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Full Length Research Paper

Pathogenicity of two seed-borne fungi commonly involved in maize seeds of eight districts of Azad Jammu and Kashmir, Pakistan

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This study was aimed to evaluate the pathogenicity of two mostly prevailing fungal species on maize (*Zea maize* L.) from different localities of Azad Jammu and Kashmir (AJK), Pakistan. Pathogenic fungi deteriorate food grains by producing mycotoxins and aflotoxins during storage consequently shedding menace on its nutritive quality. There were 12 species of mycoflora associated with maize seeds in the analyzed samples. *Fusaium* and *Aspergillus* species were the largest group of seed-borne fungal species present in all localities (87.25% and 82.50%, respectively). *Fusarium moniliforme* causes ear rot, kernel rot, stalk rot, seedling blight, seed rot, wilt and stunt and *Aspergillus niger* is responsible for rot of stored grain. These species were tested to determine their pathogenicity to maize seed germination and seedlings. Pathogenicity findings depicted that maize variety under cultivation in the area was highly susceptible to these fungal pathogens as Bhimber (61.5%) and Mirpur (60.25%) zones had more prevalence than other areas of AJK (23.5%). Results show that *Fusarium moniliforme* had 50.2% pathogenicity on seeds and 6.55% on seedlings, whilst *Aspergillus niger* had 62.87% on seeds and 11.24% on seedlings. These depicts that mycoflora had significant detrimental impacts on seeds and seedling's life of maize.

Key words: Pathogenicity, seed-borne, mycoflora, maize, germination, Azad Kashmir.

INTRODUCTION

Maize (*Zea maize* L.) is the member of *Poaceae* family and it is the world's top most cereal crop in terms of total production and productivity after wheat and rice (FAO, 2006). It is grown and cultivated in all parts of the world. It covers ca.75% of the world food supplies (Cassin and Cootti, 1979). Maize is the third most important cereal cultivated in many areas of Pakistan after wheat and rice crops (Fageria et al., 1997). It is cultivated on 0.9355 million hectares annually with production of about 1.7371 metric tones with an average yield of 1857 Kg/hectares (Anon., 2007). The share of maize in grain production is 1.8 million tons per annum (GOP, 2006). It also ranks third in acreage production and yield in Pakistan. In Pakistan, it is mainly grown in Khyber Pakhtoonkhawa (KPK) province (with 68% yield) and Punjab is second with 30% of total maize grain production. However, very little maize (2 to 3%) is produced in province of Sind, Baluchistan and Azad Jammu Kashmir (GOP, 2007).

The crop is infected and affected by different microbes which not only impede its growth but also cause loss of yield, consequently leading to starvation scenario. The microbial infestation causes a huge food loss (30% total production) in the world (Chhokar, 2001) and predominant share is due to mycofloral infection and deterioration (Agrios, 1997; Chandler, 2005). It is predicted in the previous research that more than 60 different diseases are caused by various pathogens affecting maize crop (Anon, 2007) and fungi is dominant

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in this plethora. Pathogenic fungi deteriorate food grains by producing mycotoxins and aflotoxins during storage consequently shedding menace on its nutritive quality (Park et al., 2004; Koirala et al., 2005; Domijan et al., 2005). The most predominant fungi infecting seed germplasm were *Aspergillus* and *Fusarium* species (Askun, 2006; Fandohan et al., 2003). Anne et al., (2000), Curtui et al., (1998) and Susan et al., (2005) isolated several *Fusarium* species from maize seed in previous research. *Fusarium* and *Aspergillus* species were found as common fungal contaminants of maize that produces high mycotoxins (Bakan et al., 2002; Verga et al., 2005).

F. moniliform produces gibberela ear rot, kernel rot, stalk rot, seedling blight, seed rot, wilt and stunt (Thiel et al., 1991). *Aspergillus* species affects systemically and produces aflatoxin in seedling of maize and damage stored corn (Blat, 1969). Maize is also infected by downy mildew pathogen (Adenle and Cardwell, 2000; Ajala et al., 2003; Ahmed et al., 2006). *Fusarium* spp., invade more than 50% maize grain before harvest and produces mycotoxins (Bakan et al., 2002). The rank of fungi is second after insects as the cause of deterioration and loss of maize (Ominski et al., 1994).

