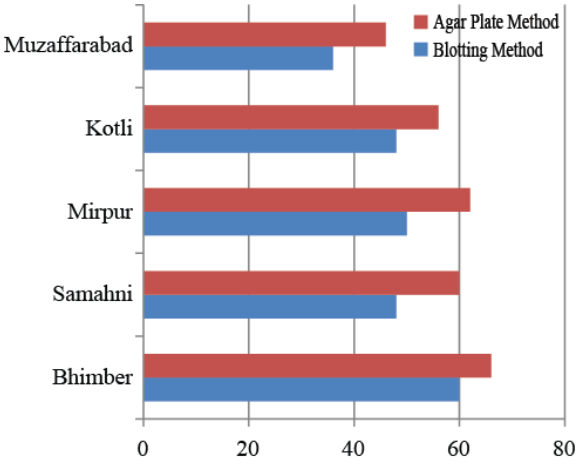

Fig. 1. Comparison of blotter method and agar plate method for seed-borne fungal analysis of *Pinus roxburghii* from different localities of Azad Jammu and Kashmir.

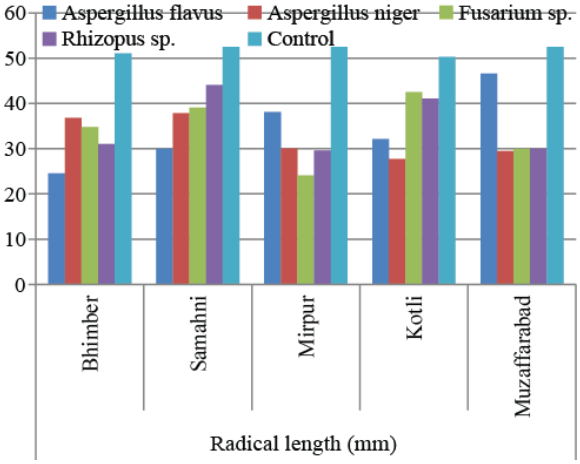

Fig. 2. Effect of fungal treatments on *Pinus roxburghii* radical length (mm) in different localities of Azad Jammu and Kashmir by Blotter method.

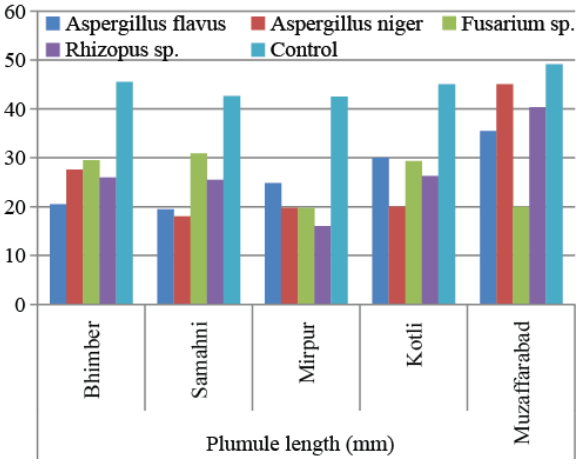

Fig. 3. Effect of fungal treatments on *Pinus roxburghii* plumule length (mm) in different localities of Azad Jammu and Kashmir by Blotter method.

In another experiment, it was analyzed that length of plumule of seedlings of pinus species growing in nature and nurseries are severely affected. There is stunt growth of plumule seen. The different ecoclimatic zones and there altitudinal and latitudinal parameters vary and how this variation do cause pathogenicity difference for tested taxa of fungi (Table 6 and Fig. 3). This retarding effects are variable due to phytogeographical variations in different samplings areas of the study. Secondly, the effect of test mycoflora on plumule length was also conducted in laboratory experiment in order to visualize pathogenicity, and it was found that *Fusarium* sp was most severe (25.90%) and it is clearly presented in Table 6.

Table 2. Comparison of blotting method and agar plate method based on seed vigour for *Pinus roxburghii* s by using DMRT.

