Turkish Journal of Agriculture and Forestry

Volume 39 | Number 1 Article 8

1-1-2015

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PARVEEN, SHABANA; SAHARAN, MAHANDER SINGH; VERMA, AJAY; and SHARMA, INDU (2015) "Pathogenic variability among Tilletia indica isolates and distribution of heterothallic alleles in the Northwestern Plains Zone of India," *Turkish Journal of Agriculture and Forestry*: Vol. 39: No. 1, Article 8. https://doi.org/10.3906/tar-1401-143

Available at: https://journals.tubitak.gov.tr/agriculture/vol39/iss1/8

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Turkish Journal of Agriculture and Forestry

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Research Article

Turk J Agric For
(2015) 39: 63-73
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doi:10.3906/tar-1401-143

Pathogenic variability among *Tilletia indica isolates* and distribution of heterothallic alleles in the Northwestern Plains Zone of India

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Received: 31.01.2014 • **Accepted:** 27.09.2014 • **Published Online:** 02.01.2015 • **Printed:** 30.01.2015

Abstract: *Tilletia indica* Mitra, the causal agent of Karnal bunt of wheat (KB), is a quarantined disease of international importance whose pathogen shows high variability due to its heterothallic nature. Pathogenic variation was studied under artificially inoculated conditions on 13 wheat genotypes including bread and dicoccum wheat. This study used 34 *T. indica* isolates collected from 6 major wheat growing states in India (Rajasthan, Haryana, Punjab, Himachal Pradesh, Uttarakhand, and Uttar Pradesh). Mean coefficient of infection of the isolates ranged from 2.11% (KBR4) to 14.60% (KBH1). Among the 13 genotypes screened, only 1 genotype was found to be highly susceptible to infection. KBR1 and KBHP3 were the most virulent and least aggressive isolates and had coefficients of infection of 79.42% and 6.22%, respectively, on the susceptible variety HD 2009. Pathogenic variability test revealed the existence of 3 different aggressive groups among the 34 selected isolates on a set of host differentials. A total of 64 monosporidial (Ms) lines were developed from the isolates, which originated from 6 states, representing 19 locations in the North Western plains zone of India. Self-paired Ms lines revealed 2 mating alleles. A total of 15 mating alleles were postulated using 46 Ms lines. This study confirmed the heterothallic nature of *T. indica* and the existence of multiple alleles controlling pathogenicity.

Key words: Heterothallic allele, host differentials, Karnal bunt, *Tilletia indica,* wheat

1. Introduction

Wheat (*Triticum* spp.) is the most prominently grown cereal in the world. It occupies an important position in the Indian economy because of its significance for food security. Wheat accounts for more than 50% of the caloric intake of the Indian diet. Its production in India accelerated after the green revolution of the 1960s. As a result of increased wheat production, India currently has surplus stock of wheat and a potential to generate additional surplus. However, due to the prevalence of the Karnal bunt (KB) disease, wheat grain exporters are facing trade barriers under the Sanitary and Phytosanitary agreement, such as restricted movement of consignment to other countries (FAO, 1996). Karnal bunt or partial bunt of wheat caused by the sporadic fungus *Tilletia indica* (syn. *Neovossia indica*) was first reported from Karnal (Haryana) by Mitra (Mitra, 1931). Since then, KB has occurred frequently in the North Western plains of India. This disease has also been reported in Pakistan, Nepal, Iraq, Iran, Afghanistan, South Africa, Mexico, and limited areas of the southwestern United States (Jones, 2007). KB mainly affects common wheat, durum wheat, triticale, and other related species. The financial losses caused by the disease are substantial, ranging from 5% to 20%.

T. indica survives in the form of diploid teliospores in or on the seed and in agricultural soil. During germination, *T. indica* divides meiotically and produces primary sporidia. Primary sporidia give rise to successive generations of secondary spores (allantoid and filiform). The sporidia/mycelium then undergo dikaryotization at a life-cycle stage prior to the production of diploid teliospores. In contrast to other smut and bunt fungi, infection occurs during the flowering or grain filling stage of wheat and is usually confined to a few kernels in the earhead, which are subsequently partially or totally destroyed as a result of infection (Krishna and Singh, 1983; Singh, 1994). The pathogen converts the infected ovary into a sorus, where a mass of dark brown teliospores are produced. Symptoms of infection only become evident when the grain fully develops. Teliospores of KB-infected wheat impart a foul odor of trimethylamine to the grain, which is unacceptable for flour production (Sekhon et al., 1980).