Azad Kashmir is beautiful vale with great potential of biodiversity and cereal crops. A huge quantity of maize is produced in different areas to meet the nutritional and other economic aspects of the communities. The purposes of this study were diverse including: firstly, to find general mycoflora associated with seeds of maize in different districts of Azad Jammu and Kashmir (AJK) region; secondly, to determine infestation mechanism of *Fusarium* and *Aspergillus* species on maize seeds, its severity and consequent impedes on yield; and thirdly, to do a comparative analysis on the severity of these taxa, determine incidence frequency in different localities of study area, and explore the correlation of mycoflora on maize cultivars in different environmental conditions.

MATERIALS AND METHODS

Sample collection

Samples of different varieties were collected spatially and temporally from the peasant's field plots from eight different districts of Azad Jammu and Kashmir (AJK): Mirpur, Bhimber, Kotli, Sudhnoti, Poonch, Bagh, Muzaffarabad and Neelum. 1 kg composite sample was taken at random from a source of size; one ton seeds from each sampling site (farmer's germplasm stock). For control experiments seeds, were purchased from National Agricultural Research Center (NARC) Islamabad. These samples were transported in polythene bags for investigation in Plant Pathology Laboratory, University of Azad Jammu and Kashmir, Muzaffarabad. These maize seed samples were further analyzed for mycoflora screening and determination of their pathogenicity.

Isolation of fungi

Infected seeds were sterilized by immersing in 10% household chlorine bleach (NaClO2) for 5 min and rinsed in double distilled water for 5 min and dried for almost 2 min (Elmer et al., 2001). Seeds were plated on potato agar dextrose (PDA) media (Tanveer et al., 2011; Onkar and Sinclair, 1985). Five seeds were placed in each Petri dish with ten replicates and incubated at 30 ± 2°C for seven days. The species and number of fungi were recorded using the Colony counter technique (CCT) as suggested by Dhingra and Sinclair (1985) and Tanveer et al. (2011). The isolated fungi were identified by using taxonomic features such as conidia and hypha (Benoit and Marthur, 1970; Tanveer et al., 2010) from manuals and herbaria specimen/slides preserved at the Department of Botany, MUST, Bhimber Campus, Bhimber Azad Kashmir.

Frequency of fungal infection

Fungal infection frequency (FIF) was calculated to determine the most susceptibility of different seeds of different cultivars to various fungal infections as per protocol of El-Awadi (1993). The total infection percentages of the component plating and seedlings tests were calculated by using following formulae:

$$\text{Infection \%} = \frac{\text{No. of plants affected by a pathogen}}{\text{Total no. of plants}} \times 100$$

$$\text{Colonization \%} = \frac{\text{No. of pieces infected by a pathogen}}{\text{Total no. of pieces tested}} \times 100$$

Pathogenicity evaluation

The pathogenicity of identified fungi isolated from maize seeds was performed *in vitro* according to Koch's postulates by using 2-factorial experiment in Randomized Completely Block Design (RCBD) with 5-replicates. Seeds were surface sterilized with Chlorox (Sodium Hypochlorite 13% v/v) for 5 min followed by thorough rinsing with sterilized water. Five seeds were blotted on sterile filter paper and thereafter inoculated by soaking them in a homogenized mycelial suspension of the test fungus (*Aspergillus flavus*). Then, five seeds per replication were placed on 1% water agar (WA) plates and subsequently incubated at 28 ± 2°C for growth to check the fungal positivity.