Localities	Blotting method	Agar plate method
Bhimber	60a ± 0.09	66a ± 0.32
Samahni	48b ± 0.74	60a ± 0.47
Mirpur	50b ± 0.16	62a ± 0.89
Kotli	48b ± 0.74	56a ± 0.48
Muzaffarabad	36c ± 0.44	46a ± 0.48

Means are not significantly different @ p≤0.05

Discussion

Mycoflora is ubiquitous in biosphere having useful as well harmful impacts on flora, fauna and human beings. Plants are also very significant for man's life in form of source of livelihood, subsistence and medicines. Gymnosperms particularly *Pinus roxburghii* is paramount part of coniferous forest's ecosystem. This species is major part of forest of Azad Jammu and Kashmir, particularly in mountainous terrains of the area (Ishtiaq *et al.*, 2012). This plant is playing significant role in daily lives of rural communities of Azad Jammu and Kashmir as it is used as natural resource for food, fodder, fuel, shelter and medicines. The present study was aimed to explore and determine the isolation of seed borne mycoflora of *Pinus roxburghii* from Azad Jammu and Kashmir. Most of forest trees proliferate through seeds and pinus is one of those. The seeds are also edible and used commercially for oil extraction (Bilgrami & Ghaffar, 1993, Storer *et al.*, 1998). The environmental conditions of forest of pine have dynamic and variable effects on seed germination and seed growth due to variable biotic and abiotic climatic parameters. Pinus seedlings are grown from seeds in local private and forest nurseries in various areas of Azad Jammu and Kashmir.

Pinus seed associated mycoflora was found comprising of nine species and its presence is very common. The quality and quantity of fungi borne on seeds was variable due to different ecological zones and these findings are in coincident with previous work researchers (Tariq *et al.*, 2005; Bhutta *et al.*, 1999). The study revealed isolation of 11 fungal species belonging to 9 genera including *Aspergillus niger, Aspergillus flavus, Botrytis* sp., *Botryosphaeria* sp., *Cladosporium cladosporioides, Drechslera* sp., *Fusarium* sp., *Penecillium* sp., *Rhizopus stolonifer, Rhizopus oryzae* and *Mucor* sp. from *Pinus roxburghii* seeds from different localities. Among these *Aspergillus* species are more toxic and pathogenic and they

deteriorate seed quality and health (Trappe, 1961). It hampers not only seed germination but also it retards the growing process of seedlings such as radical and plumule length (Tables 5 & 6; Figs. 2 & 3) and similar facts about toxicity of fungi is prescribed in past research (Orisi *et al.,* 2000; Ghiasian *et al.,* 2004; Hussain *et al.,* 2013). Bilgrami & Ghaffar (1993) detected *Aspergillus niger, Aspergillus flavus, Penecillium* sp., *Cladosporium, Fusarium* sp., and *Mucor* sp., from *Pinus geradiana* and they claimed that these fungi are causal agents of important seed diseases.

Various protocols are being used by different researchers to isolate and identify the seed borne mycoflora from various tree species. In this research, two protocols *viz*:, blotter and agar plate method were employed for analysis of seed borne fungi from seeds of *Pinus roxburghii* in order to produce infection free seeds (Ahmad *et al.,* 1993; Sahu & Agarwal, 2004). APM analysis recognizes mycoflora more precisely than blotter paper method (Table 2, Fig. 1) which coincides with previous findings (Nayyar *et al.,* 2013). It was found that investigated mycoflora has more incidence in Bhimber and Samahni areas of Azad Kashmir which also situated in sub-tropical zone and this might be due to warm and tropical environment of the section which afford suitable environment of fungal propagation (Ishtiaq *et al.,* 2012; 2013; Ghildiyal *et al*., 2009). This is a first report of fungi found on *Pinus roxburghii* seeds and its impacts on seed quality and seed growth in Azad Jammu and Kashmir.

Table 3. Determination of pathogenic fungi and their effect on seed vigour of *Pinus roxburghii* in different localities of Azad Kashmir by using DMRT.

