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Prevalence, incidence, severity and morphological characterization among isolates of karnal bunt (*Tilletia indica*) in Punjab and Khyber Pakhtunkhwa (Pakistan)

Aasma*, Shahzad Asad, Muhammad Fayyaz, Anjum Munir

Crop Diseases Research Institute, National Agricultural Research Centre, Islamabad, Pakistan

Key words: *Tilletia indica*, Karnal bunt, Prevalence, Incidence, Morphological characterization.

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Abstract

Karnal bunt is a disease of stern concern all over the world and causing economic losses in wheat growing areas of Pakistan. The disease is known to occur since long and is the main constraint in exporting the wheat in Pakistan. Scanty work on this disease is done in the past. So keeping in view its significance a study was designed to ascertain the disease situation in wheat production zones of Punjab and Khyber Pakhtunkhwa. The disease has shown its increasing trend with few available reports and the pathogen became established in the places where previously it was not known. The prevalence was calculated from both physically observed grains and wash test while isolations of the isolates were made only from damaged grains. During 2013-14 wheat season the average Prevalence, incidence and severity of Karnal bunt in Punjab were 4.87%, 0.66, 0-2 and 14.87%, 1.19 %, 0-2 respectively whereas in Khyber Pakhtunkhwa, it was found to be 11.33%, 3.02.0-2 and 15.06%, 3.49%, 0-2 respectively. In the current study detailed work on the morphological characterization of *Tilletia indica* was done as the scientific information on pathogen morphology is lacking in Pakistan. Variation was observed in the morphology of 49 isolates on the basis of teliospores size, percentage germination, size of filliform and allantiod sporida (secondary sporidia), growth pattern and colony color. Size of teliospores ranged from 53.09µm (PB-55) to 35.23 µm (PB-68). The germination ranged from 78.53 % (PB-50) to 10.82 % (KPK-17). Continuous monitoring is needed to identify the areas which are free from Karnal bunt pathogen.

* **Corresponding Author:** Aasma ✉ aasmafahad@outlook.com

Introduction

Wheat (*Triticum aestivum* L.) is globally an important crop. During 2017-18 it was planted on an area of 8,734 thousand hectares in Pakistan with an average production 25,492 million tons (Govt. of Pakistan, 2017-18). Diseases are one of the main reasons of wheat yield loss (Jones and Clifford, 1978). Karnal Bunt is one among five smut and bunt diseases that affect wheat worldwide. Common bunt, flag smut and loose smut occur in most wheat growing countries, whereas Karnal bunt and dwarf bunt are less distributed around the world. The disease is subjected to quarantine regulations in many countries of the world. Smut and bunts diseases of wheat are not toxic to humans and livestock (Wright *et al.,* 2013). Pathogen not only reduces the weight but also seriously damages the seed vigor and due to the production of trimethylamine a volatile compound with a characteristic astringency ultimately the quality of the flour deteriorated, which inflicted huge economic loss to the producer (Singh *et al.,* 1983).

In Pakistan, the Karnal bunt has become an alarming disease in recent years due to its prevalence in the main wheat-growing areas of Punjab and Khyber Pakhtunkhwa, Karnal bunt was considered a minor disease in Pakistan, but its incidence has increased up to 20% (Khan *et al.,* 1992). Saleem and Akhtar (1988) reported KB from Sialkot district of Punjab. Mundkur, 1943; Bedi *et al.*, 1949 also reported the disease from Punjab and Khyber Pakhtunkhwa. Previously it was also reported from northern area of Pakistan and later it was found prevalent in Muzaffargarh, Jhang and Khanewal district of the Punjab (Bhatti and Ilyas, 1986).The annual incidence of disease varies widely, depending on favorable climatic conditions during heading. Favorable temperature (20 to 25°C), (Bonde and Smilanick, 1998) in concurrence with high relative humidity allows secondary sporidia to cause disease (Bansal *et al.*, 1984). In Punjab, the incidence of Karnal bunt has multiplied over a time. Khan *et al.* (2010) studied two years climatic data (2006-2007) to ascertain the conditions conducive for Karnal bunt development. They found 84% variability during two years when employed independent variables (max and min air temperature, relative humidity, rainfall and wind speed). A positive correlation existed between the incidence of the disease and secondary spore production. The fungal spores expressed variation in their germination percentage, changes in primary and secondary spore production (Pannu and Chahal, 2000), spore size, and of secondary (filform, allantoid) sporidia (Aasma *et al.*, 2012).Trade in the international market has been badly affected after its outbreak in wheat growing regions of Pakistan. Due to its quarantine importance and since Pakistan has export potential of its surplus wheat, the present study was designed to find out its prevalence, severity, incidence in Punjab and Khyber Pakhtunkhwa.