A pathogenicity test on seedling was conducted using 4 to 5 days old seedlings grown under aseptic conditions. Seedlings were aseptically transferred to slants of water agar (WA) in test tubes (1/tube). Five seedlings were inoculated per test tube and then incubated in the growth chamber at 25 ± 2°C. Seeds purchased from NARC were grown as seedlings and propagated on same experimental conditions for control comparison. The control samples were sprayed only with distilled water. The symptoms developed on the inoculated maize seeds and seedlings were compared with those of control run experiment. The germination rate of seeds of different cultivars of different districts was determined by keeping all records on paper/computer. The data were formulated in matrix form and proceeded for statistical analysis and evaluation according to the ISTA rules (1996).

Data analysis

The collected data was formulated in tabular format and subjected to statistical analysis according to "Analysis of Variance (ANOVA)" technique by using MVSP and Matlab softwares and means were compared by using least standard deviation (LSD) at $P < 0.05$ level

Table 1. Percentage of mycoflora by using blotter paper on Maize seeds.

Fungi detected	Locality								Total infection
	Mirpur	Bhimber	Kotli	Sudenoti	Poonch	Bagh	M-abad	Neelum	
Fusarium moniliforme	27.25	27.75	5.0	1.25	0.75	4.0	14.5	0.25	80.75
F. semitectum	-	-	0.25	0.75	-	-	1.25	-	2.25
F. graminearum	0.75	0.75	-	0.25	-	-	-	-	1.75
F. equisetti	-	-	0.75	1.25	0.25	-	-	0.25	2.50
Total *Fusarium species*	28.0	28.50	6.0	3.50	1.0	4.0	15.75	0.50	87.25
Aspergillus niger	6.25	7.5	4.0	8.25	7.0	1.5	13.75	15.0	63.25
A. flavus	1.5	3.75	4.25	-	-	1.0	1.5	-	12.0
A. clavatus	-	-	-	-	5.25	-	-	0.75	6.0
A. Fumigatus	-	-	-	-	-	-	-	1.25	1.25
Total *Aspergillus species*	7.75	11.25	8.25	8.25	12.25	2.50	15.25	17.0	82.50
Alternaria alternata	2.5	3.0	3.75	1.5	2.5	3.5	0.75	3.25	20.75
Drechslera maydis	6.75	3.0	3.5	1.5	3.25	0.25	0.5	4.5	23.50
Curvularia lunata	5.75	3.75	4.25	1.25	2.25	4.25	0.25	1.25	23.0
Rhizopus stolonifera	3.5	3.5	5.25	6.25	-	4.5	6.25	3.5	32.75
Penicillium galaucum	3.0	2.75	-	2.0	2.75	4.25	-	1.0	15.75
Mucor spp.	2.25	2.5	3.5	-	1.25	-	1.0	3.25	13.75
Macrophomina phaseolina	0.75	1.5	2.75	-	-	-	0.25	-	5.25
Cephalosporium acremonium	-	1.0	0.75	1.5	1.25	-	-	-	4.50
Nigrospora oryzae	-	0.75	0.75	-	-	0.25	-	-	1.75
Diplodia maydis	-	-	-	-	1.25	-	-	1.75	3.0
Total infections	60.25	61.5	38.75	25.75	27.75	23.5	39.03	36	

(Steel et al., 1996).

RESULTS AND DISCUSSION

The study was designed to determine the different types of maize pathogens. In the analysis, different types of fungi were found with diverse infection severity. The experiment produced a diverse nature of fungal isolates associated with maize seeds sample collected from different localities of eight districts of Azad Kashmir (Table 1). However, two most commonly prevailing seed-borne fungi were studied to determine their pathogenicity on maize seed germination and seedlings. These two pathogenic isolates include *F. moniliforme* and *A. niger* with 80.75% and 63.25% infection, respectively. But in three localities *viz*. Muzaffarabad, Mirpur and Bhimber, these were most prevalent with high infection rate that is 39.05, 60.25 and 61.50%, respectively (Table 1 and Figure 1). Research findings of experiment depict that analysis of variance of the effects of fungal treatment on seeds germination (%) and seedling length (%) have been collected for three localities of AJK, Pakistan which is quite different from others (Table 3). These findings coincide with literature that *Fusarium, Aspergillus* and *Penicilliun* are predominantly found in regions like Pakistan and other tropical areas (Orisi et al., 2000; Ghiasian et al., 2004). The prevalence and severity graph of these genera that is *Fusarium* and *Aspergillus* also proves the theory because the study area (AJK) is part of the sub-tropic zone. So, this is the reason these common fungal isolates from three localities were taken into accounts by preparing PDA slants (Figures 4a, b, c and d) for comparative pathogenicity studies.