Fungus infested	Germinated seeds	Rotted seeds	Abnormal seedling	Healthy seedling
Aspergillus flavus	20.50d ± 0.32	79.50a ± 0.91	5.50b ± 0.49	15.0d ± 0.17
Aspergillus niger	25.75c ± 0.42	74.25b ± 0.67	7.0c ± 0.57	18.75c ± 0.24
Fusarium sp.	15.75e ± 0.54	84.25a ± 0.81	10.0a ± 0.36	5.75e ± 0.21
Rhizopus sp.	35.75b ± 0.12	64.75c ± 0.47	6.0b ± 0.81	29.25b ± 0.78
Control	65.50a ± 0.25	34.5d ± 0.78	2.0d ± 0.95	63.50a ± 0.80

Means within column followed by the same letter(s) are not significantly different (p≤0.05)

Table 4. Effect of fungal treatments on *Pinus roxburghii* seed germination (%) in different localities of Azad Jammu and Kashmir.

S. #.	Treatments	% Age of seed germination					Means
		Bhimber	Samahni	Mirpur	Kotli	Muzaffarabad	
1.	*Aspergillus flavus*	24b ± 0.59	36b ± 0.46	28b ± 1.34	28b ± 0.78	56a ± 1.45	35.20
2.	*Aspergillus niger*	40a ± 0.32	32b ± 0.19	32b ± 0.89	40ab ± 0.18	56a ± 0.98	40.0
3.	*Fusarium* sp.	28b ± 0.87	44ab ± 0.43	36ab ± 0.65	52a ± 0.74	32b ± 0.25	37.6
4.	*Rhizopus* sp.	36a ± 1.23	40a ± 0.74	32a ± 0.25	40a ± 0.39	52a ± 0.28	40.0
5.	Control	52a ± 0.54	60a ± 0.49	64a ± 0.95	68a ± 0.67	64a ± 0.16	61.6
	Means	**36.0**	**42.40**	**38.4**	**45.6**	**52.0**	

Means within rows followed by the same letter(s) are not significantly different (p≤0.05) by using DMRT

Table 5. Effect of fungal treatments on radical length (mm) of *Pinus roxburghii* in different localities of Azad Jammu and Kashmir.

S. #	Treatments	Radical length (mm)					Means
		Bhimber	Samahni	Mirpur	Kotli	Muzaffarabad	
1.	*Aspergillus flavus*	24.6e ± 0.24	30.0d ± 0.28	38.1b ± 0.16	32.1c ± 0.16	46.6a ± 0.44	34.28
2.	*Aspergillus niger*	36.8a ± 0.44	37.8a ± 0.44	30.1b ± 0.44	27.8c ± 0.44	29.5b ± 0.28	32.40
3.	*Fusarium* sp.	34.8c ± 0.44	39.1b ± 0.60	24.1e ± 0.16	42.5a ± 0.28	30.0d ± 0.57	28.70
4.	*Rhizopus* sp.	31.0c ± 0.28	44.1a ± 0.72	29.6c ± 0.33	41.1b ± 0.12	30.0c ± 0.57	35.16
5.	Control	51.1ab ± 0.60	52.5a ± 0.60	52.5a ± 0.28	50.3bc ± 0.33	52.5a ± 0.50	51.04
	Means	**35.68**	**39.96**	**34.88**	**38.76**	**37.72**	

Means within rows followed by the same letter(s) are not significantly different (p≤0.05) by using DMRT

Table 6. Effect of fungal treatments on plumule length (mm) of *Pinus roxburghii* in different localities of Azad Kashmir by water agar method.

S. #	Treatments	Plumule length (mm)					Means
		Bhimber	Samahni	Mirpur	Kotli	Muzaffarabad	
1.	*Aspergillus flavus*	20.5d ± 0.28	19.5d ± 0.28	24.8c ± 0.44	30.0b ± 0.57	35.5a ± 0.28	26.06
2.	*Aspergillus niger*	27.6b ± 0.44	18.0d ± 0.50	19.8c ± 0.16	20.0c ± 0.28	45.0a ± 0.28	26.08
3.	*Fusarium* sp.	29.5b ± 0.28	30.9a ± 0.45	19.8c ± 0.44	29.3b ± 0.33	20.0c ± 0.28	25.90
4.	*Rhizopus* sp.	26.0b ± 0.28	25.5b ± 0.28	16.0c ± 0.50	26.3b ± 0.33	40.3a ± 0.16	26.80
5.	Control	45.5c ± 0.28	42.6c ± 0.66	42.5b ± 0.28	45.0b ± 0.28	49.1a ± 0.16	44.94
	Means	**29.82**	**27.30**	**24.58**	**30.12**	**37.98**	