Materials and methods

Survey and Collection of wheat samples

Major wheat growing agro-ecological zones of Punjab and Khyber Pakhtunkhwa were surveyed during 2013-2014 crop season to find out the prevalence, incidence and severity. Two surveys were conducted in the months of April-June in Punjab zone 5, 6 and 7 and in Khyber Pakhtunkhwa zone 9 and 10. During the first survey, 21 wheat growing districts of Punjab (Fig.1 A) and 8 districts of Khyber Pakhtunkhwa (KPK) (Fig.1 B) were visited while in the second survey 24 districts of Punjab (Fig.2 A) and 11 districts of Khyber Pakhtunkhwa (Fig. 2 B) were visited for the collection of samples. A total of 839 samples from different districts of Punjab and Khyber Pakhtunkhwa were collected in 2013while 1542 samples were collected in 2014(Fig .1 A& B; Fig 2 A & B).

General Protocol for sampling

Each sample consisted of 2kg seeds and was collected randomly from stores and piles before transporting to the silos after harvesting and threshing. The seed samples were properly packed and labeled and were brought to mycology laboratory of Crop Diseases Research Institute (CDRI), National Agricultural Research Centre (NARC) Islamabad for further identification and experimentation.

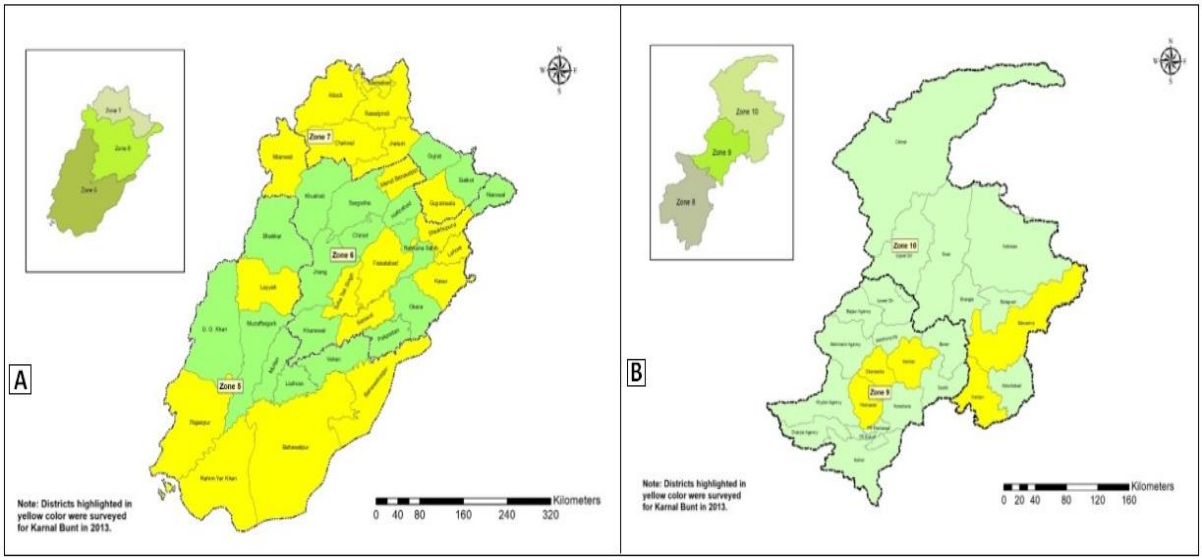

Fig. 1. Map showing the locations surveyed in (A) Punjab and (B) Khyber Pakhtunkhwa for Karnal bunt during 2013.

Identification of Karnal bunt

Karnal bunt disease cannot be identified on the basis of visual symptoms in the field. Initially 1500 seeds per lot were examined visually for the presence of *Tilletia indica* (Fig. 3 A&B; Fig 4A). For further confirmation the seed samples were analyzed for the presence of spores by using the wash test method (Shetty *et al.*, 1988; Begum and Mathur, 1989 and Mathur and Cunfer, 1993). On the basis of wash test, disease distribution was assessed.