Teliospores of *T. indica* are very resistant to chemical treatments (Hoffman, 1982; Smilanick et al., 1988). Once teliospores are established in the soil, they can survive for 36–43 months (Joshi et al., 1983; Warham, 1986; Bonde et al., 2004) and have been reported to survive under laboratory conditions for at least 16 years (Bonde et al.,

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1997). There are various conventional approaches for the management of the disease, such as cultural practices, adjustment of time of irrigation, controlling the nitrogen balance of the soil (Goel et al., 1977; Aujla et al., 1981), and mulching the soil with plastic (Singh et al., 1992). However, these are not sufficient to fully control the incidence of KB. Chemical control, however, can be accomplished with fungicidal applications during anthesis, but these are often not economically feasible on a commercial scale. Cultivation of resistant genotypes is currently one of the most economical and environmentally safe options for the management of the disease.

For the development of KB-resistant wheat varieties, accurate knowledge of the plant pathogen population structure and epidemiology is essential. The screening of host lines with a diverse population of pathogenic strains is an important requirement for the development of varieties with a wider genetic base. Similar to other major wheat diseases, the evaluation of KB resistance is an essential part of the Indian wheat crop improvement program. According to Nagarajan et al. (1997), these cultivars do not maintain the desired level of resistance when cultivated in farmers' fields. The susceptibility of these cultivars can be attributed to the inoculation of breeding materials that are derived from teliospores extracted from infected grains belonging to random field samples. However, the teliospore itself is the product of a dikaryotic mycelium, which is formed prior to infection by the fusion of a compatible strain of allantoid sporidia, a condition termed as heterothallism (Duran and Cromarty, 1977; Krishna and Singh, 1983). Therefore, pathogenic variability demonstrated on the basis of host differentials using teliospores is incomplete unless the diversity of their predecessors is taken into consideration. This study was carried out to identify the pathogenic variability and distribution of heterothallic alleles among isolates of *T. indica* in the major wheat growing regions of India.

2. Materials and methods

2.1. Collection of KB-infected grain samples, culturing, and maintenance

KB-infected wheat samples were collected from 6 Indian states (Rajasthan, Haryana, Punjab, Uttar Pradesh, Uttarakhand, and Himachal Pradesh) in 2010–2012. Teliospores of each isolate of KB were germinated separately at 12 °C over a thin layer of 2% water agar (HiMedia) in petri plates after extraction from a punctured sorus of an infected grain. A single germinating teliospore was randomly selected from a water agar plate using a sterilized needle and was inoculated separately in a liquid and solid potato dextrose media (HiMedia). The cultures were incubated in a biological oxygen demand (BOD) incubator at 20 ± 2 °C under alternating light and dark conditions for 20 days.

2.2. Host differentials used

Pathogenicity tests were conducted on 13 different hosts belonging to *Triticum aestivum* and *T. dicoccum*, including susceptible, moderately susceptible, and resistant lines. The differentials were sown in a 1-m row each with 25 cm spacing during 3 (2010–2013) crop seasons at the Directorate of Wheat Research, Karnal, India. Two replicates were maintained for each host. Sowing was conducted in mid-November each year, matching the timing of normal wheat sowing. Fertilizer was applied according to the recommendations of Tandon and Sethi (1991), and water was provided on a needs basis.

2.3. Inoculation procedure for the isolates

In this study, procedures for the inoculation of different host lines with teliosporic culture and incubation requirements were used as described by Aujla et al. (1982, 1987). Bulk inoculum of each isolate of *T. indica* containing allantoid sporidia was raised on potato dextrose agar (PDA). Sporidial suspension of 10^4/mL concentration was prepared using a hemocytometer. Five main tillers of individual host differentials were inoculated at boot leaf stage (Z-49) (Zadocks et al., 1974) for each isolate. One milliliter of sporidial suspension per earhead was inoculated using a hypodermic syringe. To avoid contamination of the isolates, a separate syringe was used for each isolate. The inoculated earheads were tagged with all the necessary information. The experiment was repeated for 2 crop seasons under identical conditions for each isolate. After inoculation, a high-humidity environment was maintained to ensure infection by using a mist sprayer for at least 4 h. Inoculation was conducted during evening hours. Uninoculated negative controls for each differential were also maintained.