Means within rows followed by the same letter(s) are not significantly different (p≤0.05) by using DMRT

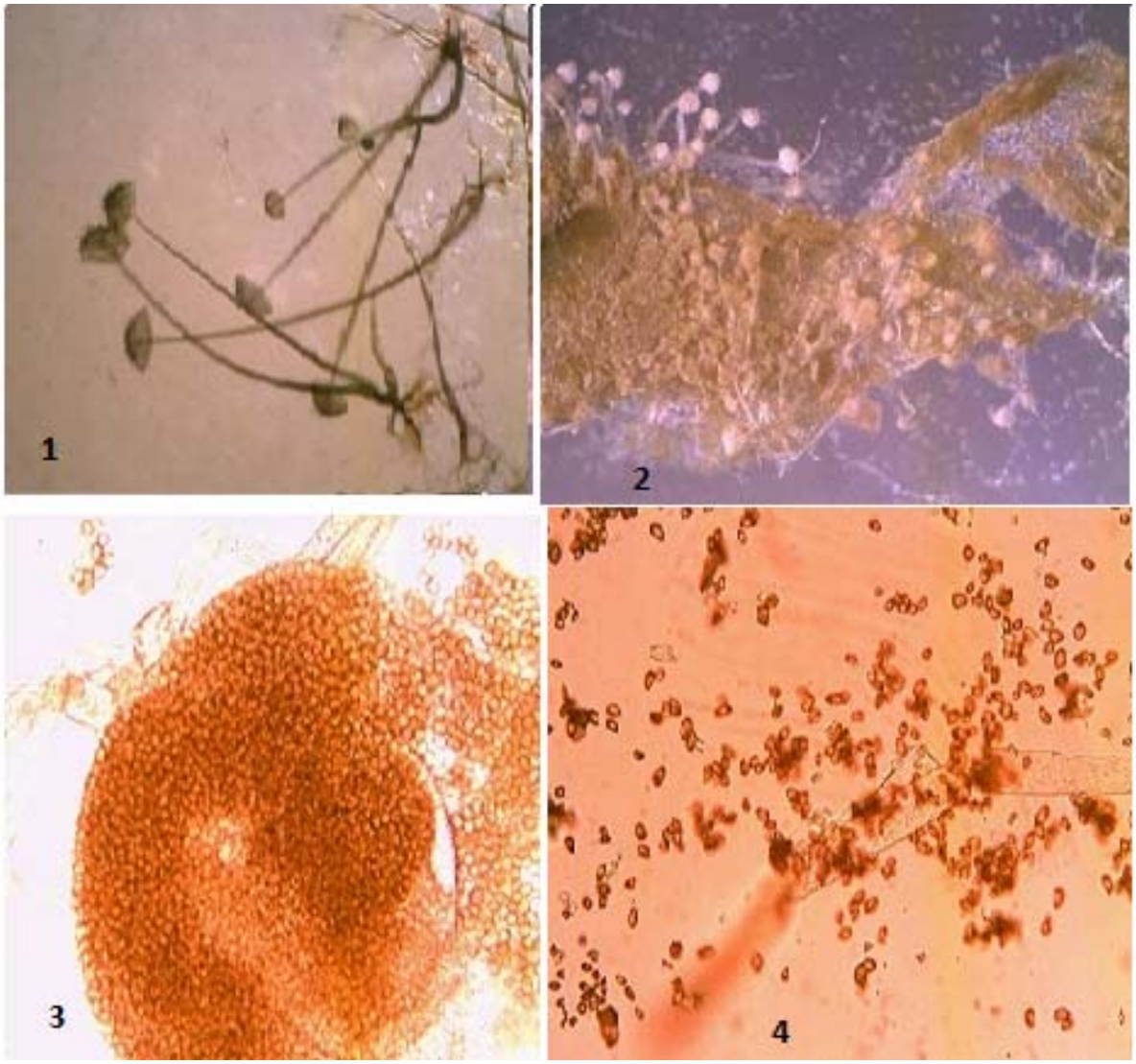

Fig. 4. Different species of fungi isolated from *Pinus roxburghii* seeds from Azad Kashmir.
Key: 1: *Rhizopus oryzae;* 2: *Aspergillus* sp; 3: Sporangium of *Aspergillus* sp; 4: *Cladosporium cladosporioides*

The high incidence of *Aspergillus niger, Aspergillus flavus, Fusarium* and *Cladosporium cladosporioides* species are found in all areas of temperate, tropical and subtropical zonation. Due to their multiplicative mechanism these taxa are found to be prevalent in many forest areas of world (Renata *et al.,* 2004). *Fusarium* sp. is frequently occurring in seed borne fungi and also blamable for seed decline and seedling death. Various workers testified the association of different *Fusarium* spp. with seeds of forest trees such as *Cedrus deodara* (Mittal *et al*., 1993).

Different species of *Aspergillus*, *Penicillium* and *Rhizopus* are testified and reported to decrease the germination of seeds and impairment the seeds in storing. *A. flavus* and *A. niger* were the largest storage fungi of groundnut seeds (Mukherjee *et al*., 1992), bottle gourd seeds (Sultana & Ghaffar, 2009), sunflower seeds (Sharfun *et al*., 2005) and soybean seeds (Tariq *et al.,* 2005). *Rhizopus* has been stated on ground nut seeds (Rasheed *et al.,* 2004). These species have been reported to decrease the propagation of seed and damage the seeds in storage.

It may be attributed to seeds as extensive loss during germination by influence inhibition of seed germination, seed rot, root rot, collar rot, and damping off of seedlings which have been reported by other workers (Ghiasian *et al.,* 2004). In these studies, the culture deposit of most fungi *Aspergillus*, *Fusarium and Rhizopus* reduced seed germination, weakened seedling vigour and length of radical and plumule was inhibited to various extents. Ghildiyal *et al.