The pathogenicity test with two most frequently isolated fungi that is, *F. moniliforme* and *A. niger* was carried out and showed pathogenic effects on seeds germination. These are highly pathogenic seeds-borne fungi that were frequently recorded almost with all samples from all localities and were also reported pathogenic by several other studies (Richardson, 1979; Iftikhar, 1991; Fakhrunnisa and Hashmi, 1992; Ahmad et al., 1993). *F. moniliforme* was found to be highly infective by producing mycotoxins that are involved in retarding seed germination and seedlings growth as also reported by Yates et al. (1997). Results based on the *in vitro* seeds inoculation tests, showed that *F. moniliforme* and *A. niger* had significant effect on the seeds germination and seedlings health due to high infection and subsequent production of mycotoxins (Figures 1, 2 and 3). *F.*

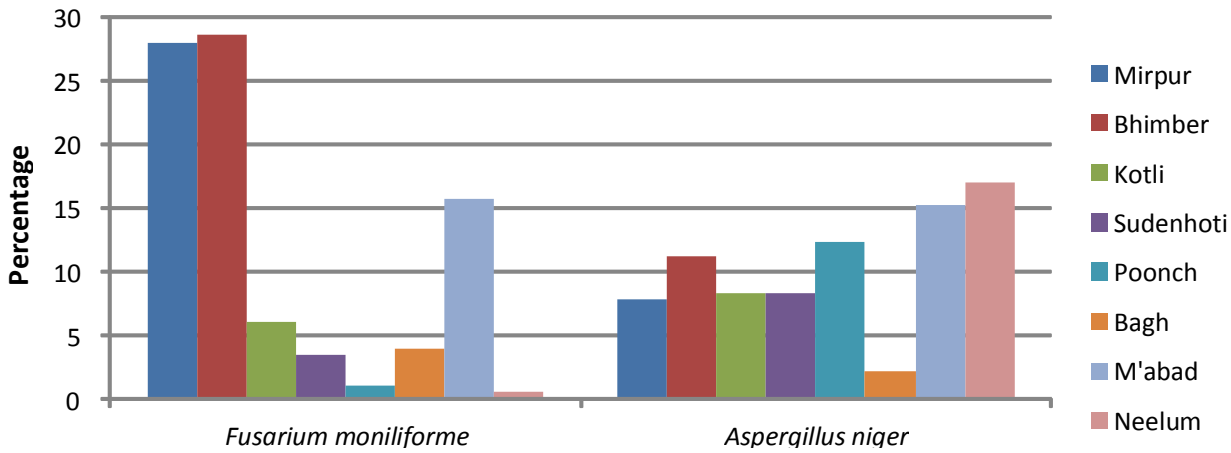

Figure 1. Percentage infection by the two pathogens on maize seeds in different localities of AJK.