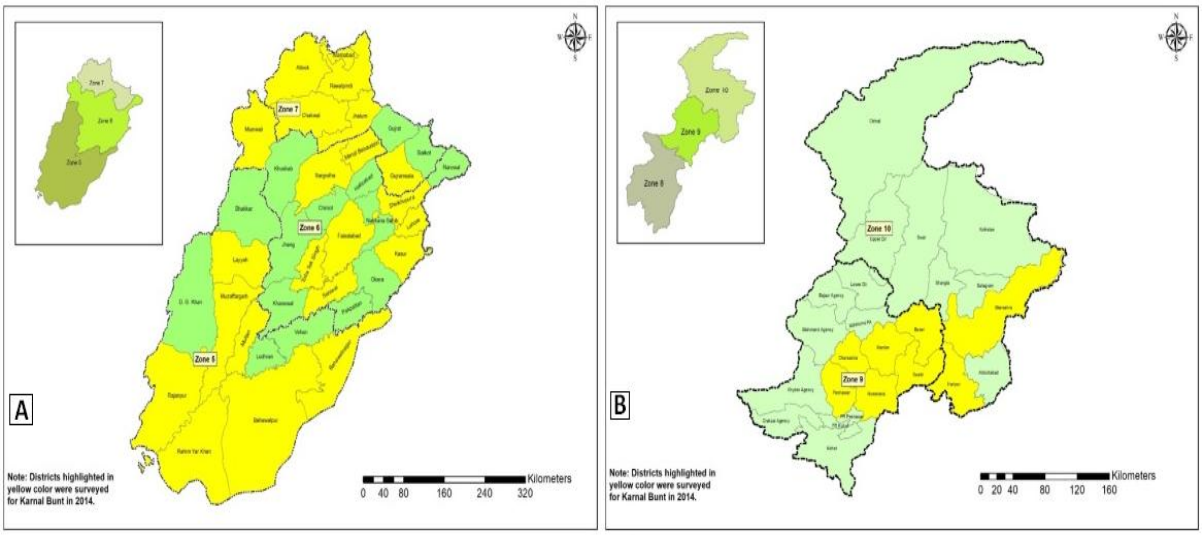

Fig. 2. Map showing the locations surveyed in (A) Punjab and (B) Khyber Pakhtunkhwa for Karnal bunt during 2014.

For the calculation of incidence, prevalence and severity of the diseased wheat seed samples the following formula was used:

$$\text{revalence (\%)} = \text{Locations showing KB} \div \text{Total no. of locations} \times 100$$

$$\text{Incidence (\%)} = \text{No. of infected seeds in sample} \div \text{Total no. of seeds in sample} \times 100$$

The severity was calculated on 0-5 severity scale (Warham *et al.*, 1986; Aujla *et al.*, 1989; Table 1).

Isolation of Tilletia indica

To release teliospores, single infected seed was taken from every sample and was vortexed for 2 min. in 10 ml of sterilized distilled water in 15 ml eppendrof

tube. This spore suspension was passed through a 60 µm sieve and was transferred into the new eppendrof tube and subjected to centrifuge at 12000 rpm for 2 minutes. The pellet was again suspended in one percent sodium hypochlorite solution for 30 seconds to surface sterilized the seed. Sodium hypochlorite was removed by washing spores with sterilized distilled water and centrifuged. Washing was done three times. *Tilletia indica* spores (five drops from the washed spore suspension) of each isolates were plated individually on the solidified water agar with macro-pipette, spread with the help of L shape spreader and incubated at 20± 2° C for 10 days. After incubation, the Teliospores of *Tilletia indica* were germinated and were observed under stereoscope for confirmation. Presence of the star and thread like structure on the medium confirmed the presence of Primary sporidia (Fig.4 B). Later the single teliospores from the colonies of *Tilletia indica* were cultured on Potato Dextrose Agar medium to produce secondary spordia to obtain the pure cultures (Fig. 6 A&B). The mother culture thus produced was labeled and stored in the refrigerator for further use (Pannu and Chahal, 2000). The colonies of *Tilletia indica* developed on water agar from single teliospores of each isolate were multiplied on potato dextrose agar medium for mass culturing. These flasks were incubated at 20± 2 °C for 15 days and later labeled for each individual isolate and placed in refrigerator for further use.

Morphological characterization of Tilletia indica isolates

Isolates collected during the study were purified and morphological data was noted. The shape and color of teliospores was observed from the infected seeds. Firstly, seeds were ruptured with the help of sterilized forceps and were observed under stereo and compound microscope at various magnifications. Ocular and stage micrometer was used to measure the size of teliospores, by taking 50 teliospores randomly from each isolate. Teliospores that were germinated on the water agar plates were calculated for the percent teliospores germination. Total and germinated spores were counted under stereoscope from each isolates after 15 days of incubation period and the percentage germination was calculated by using the following formulae below.

$$\text{Teliospores germination} = \text{No. of germinated teliospores} \div \text{Total teliospores} \times 100$$

Teliospores after germination produced primary sporidia which were transferred to PDA medium. After 10 days of incubation at 20 °C, primary sporidia produced colonies of secondary sporida. The size of secondary sporidia (filiform and allantoid) ;(Fig. 5 A&B) was measured using ocular and stage micrometer by taking 50 sporidia at random from each isolate. The color and growth pattern of all colonies were recorded from 10 days after incubation onwards.

Results

Prevalence, incidence and severity of Karnal bunt in Punjab and Khyber Pakhtunkhwa during 2013

In different districts of Punjab and Khyber Pakhtunkhwa varying degree of Karnal bunt prevalence, incidence and severity was observed.

Table 1. Rating scale used to assess wheat cultivars for Karnal bunt (*Tilletia indica*).

Infection Category/Grade	Symptoms	Assigned numerical value for calculation of (CI)
0	Healthy	0
1*	Inconspicuous point infection (trace) 5% seed bunted	0.25*
2*	Well-developed point infection 25% seed bunted	0.25*
3	Infection spreading along the groove 50% seed bunted	0.5
4	Three-quarters of seed converted to sorus 75% seed bunted	0.75
5	Seed completely converted to sorus 100% seed bunted	1.0

* Categories combined to calculate Coefficient of Infections for comparison.

The prevalence of Karnal bunt on an average in Punjab was calculated 4.87% with the maximum prevalence was calculated in district Jhelum (18.51 %) followed by Chakwal (15.91 %) while the minimum (2%) in Sahiwal. No infection was found in the samples collected from districts Rahim Yar Khan, Attock, Mandibhaudin, Sheikhupura, Gujrat, Kasur, Khusab, TobaTek Singh, Rajanpur, Islamabad, Layyah and Mianwali.