* (2007) determined the consequences of many fungi such as *Alternaia, Curvularia, Fusarium and Phoma* on plant species falling seed germination and seedling vigour which corroborated with our research results. The identified species exposed variable stunted growth rate with differences but formerly do block the germination of seeds and seedlings growth, and comparable results have been earlier reported in several studies (Ahmad *et al*., 1993; Mittal, 1983). The results of *Pinus roxburghii* seed analysis revealed that *Fusarium*

moniliforme has maximum infestation and septicity rate in the area of Azad Kashmir which severely obstructs the seed germination and seedling growth due to assembly of mycotoxins and these findings corroborates with past work of scientist (Domijan *et al.,* 2005.

As outcomes of this research reveal that *Fusarium* and other species do inhibit damaging influences on seed germination of Pinus and same result was expressed by Mathur & Seghal (1964) in their study on other species. The results also showed that Bhimber has the most infection rate that may be due to its damp and tropical geography and plain area helping spore dispersion by air stream mechanism (Tanveer *et al*., 2010; Ishtiaq *et al.,* 2008; 2010). Results collected from different nurseries of Azad Jammu and Kashmir showed there is a huge loss during the germination of Pinus seed and after growth too seedlings are affected. This loss is due to fungal pathogens or other entities which may be insects or protozoans. Mostly inhibition of seed germination is due to fungal species or spores which are present on surface or inside the seeds. But it is further recommended comprehensive research may be arranged consisting of more number of taxa from various populations of all major areas of Azad Jammu and Kashmir. This type of study will produce better recommendatory findings on gymnosperms of Samahni and other coniferous forest areas of Azad Kashmir and Pakistan.