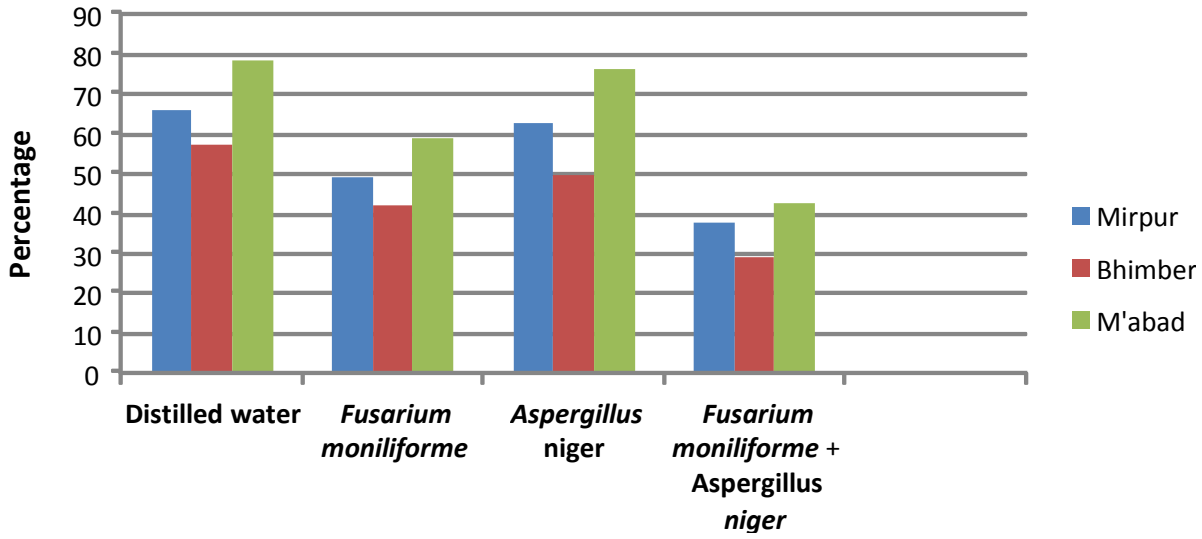

Figure 2. Percentage seeds germination after pathogenic inoculums in different localities of AJK.

moniliforme and *A. niger* affects maize seeds germination greatly due to the high production of mycotoxins (Sinha, 1990).

The comparison of LSD depicted that distilled water (control) treatment on germination had interactive role among three locality cultivars. The control showed significant difference with all other treatments except *A. niger* on Mirpur locality. *F. moniliforme* treatment and *A. niger* showed no interaction at Bhimber locality with Muzaffarabad locality. When the effect on seedling length was compared, it was observed that Mirpur and Muzaffarabad localities with distilled water treatment showed no significance with each other but Bhimber locality was significantly different from all others. Distilled water also showed no significance at Mirpur and Muzaffarabad localities with *A. niger* treatment. *F. moniliforme* treatment showed significant difference with each other but Bhimber locality depicted no major difference. However, *A. niger* were no significantly different from each other in all samples (Table 2). These results are inconformity with those of Arif and Ahmed (1969). *F. moniliforme* is a more deteriorating pathogen as compared to *A. niger* that damaged the roots of the emerging germ-lines. This also caused rotting of the seeds and browning/necrosis of the lateral roots of the seedlings. The seed samples inoculated with *A. niger* showed better growth (62.87%) as compared to *F. moniliforme* (50.20%) however, in the combination form of *F. moniliforme* and *A. niger* sample, the percent germination become decreased (36.53%). Similarly, in seedlings length measurement, it was 11.24, 6.55 and 4.84%, respectively (Figures 5a, b, c and d). Ibrahim and Farag (1965) recorded poor seedling emergence due to *F. moniliforme, Aspergillus* spp., *Penicillium spp., Alternaria alternata* and others. CaldWell et al. (1981) reported *F. moniliforme* as the best pathogenic in maize