Table 2. Prevalence, incidence and severity of Karnal bunt in different districts of Punjab during year 2013.

Sr. No.	Districts	No. of Samples	Prevalence (%)	Incidence (%)	Severity (0-5)
1	Rawalpindi	77	15.58	1.98	0-2
2	Faisalabad	91	5.49	0.81	0-1
3	Bahawalnagar	60	11.67	1.24	0-2
4	Bahawalpur	54	9.26	1.65	0-2
5	Chakwal	44	15.91	3.31	0-3
6	Rahim Yar Khan	18	0	0	0
7	Attock	18	0	0	0
8	Mandibhaudin	12	0	0	0
9	Sheikhupura	18	0	0	0
10	Gujrat	10	0	0	0
11	Jehlum	27	18.512	1.55	0-2
12	Kasur	10	0	0	0
13	Lahore	35	11.43	0.97	0-1
14	Sahiwal	50	2	0.87	0-1
15	Gujranwala	24	12.5	1.4	0-2
16	Khushab	10	0	0	0
17	Toba Tek Singh	57	0	0	0
18	Rajanpur	9	0	0	0
19	Islamabad	12	0	0	0
20	Layyah	15	0	0	0
21	Mianwali	12	0	0	0
	Total	663	4.87	0.66	0-2

The average disease incidence was 0.66% with the highest (3.31%) in district Chakwal and the lowest (0.81%) in Faisalabad. The average disease severity was ranged (0-2) on 0-5 rating scale. Maximum severity 3 was calculated in the Chakwal whereas the minimum in Faisalabad, Lahore, and Sahiwal (Table 2).

Table 3. Prevalence, incidence and severity of Karnal bunt in different districts of Punjab during year 2014.

Sr. No.	Districts	No. of Samples	Prevalence (%)	Incidence (%)	Severity (0-5)
1	Rawalpindi	82	21.95	2.19	0-2
2	Faisalabad	144	16.67	2.71	0-2
3	Bahawalnagar	57	26.32	3.32	0-3
4	Bahawalpur	73	10.96	2.51	0-2
5	Chakwal	54	27.78	3.04	0-2
6	Rahim Yar Khan	44	27.27	2.49	0-2
7	Attock	39	0	0	0
8	Mandibhaudin	25	0	0	0
9	Sheikhupura	47	0	0	0
10	Gujrat	22	0	0	0
11	Jehlum	34	35.29	2.11	0-2
12	Kasur	15	0	0	0
13	Lahore	31	32.26	2.59	0-2
14	Sahiwal	47	19.15	2.29	0-2
15	Gujrawala	27	70.37	2.06	0-2
16	Khushab	15	0	0	0
17	Toba Tek Singh	12	0	0	0
18	Rajanpur	19	0	0	0
19	Islamabad	18	22.22	1.58	0-1
20	Laiyah	26	0	0	0
21	Mianwali	19	0	0	0
22	Muzfarghar	15	46.67	1.67	0-1
23	Multan	18	0	0	0
24	Sargodha	15	0	0	0
	Total	898	14.87	1.19	0-2

The average prevalence of Karnal bunt in Khyber Pakhtunkhwa was calculated (11.33%). With maximum prevalence was calculated in district Kohat (40%) followed by Charsadda (33.33%) and Mansehra (13.33 %) whereas the minimum was in Mardan (1.23%) Samples from Haripur, Malakand, and Mingora showed no visual infection.

The average disease incidence was 3.02% with highest incidence was found in district Charsadda (11.523%) followed by Peshawar (6.67%) while lowest (1.63%) in district Kohat. The average severity ranged 0-2 with the highest disease severity 3 was calculated in the sample collected from Charsadda while the lowest 1 was recorded from Mardan and Mansehra (Table 4).

Table 4. Prevalence, incidence and severity of Karnal bunt in different districts of Khyber Pakhtunkhwa during year 2013.

Sr. No.	Districts	No. of Samples	Prevalence (%)	Incidence (%)	Severity (0-5)
1	Charsadda	9	33.33	11.523	0-3
2	Haripur	8	0	0	0
3	Mardan	81	1.23	1.733	0-1
4	Peshwar	36	2.78	6.67	0-2
5	Mansehra	15	13.33	1.63	0-1
6	Kohat	5	40	2.6	0-2
7	Malakand	10	0	0	0
8	Mingora	12	0	0	0
Total		176	11.33	3.02	0-2

Prevalence, incidence and severity of Karnal bunt in Punjab and Khyber Pakhtunkhwa during 2014

During 2014 survey in Punjab, the average prevalence was calculated 14.87% with maximum prevalence was calculated in district Gujranwala (70.37%) followed by Muzfarghar(46.67%) and Jhelum (35.29%) while the minimum in Bahawalpur (10.96%). No infection was found in the samples collected from districts Attock, Mandibhaudin, Sheikhupura, Gujrat, Khushab, Toba Tek Singh, Rajanpur, Layyah, Mianwali, Multan, and Sargodha. The average disease incidence was 1.19% with highest disease incidence was in district Bahawalnagar (3.32%) while the lowest (1.58 %) in Islamabad.