Conclusion

Seed borne mycoflora can impact *Pinus roxburghii* germination, seedling growth and population density through different sources. *Aspergillus* and *Fusarium* species are most prevalent than others species on seed surface of pinus. Seed vigor testing is a privilege for the controlling of seed borne infection by determining its severity and it confirms that fungi do hither it. Analysis reveal that agar plate method was some better than blotter method. The climate of an area also controls type and populations of fungi on seeds of *Pinus roxburghii* and fungi density and its infection rate do vary with climatic changes.

References

Ahmad, D., S. Iftikhar and A.R. Bhutta. 1993. Seed-borne microorganisms in Pakistan. Checklist, 1991. PARC, Islamabad, Pakistan. 32.

Ahmed, M.U., M.N. Wahab, K. Khanand and S. Hussain. 2009. Phytosociology of *Pinus roxburghii* Sargent (chirpine) in lesser Himalayan and Hindukadh range of Pakistan. *Pak. J. Bot.,* 41(5): 2357-2359.

Barnett, H.L. and B.B. Hunter. 1998. Illustrated genera of imperfect fungi 4th Edition. St. Paul, MN, APS Press. 218.

Bhutta, A.R., R.M Bhatti and I. Ahmad. 1999. Effect of seed diffusates on growth of seedborne fungi of sunflower. *Helia*., 22(31): 143-50.

Bilgrami, Z. and A. Ghaffar. 1993. Detection of seed-borne mycoflora in *Pinus gerardiana. Pak. J. Bot.,* 25(2): 225-31.

Domijan, A.M., M. Peraica, V. Zlender, B.Z. Jurjevic, P.S. Topolovecand and D. Ivic. 2005. Seed-borne fungi in common bean seeds. *J. Food and Chem. Toxicol.,* 43(3): 427-432.

Domsch. K.H., W. Games and T. Anderson. 1980. Compendium of soil fungi. Vol. 1. Academic Press, pp. 859.

Edwards, D.G. 1981. The role seeds and seed research in combating the exploitation of the world's forest resources. *Seed Science and Technology*, 12: 757-765.

Elmer, W.H., H.A. Yang and M.W. Sweetingham. 2001. Characterization of *Colletotrichum gloeosporioides* isolates from ornamental lupines in Connecticut. *Plant Disease*, 85: 216-219.

Ghiasian, S.A., B.P. Kord, S.M. Rezayat, A.H. Maghsood and K.H. Tahir. 2004. Mycoflora of Iranian maize harvested in the main production areas. *J. Mycopathol.*, 158: 113-121.

Ghildiyal, S.K., C.M. Sharma and S. Gairola. 2009. Environmental variation in seed and seedling Characteristics of *Pinus roxburghii* Sarg. *Apl. Eco. Envir. Res.,* 7(2): 121-129.

Ghildiyal, S.K., C.M. Sharma and V.P. Khanduri. 2007. Improvement in germination of chirpine (*Pinus roxburghii*) by a pre sowing treatment with hydrogenperoxide. *J. Trop. Fors. Sci.,* 19(2): 113-118.

Habib, A., S.T. Sahi, M.U. Gh. Gzanfar and S. Ali. 2009. Location of seed-borne mycoflora of Eggplant (*Solanum melongena* L.) in different seed components and impact on seed germinability. *Inter. J. of Agri. & Bio*., 3: 514-516.

Hussain, N., A. Hussain, M. Ishtiaq, S. Azam and T. Hussain. 2013. Pathogenicity of two seed-borne fungi commonly involved in maize seeds of eight districts of Azad Jammu and Kashmir, Pakistan. *Afri. J. Biotech*., 12(12): 1684-5315.

Hussain, T., M. Ishtiaq, A. Hussain, K. Sultana and M. Ashraf. 2011. Incidence of fungi in water springs of Samahni Valley, District Bhimber, Azad Kashmir, Pakistan. *Int. J. Bio. Cand.*, 2(2): 94-101.

Ishtiaq, M. and M.A. Khan. 2008. An Ethnopharmacological Inventory of plants used in midwifery in Tehsil Samahni, District Bhimber Azad Kashmir, Pakistan. *Ind. J. Trad. Knowl*., 7(2): 277-283.