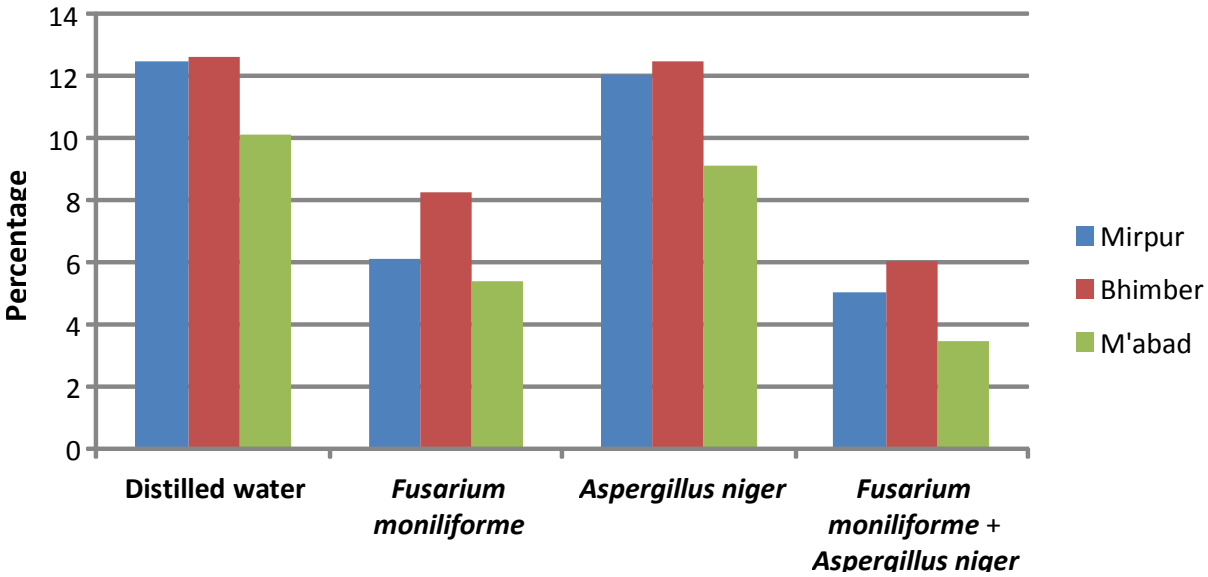

Figure 3. Percentage seedlings length (cm) after pathogenic inoculums in different localities of AJK.