Table 5. Prevalence, incidence and severity of Karnal bunt in different districts of Khyber Pakhtunkhwa during year 2014.

Sr. No.	Districts	No. of Samples	Prevalence (%)	Incidence (%)	Severity (0-5)
1	Charsadda	12	33.33	3.17	0-2
2	Haripur	27	0	0	0
3	Mardan	120	20	8.81	0-3
4	Peshwar	81	43.21	5.05	0-2
5	Mansehra	16	0	0	0
6	Kohat	10	20	1.8	0-1
7	Malakand	15	0	0	0
8	Mingora	18	0	0	0
9	Noshera	72	25	7.89	0-3
10	Buner	121	11.57	5.51	0-2
11	Sawabi	152	12.5	4.56	0-2
	Total	644	15.06	3.49	0-2

The average severity ranged among all fields in Punjab was observed (0-2) and maximum disease severity (0-3) was from Bahawalnagar, whereas the minimum 1 from Muzfarghar and Islamabad (Table 3). The average prevalence of Karnal bunt in Khyber Pakhtunkhwa was calculated (15.06 %) with the maximum prevalence was recorded in district Peshawar (43.21%) followed by Charsadda (33.33%) and Nowshera (25 %) and the minimum was in district Buner (11.57%).

Table 6. Morphological and physiological characteristics of different isolates of *Tilletia indica*.

Sr. No.	Districts	Isolates	Teliospore		Size of secondary sporidia(µm)	
			size (µm)	Germination (%)	Allantoid	Filliform
1	Faisalabad	PB-25	43.50	34.63	39.75	58.51
2	Rawalpindi	PB-41	41.59	59.66	33.07	53.11
3	Bahawalpur	PB-42	49.74	20.60	37.84	59.78
4	Bahawalpur	PB-43	37.40	54.05	31.16	50.88
5	Rawalpindi	PB-44	39.81	49.21	32.44	52.15
6	Rawalpindi	PB-45	38.80	37.06	32.12	52.79
7	Rawalpindi	PB-46	45.28	11.80	35.93	57.88
8	Chakwal	PB-47	45.03	21.54	34.98	57.56
9	Chakwal	PB-48	43.76	50.77	34.34	55.97
10	Sahiwal	PB-49	43.50	43.06	33.71	55.33
11	Chakwal	PB-50	41.34	78.53	35.62	52.79
12	Chakwal	PB-51	51.90	18.91	38.80	62.33
13	Faisalabad	PB-52	40.83	61.52	33.39	52.15
14	Chakwal	PB-53	48.72	27.69	37.21	59.47
15	Jehlum	PB-54	42.99	16.16	33.39	55.01
16	Faisalabad	PB-55	53.09	22.25	39.43	63.60
17	Chakwal	PB-56	44.01	54.12	35.93	56.60
18	Bahawalnagar	PB-59	41.85	78.24	33.39	53.42
19	Bahawalnagar	PB-60	37.14	69.22	30.21	50.56
20	Rawalpindi	PB-61	40.32	67.89	34.34	52.47
21	Rawalpindi	PB-62	37.78	21.05	30.53	51.52
22	Rawalpindi	PB-63	44.75	32.61	35.62	57.24
23	Bahawalnagar	PB-64	37.52	29.37	31.80	51.20
24	Jehlum	PB-65	42.46	33.36	35.62	54.06
25	Jehlum	PB-66	38.92	68.64	32.75	53.74
26	Jehlum	PB-67	51.24	24.02	38.48	61.37
27	Jehlum	PB-68	35.23	38.54	30.53	50.24
28	Lahore	PB-69	39.69	57.58	33.39	51.83
29	Lahore	PB-70	44.65	56.69	36.25	56.92
30	Lahore	PB-71	41.98	60.20	34.34	55.33
31	Lahore	PB-72	43.63	32.68	34.03	55.65
32	Gujrawala	PB-73	42.74	39.26	32.75	54.70
33	Gujrawala	PB-74	40.07	44.15	32.44	51.83
34	Gujrawala	PB-75	47.32	33.39	36.57	58.83
35	Rawalpindi	PB-76	42.74	55.19	34.66	54.38
36	Rawalpindi	PB-77	43.88	53.79	34.98	56.29
37	Rawalpindi	PB-78	41.57	63.10	32.44	53.42
38	Rawalpindi	PB-79	40.70	46.86	32.44	51.83
39	Faisalabad	PB-80	38.67	36.72	31.48	52.15
40	Faisalabad	PB-82	38.67	51.07	32.12	52.47
41	Charsada	KPK-11	49.81	20.90	37.52	60.74
42	Peshwar	KPK-12	41.87	63.52	33.71	53.74
43	Kohat	KPK-13	40.20	28.51	32.75	54.70
44	Mardan	KPK-14	38.92	32.85	33.07	51.52
45	Charsada	KPK-15	48.34	33.04	36.89	59.15
46	Charsada	KPK-16	45.92	65.48	36.25	58.19
47	Mansehra	KPK-17	46.43	10.82	37.21	58.51
48	Mansehra	KPK-18	39.94	75.34	34.03	54.38
49	Kohat	KPK-19	52.66	32.78	39.11	62.96

The samples collected from Haripur, Mansehra, Malakand and Mingora were found free of infection. The average disease incidence was 3.49% with highest incidence was found in district Mardan (8.81 %) followed by Nowshera (7.89 %) and the lowest (1.8 %) was in Kohat. The average severity was ranged to (0-2) with the highest disease severity range (0-3) found in Mardan and Nowshera (0-2) and lowest (0-1) in Kohat (Table 5).