Ishtiaq, M., H. Tanveer, H. Altaf, K. Sultana and M. Ashraf. 2010. Incidence of fungi in water springs of Samahni Valley, District Bhimber, Azad Kashmir, Pakistan, *International Journal of Biology Canada*, 2(2): 94-101.

Ishtiaq, M., S.A. Mumtaz, T. Hussain and A. Ghani. 2012. Medicinal plant diversity in the flora of Leepa Valley, Muzaffarabad (AJK), Pakistan. *Afr. J. Biotech.*, 11(13): 3087-3098.

Ishtiaq, M., W. Hanif, M.A. Khan, M. Ashraf and A.M. Butt. 2007. An ethnomedicinal survey and documentation of important medicinal folklore food phytonims of flora of Samahni Valley, (Azad Kashmir) Pakistan. *Pak. J. Biol. Sci*., 10(13): 2241-2256.

Kirti, J., N.S.K. Harsh, P.S. Rauatand and A.N. Shukla. 2004. Mycoflora associated with different seed sources of *Pinus wallichiana*. *Ind. Forester,* 130(12): 1410-14.

Langenheim, L.H. 2003. Plant resins chemistry, evaluation, ecology and ethnobotany. Timber press, Auckland, New Zealand, pp. 453-54.

Marsh, S.F. and G.A. Payne. 1984. Preharvest infection of corn silks and kernels by *Aspergillus flavus*. *Phytopath*., 60: 1775-77.

Martín, P., J. Pajares and J. Díez. 2008. Pathogenicity of *Fusarium verticillioides* and *Fusarium oxysporum* on *Pinus nigra* seedlings in northwest Spain. *Forest Path*., 38(2): 78-82.

Mathur, R.L. and S.P. Sehgal. 1964. Fungal mycoflora of seeds of jowar (*Sorghum vulgare*) its role in reduced emergence and vigor of seedling and control. *Ind. J. Phytopath.,* 17: 227-233.

Mittal, R.K. 1983. Studies on the mycoflora and its control on the seeds of some forest trees.*Cedrus deodara* I. *Canadian J. Bot.,* 61(1): 197-201.

Mittal, R.K. and B.P. Wang. 1993. Effects of some seed-borne fungi on *Picea glauca* and *Pinus strobes* seeds. *Europ. J. Forest Path.,* 23: 138-146.

Mittal, R.K., S.P. Wang and D. Harmsworth. 1993. Effects of extended prechilling on laboratory germination and fungal infection in seeds of white spruce and eastern white pine tree. *Planters' Notes,* 38(4): 6-9.

Mohanan, C., K.C. Chacko, A. Chandran and G. Varma. 2005. Seed health problems in tropical forest tree seeds and their impact on seedling production.

Muhammad, S.A., Z.H. Malik, N.Z. Malik and M.A. Sadia. 2012. The position of *Pinus roxburghii* in the forests of Kotli hills, Azad Jammu and Kashmir. *Afri. J. Pl. Sci.,* 6(3): 106-112.

Mukherjee, P.S., S.K. Nandi and B. Nandi. 1992. Changes in groundnut seeds in storage. *J. Mycopath. Res.,* 30(2): 113-119.

Mustafa, A. 2009. Seed mycoflora of Shisham (*Dalbergia sissoo* Roxb.) and their integrated management. Ph.D. Thesis, Univ. of Faisalabad.

Nayyar, B.G., A. Akram, M. Arshad, S M. Mughal, S. Akhund and S. Mushtaq. 2013. Mycoflora detected from seeds of *Sesamum indicum* L. in Sialkot, Pakistan. *J. Pharm. and Bio. Sci.,* 2319-7676, 7: 99-103.

Nazar, H., A. Hussain, M. Ishtiaq, S. Azam and T. Hussain. 2013. Pathogenicity of two seed-borne fungi commonly involved in maize seeds of eight districts of Azad Jammu and Kashmir, Pakistan. *Afri. J. of Biotechnol.*, 12(12):1363-1370.