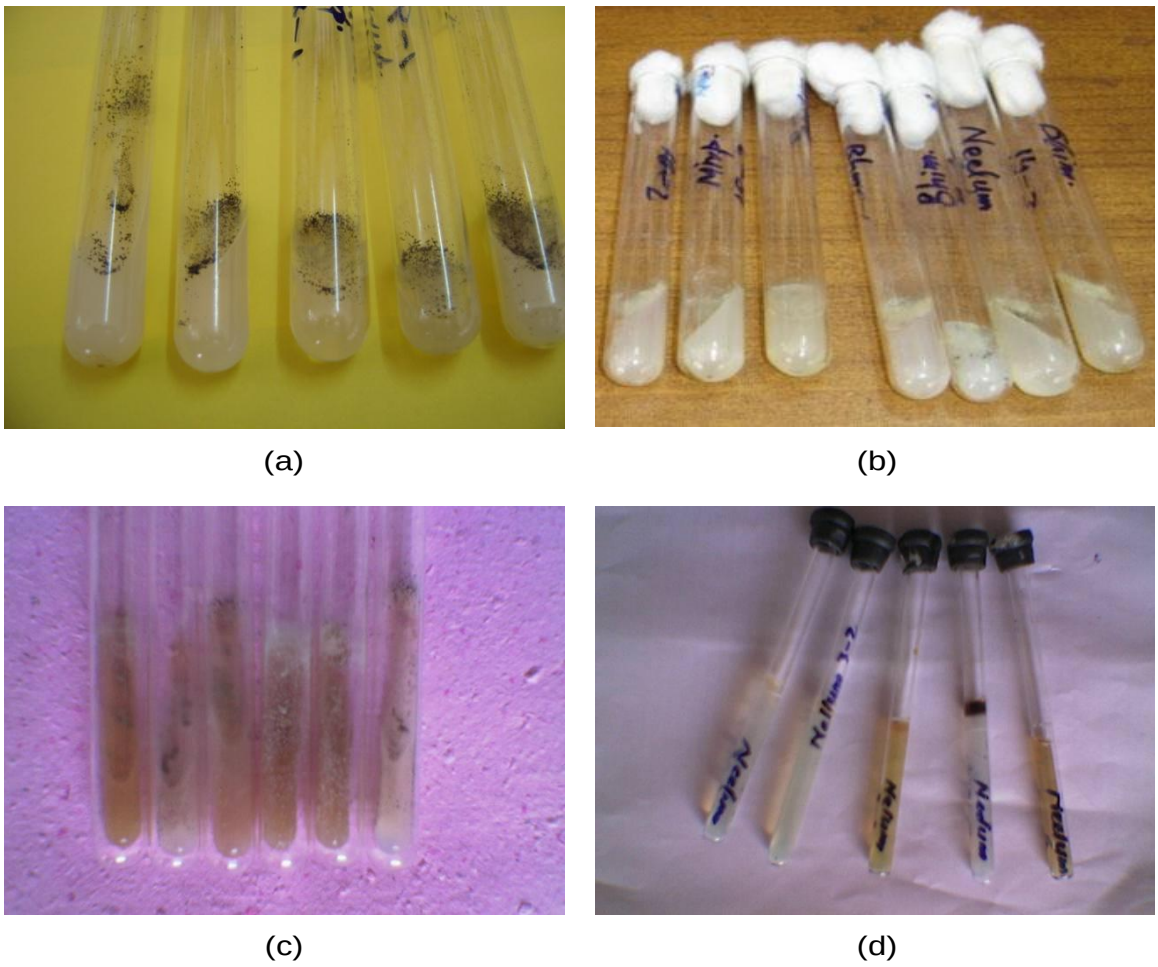

Figure 4. Plates showing PDA cultured slants prepared for testing pathogenicity in maize seeds. (a) *Aspergillus niger* PDA culture in test tubes slants; (b) *Fusarium moniliforme* PDA culture in test tubes slants; (c) Mycoflora culture of different strains; (d) Mycoflora culture of different strains.

Table 2. Effects of fungal treatments on the seeds germination (%) and seedling length (%) in three localities of AJK by using L.S.D.

S/N	Treatment	Germination %			Mean	Seedling % Length (cm)			Mean
		Mirpur	M'abad	Bhimber		Mirpur	M'ab	Bhimbr	
1	Distilled Water	66.20	78.40	57.20	67.27 A	12.48	12.62	10.12	11.74 A
2	*F. moniliforme*	49.40	59.00	42.20	50.20 B	6.08	8.22	5.36	6.55 B
3	*Aspergillus niger*	62.80	76.20	49.60	62.87 A	12.08	12.50	9.14	11.24 A
4	*F. moniliforme and Aspergillus niger*	37.80	42.80	29.00	36.53 C	5.04	6.06	3.44	4.84 C
	Mean	54.05 B	64.10 A	44.50 C		8.92 A	9.85 A	7.01 B	

Values in the same rows and column followed by the same letter are not significantly different (P = 0.05).

Table 3. Analysis of variance of the effects of fungal treatment on seeds germination (%) and seedling length (%) in three localities of AJK.

S/N	Factor		Seed germination (%)	Seedling length (%)
		Df	Mean square	Mean square
1	Treatments (A)	3	2869.794*	175.527*
2	Localities (B)	7	1921.217*	41.771*
3	AB	21	42.861	1.546