Morphological Characterization of Tilletia Indica isolates

The results of the forty nine isolates on morphological characteristics are elucidated in Table 6. The teliospores of all the isolates were light to dark brown in color and circular to globose in shape. The size of the teliospore of the isolates ranged from 53.09µm (PB-55) to 35.23 µm (PB-68). Highest teliospore germination percentage was observed in isolate PB-50 (78.53) while the lowest was recorded in isolate KPK-17 (10.82) (Table 6). The size of secondary (filiform and allantoid sporidia) of the isolates revealed the maximum size of filiformsporidia was recorded in isolate PB-55 (63.6 µm) followed by KPK-

19 (62.96 µm) whereas the minimum (50.24 µm) was observed in PB-68 (Fig. 5B).

The maximum Size of allantoid sporidia was observed in isolate PB-25 (39.75µm) followed by PB-55 (39.43 µm) while the minimum was recorded in isolate to PB- 60 (30.21 µm) (Table 6; Fig. 5A). All isolate developed irregular creamy to white colonies (Fig. 6B) within 7 to 10 days after incubation which turns crustose type (Fig. 6 A) with the passage of time.

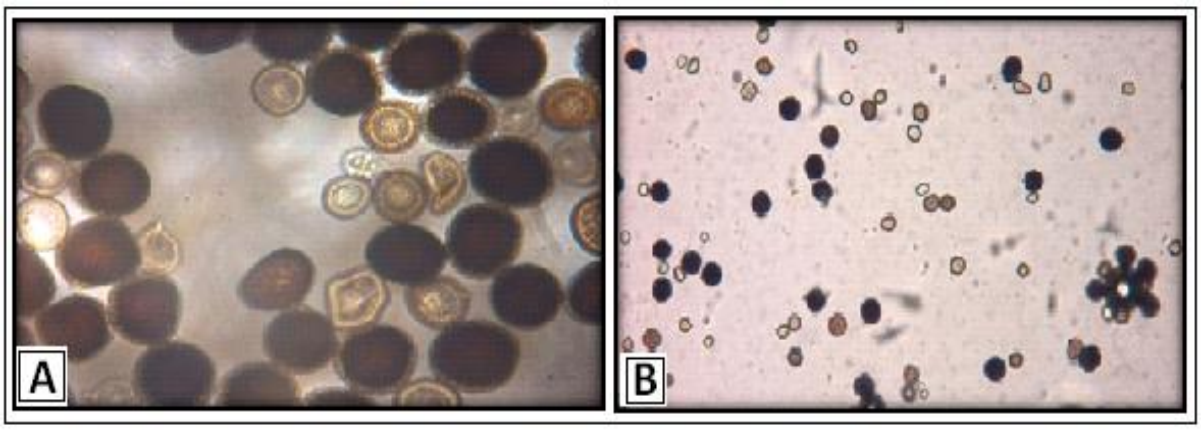

Fig. 3. Teliospores of *Tilletia indica* Observed at (A) 40X (B) 10X.

Discussion

Karnal bunt is the disease of quarantine importance and seriously damage the quality of wheat. The sowing of the diseased wheat seeds severed as a source of inoculums and ultimately became a source of introduction in those areas which are previously clean. In Pakistan very little work is done and scanty literature is available mainly focused on few surveys, conventional screening for resistance and chemical control. Keeping in view the importance of the disease, surveys was conducted to identify the disease free zones in Punjab and Khyber Pakhtoonkhwa.