Orisi, R.B., B. Correa, C.R. Possi, E.A. Schammass, J.R. Nogueira, S.M. Dias and M.A. Malozzi. 2000. Mycoflora and occurrence of fumonisins in freshly harvested and stored hybrid maize. *J. Stor. Prod. Res.,* 36: 75-87.

Puri, A., A.K. Srivastava, B. Singhal, S.K. Mishra, S. Srivastava and V. Lakshmi. 2011. Antidysentery and antioxidant activity of *Pinus roxburghii* needles. *Med. Chem. Res.,* 20(9): 1589-93.

Rasheed, S., S. Dawar, A. Ghaffar and S.S. Shaukat. 2004. Seed borne mycoflora of groundnut. *Pak. J. Bot*., 36: 199-202.

Rawat, P.S., N. Punj, K. Joshi and N.S. Harsh. 2006. Evaluation of seed borne fungi of *Pinus roxburghii. The Ind. Fors. J.,* 132: 0019-4816.

Renata, P., J. Culig and I. Hrga. 2004. Atmospheric concentrations of *Cladosporium* sp. and *Alternaria* sp. spores in Zagreb and effects of some metrological factors. *Agric. Envir. Med*., 11: 303-307.

Runion, G.B. and W.D. Kelley. 1991. Effects of triadimefon and thiram seed treatments on emergence of southern pines. *Seed Science & Technology*, 19: 57-66.

Sahai, A. and B.S. Mehrotra. 1982. Mycoflora associated with the seeds of forest trees and their effect on germination. *Pro. Indian Sci. Acad.*, 5: 706-713.

Sahu, R.K. and V.K. Agarwal. 2004. Mycoflora associated with the seeds of *Leucaena leucocephala*. *Ind. Forester,* 130(9): 1040-1044.

Sesan, T.E. and I. Taut. 1998. Mycoflora associated with conifer seeds and seedlings. *Revista-Padurilor*, 113(1): 7-16.

Sharfun, N., M. Mushtaq and M.H. Hashmi. 2005. Seed-borne mycoflora of sunflower (*Helianthus annuus* L.). *Pak. J. Bot.,* 37: 451-457.

Shuaib, M., M. Ali, J. Ahmed, K.J. Naquvi and M.I. Ahmed. 2013. Pharmacogonacy of *Pinus roxburghii*: A review. *J. Pharm. Phytochm*., 2278- 4136, 2(1): 262-268.

Steel, R. G.D. and J.H. Torrie. 1980. Principles and procedures of statistics. 2nd Ed. Mcgraw Hill Booknco., New York, USA.377-398.

Steel, R.G.J., H. Torrie and D. Dickey. 1996. Principles and procedures of statistics*: A Biometrical Approach,* 3rd edition. Mc Graw-Hill, New York, U.S.A.

Storer, A.J., T.R. Gordon and S.L. Clark. 1998. Association of pitch canker fungus, *Fusarium subglutinans*f. sp. *pini*, with Monterey pine seeds and seedlings in California. *Plant Path.,* 47: 649-56.

Sultana, N. and A. Ghaffar. 2009. Seed borne fungi associated with bottle gourd *Lagenaria siceraria*. *Pak. J. Bot*., 41: 435-442.

Sutherland, J. R. 1995. Seed borne fungi of conifers. http://www.rngr.net/Publications/ttsm/ch6 177-90.

Sutherland, J.R., T. Miller and R.S. Quenard. 1987. Cone and seed disease of North American conifers. North American Forestry Commission Pub Number 1; Publication 1.

Tanveer, H., M. Ishtiaq, A. Hussain, K. Sultana and M. Ashraf. 2010. Incidence of fungi in water springs of Samahni Valley, District Bhimber, Azad Kashmir, Pakistan, *International Journal of Biology Canada*, 2(2): 94-101.

Tariq, M., S. Dawar, M. Abid and S. Shaukat. 2005. Seed-borne mycoflora of soyabean. *Int. J. Biol. Biotech.,* 2: 711-7113.

Trappe, J.M. 1961. Strong hydrogen peroxide for sterilizing coats of tree seed and stimulating germination, *J. of Forestry,* 59: 828-829.

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