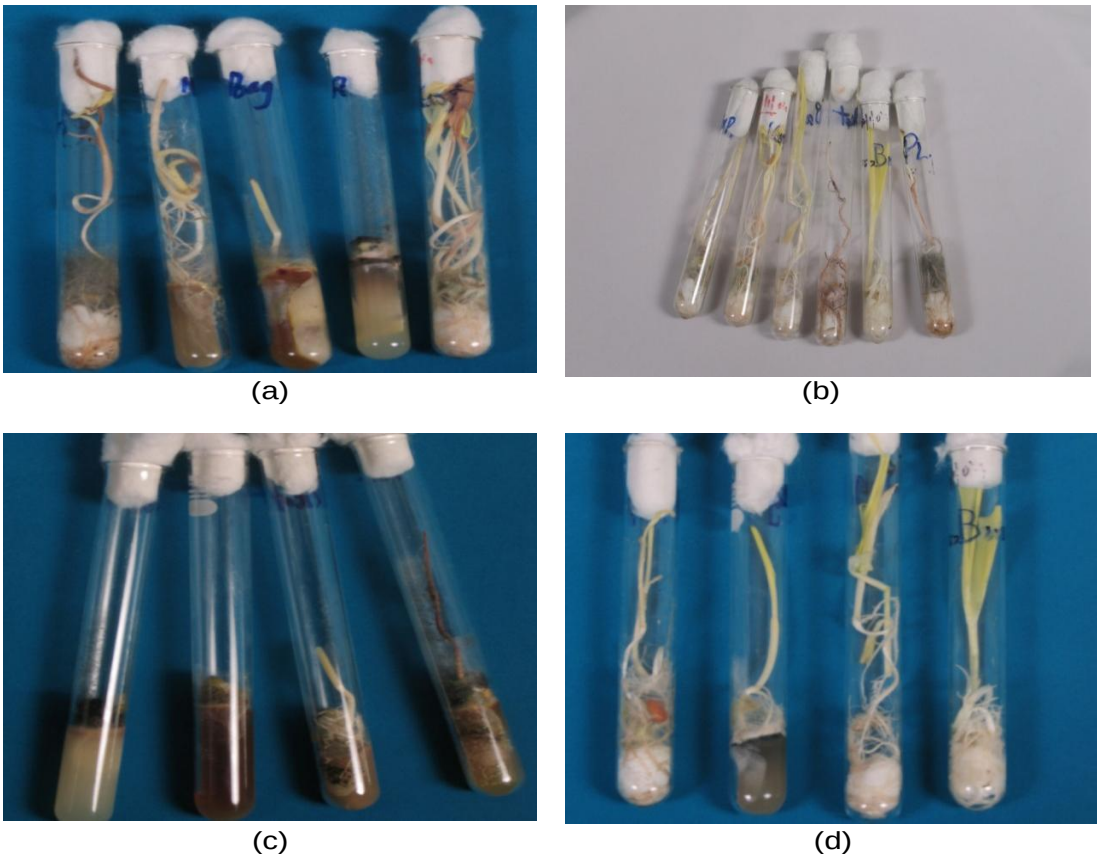

Figure 5. Plates of PDA slants after inoculation with maize seeds and seedlings of pathogenicity testing. (a) *Fusarium moniliforme* seedling inoculation. (b) *Aspergillus niger* seedling inoculation; (c) *Fusarium monliforme+Aspergillus niger* Inoculation on Seedling; (d) *Aspergillus* and *Fusarium* controlled treatment with distilled water.

as others. Randhawa et al. (1998) also reported the pathogenicity of five fungi viz. *F. moniliforme, A. niger, Curvularia lunata, Alternaria alternata* and *Helminthosporium* sativum with percentage germination of 51.66, 81.66, 66.66, 62.50 and 60.1%, respectively against the controlled (89.16%) and recorded maximum pathogenic effect of *F. moniliforme* (37.5%) and minimum of *A.* niger (7.5%) on sorghum seed germination. Mathur and Seghal (1964) reported that in the presence of *Fusarium* species especially *F. moniliforme*, other infections resulted in the inhibition of seed germination.

The study demonstrates that Bhimber (60.25%) and Mirpur (61.5%) areas had more prevalence of fungal species as compared with other areas (AJK) (Table 1) and that might be due to high temperature and humid climate in those districts (Ishtiaq and Khan, 2008). The least germination was in Bhimber (44.5%) with gradual increase in Mirpur (54.05%) and Muzaffarabad (64.01%) due to the favorable climate conditions for plant biodiversity and cereal crops in Muzaffarabad areas (Ishtiaq et al., 2012). As the study was based on laboratory experiments and conclusions drawn are *in vitro* and in controlled parameters *Ipso facto*, the field based pathogenicity of these fungi on maize cultivars should also be subjected to comprehensive research and explored to corroborate or rectify these findings.

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