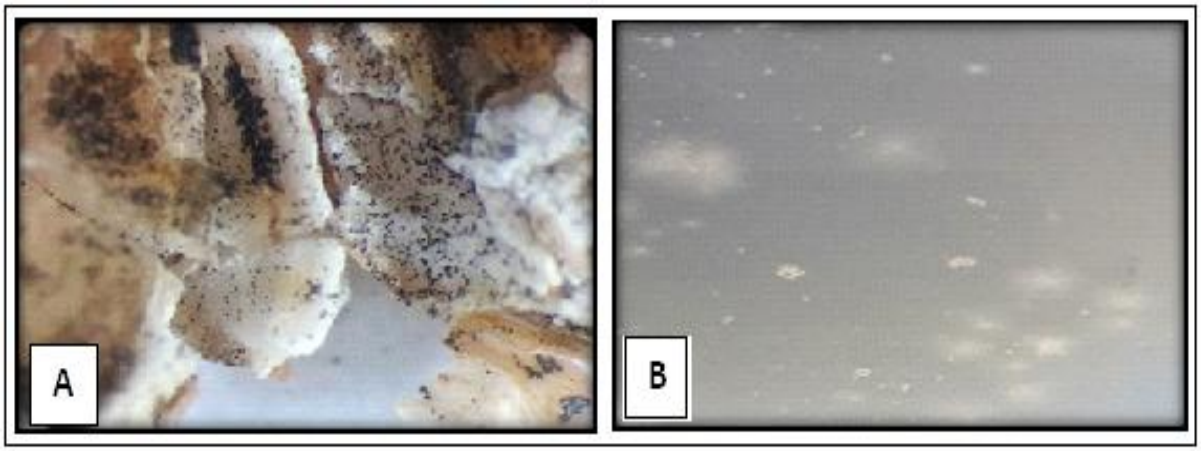

Fig. 4. (A) Teliospores *Tilletia indcia* under stereoscope(B) Star shape appearance of teliospores after germination.

Prevalence of Karnal bunt in Punjab and Khyber Pakhtoonkhwa

According to the previous reports the disease was confined in the past in the rain fed areas only in wheat growing areas and reported its presence up to 50% (Raees *et al.*, 2013). Aasma *et al.* (2012) also monitored the disease status in wheat growing areas during 2011-12 in some districts of Punjab and Khyber Pakhtunkhwa provinces of Pakistan where previously some of these areas were free from disease under rain fed conditions. The present studies revealed that the disease was on higher side in Khyber

Pakhtunkhwa compared to northern Punjab of Pakistan. The similar trend of disease was reported from India where a survey report of various western, northern hill and southern dry regions of India showed the disease severity much higher in the areas of north (Singh, 1994).

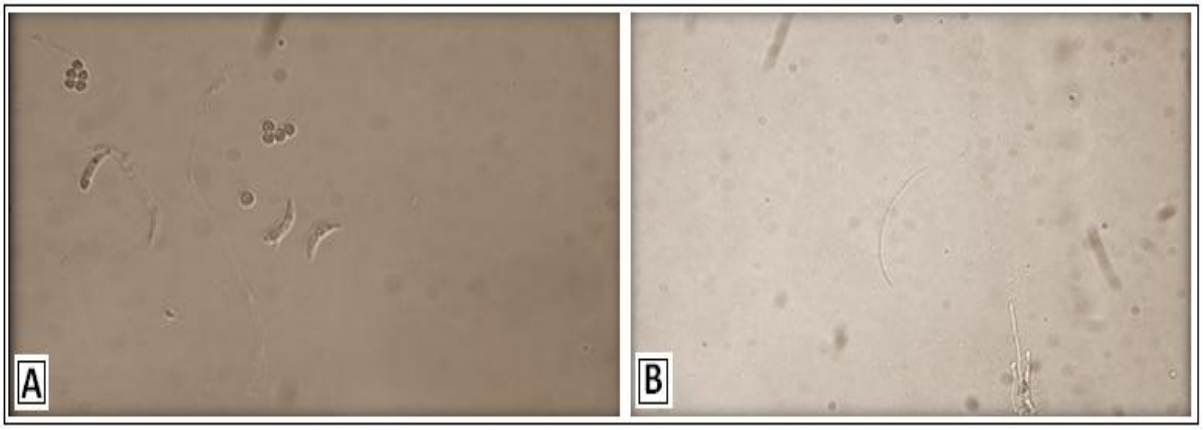

Fig. 5. Secondary sporidia of *Tilletia indica* (A) Allantoid, (B) Filiform.

The present study results indicated that the disease was more prevalent during 2014 as compared to year 2013 which is a reflection of an alarming situation. Since the disease is highly dependent on climatic factors therefore year to year variation in disease may likely to occur (Singh *et al.,* 1985; Singh *et al.,* 1996). The other aspect could be that in Pakistan the recommendation for the cultivation of a wheat variety at farmer's field is mainly focused on keeping in view its reaction against rusts without considering its status against Karnal bunt. The Similar results were also reported by other scientists in their studies (Bedi *et al.,* 1949; Joshi, 1980; Singh *et al.,* 1986; Aujla *et al.,* 1986, 1987; Joshi, 1988; Sharma *et al.,* 1998).

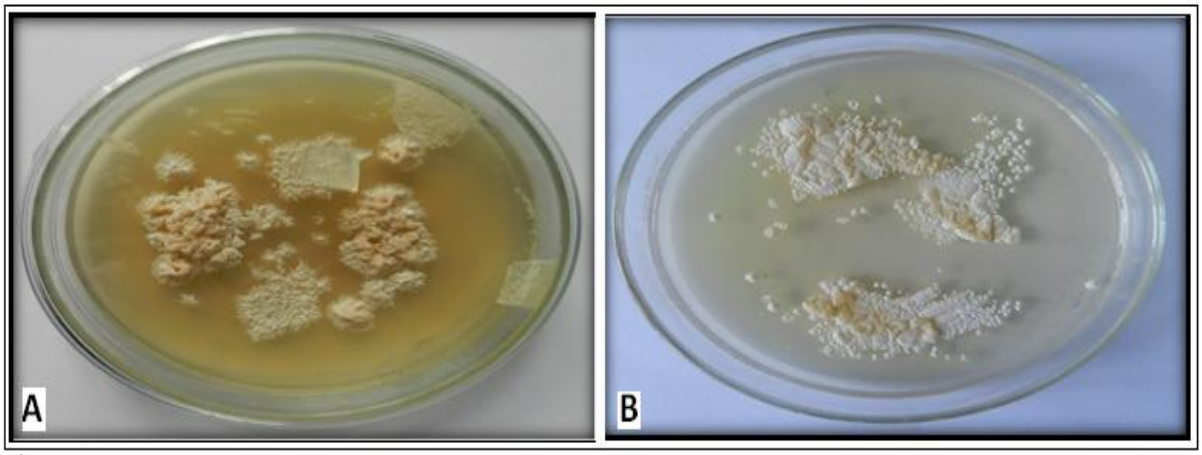

Fig. 6. Colonies of *Tilletia indica* on PDA medium (A) old culture crustose in appearance), (B) Fresh culture.

Incidence and Severity of Karnal bunt in Punjab and Khyber Pakhtoonkhwa

The incidence of disease is also greatly depends on the cultivars planted. Previously, karnal bunt had been reported from Punjab with different intensities (Mirza, 2005; Shakoor, 2009). Sajjad *et al.* (2018) conducted surveys in the southern Punjab and collected the samples from the six districts from the seventeen wheat varieties and found different level of incidence on different wheat varieties(Kohinoor-83, Inqlab-91, AS-2002, TD-1, Sehar-06, Shafaq-06, Lasani-08, Faisalabad-08, Fareed-08, AARI-11, Punjab-11, Millat-11, Aas-11, Galaxy-13, Ujala-16, Gold-16 and Johar-16). These districts of the province are considered to be the high yielding areas in terms of seed production. All these varieties were released

on the basis of their resistant status against rusts. However they reported Karnal bunt incidence on mega wheat varieties like Seher-06, TD-1 and Galaxy-13 which showed a trend towards susceptibility and deemed to be the future threat in the country as these varieties are planted over a larger area.

Disease free area or restricted distribution of Tilletia indica

There were many locations during the surveys which were found free from the disease supported the narration that the pathogen has restricted distribution (Sharma *et al.,* 2017) and the disease normally occurs sporadically and greatly influenced by prevailing weather conditions and the movement of the diseased seed which impregnated the soil with inoculums. As a result, the prevalence and severity of infection fluctuate greatly (Singh *et al*., 1996).

Morphological characterization of Tilletia indicia isolates

The analysis of the forty nine isolates revealed that various strains within species of a fungus may differ in morphology, physiology, cultural characteristics, and number of chromosomes. The pathogen possesses a high level of variation in terms of morphological characteristics and response to climatic conditions and pathogenicity tests. The study of pathogenic variability helped out to design effective strategy for its control (Gill *et al*., 1993). During the present study *Tilletia indica* was characterized by teliospore color, shape, size, germination percent, secondary sporidia, the colony color and growth pattern of the different isolates belonged to different ecologies. The teliospores of all the isolates indiscriminately were light to dark brown in color and globose to subglobose in shape and developed irregular creamy to white colonies and within 7 to 10 days after incubation with the passage of time turns crustose type. The size ranged from35.23 µm (PB-68) to 53.09µm (PB-55). This proved the existence of variability in our isolates compared to the studies conducted by Carris *et al*. (2006) who reported pale orange / brown to dark, reddish brown teliospores with mostly 22–47 µm in diameter, occasionally larger (mean 35–41 µm). The germination percentage of all the isolates were studied, revealed highest teliospore germination percentage was observed in isolate PB-50 (78.53 %) while the lowest was recorded in isolate KPK-17 (10.82 %).During the present studies it was observed that the isolates varied in respect of secondary (filiform and allantoid sporidia) size. The maximum size was recorded in isolate PB-55 (63.6 µm) while the minimum (50.24 µm) from the isolate PB-68. The maximum size of allantoid sporida was observed in isolate PB-25 (39.75µm) while the minimum from PB- 60 (30.21 µm). Duran and Fischer (1961), Khanna *et al.* (1968), Waller and Mordue (1983) reported primary sporidia on average 64-79 × 1.6-1.8 µm while the secondary sporidia on average 11.9-13 × 2 µm. Production of two types of secondary sporidia i.e. filiform and allantoid have also been reported previously and showed correlation with disease incidence and coefficient of infection (Ingold, 1997; Mitra, 1935; Aujla *et al.,* 1987; Singh *et al.,* 1998; Kumar *et al.,* 2004). Morphological and physiological variations of *Tilletia indica* isolates exist in different geographical locations, which can be correlated with environmental conditions in the region (Pannu and Chahal, 2000; Aasma *et al.,* 2012). Since the disease persists in Punjab and Khyber Pakhtunkhwa zones of wheat growing areas, it is necessary to continuously monitor the disease on a regular basis to identify areas where Karnal bunt is still not available so that production in these areas can be used for seed production and export.

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