2.4. Development of monosporidial (Ms) lines

A single teliospore with attached basidiospores (primary sporidia) was selected from water agar plates, picked up with the help of a sterilized needle, and transferred to drops of sterile water on the surface of separate agar plates. The germinating teliospore was teased over the surface of the water agar to spread the basidiospores. Each basidiospore was marked on the reverse side of the petri plate, and a water agar block containing a single germinating spore (allantoid) was cut and removed from the plate aseptically. This block was transferred to a new petri plate containing PDA media. The new culture was observed under a microscope to confirm that it contained a single spore. Petri plates were sealed with Parafilm and the cultures were incubated in a BOD incubator at 20 ± 2 °C under alternate light and dark conditions for 20 days. After the formation of colonies, the Ms lines were multiplied on PDA slants and were assigned numbers to record their identity. All Ms lines were subjected to a pathogenicity test to confirm their monosporidial identity. Those lines that could not produce

infection individually on susceptible host HD 2009 under highly conducive conditions for disease development were considered true Ms lines.

2.5. Virulence analysis of pairing of *T. indica* Ms lines on susceptible wheat varieties

During the 2010–2013 crop seasons, a compatibility assay was employed for the identification of mating types and their corresponding alleles present in the samples. The compatibility interactions of Ms lines derived from isolates were studied both separately and in all possible combinations on susceptible host HD 2009 in order to confirm compatible pairs and their pathogenic potential. For the preparation of the inoculate, 5000 allantoid sporidia of different Ms lines were dissolved separately in 1 mL of distilled water. For pairing, 2 different Ms lines were mixed in equal amounts just before inoculation. The identity of all inoculated spikes was maintained by adding tags with the name of the Ms line/combination and the date of inoculation. Between 10 and 12 spikes were inoculated for each Ms line combination. The same experiment was repeated under the same growing conditions during the next crop season. Inoculations were carried out in the late evening hours and high humidity was maintained for disease development. In the initial experiments, the inocula consisted of Ms lines that were self-paired (developed from single teliospore) as well as cross-paired. In the later experiments, Ms lines with previously identified compatible alleles were used as baselines (base maters) to determine the compatibility of additional untested lines. Each experiment was repeated for 2 successive crop seasons.

2.6. Data analysis

The inoculated earheads were harvested after maturity, and the threshed and infected grains were visually scanned for symptoms of disease. The number of grains infected in an ear and the severity of infection in the grain were recorded and a coefficient of infection (CI) was calculated according to Aujla et al. (1989). The response of the host genotype was designated into 1 of 5 categories: 1R (highly resistant), 2R (resistant), 1S (moderately susceptible), 2S (susceptible), and 3S (highly susceptible), with each category defined by a CI of 0, 0.1–5.0, 5.1–10, 10.1–20, and >20.1, respectively. The average CI for each inoculum was taken as the host reaction as described by Aujla et al. (1989). CI data were subjected to a least significance difference analysis of variance (ANOVA) and analyzed using SAS version 9.3. Differences between the isolates, genotypes, and their interactions were analyzed. Means were compared by the Tukey test. Disease reaction values, i.e. CI 0–5.0, 5.1–10, 10.1–20, and >20 of the isolate on the differential line, were scored as 0, 1, 2, and 3, respectively, and were used for the construction of a dendrogram.

3. Results

3.1. Pathogenic variation of *T. indica* on a set of host differentials

A total of 34 isolates were obtained in the 2010–2012 crop seasons in 6 states of the North Western plains zone of India (Table 1). Host pathogen interaction on differential hosts indicated the existence of pathogenic variability. Isolates induced differential disease reactions on 10 host genotypes except for the 3 genotypes of HD 29, W 485, and HP 1531, where immune (I) to resistant (R) reactions for all isolates were obtained. The standard deviation (obtained from the average of the CI) was relatively low for most of the resistant and susceptible cultivars (Figure 1). Among all the genotypes screened, only one cultivar, HD 2009, was highly susceptible to KB infection, producing a 3S reaction (an average CI of 20.13). Only one wheat genotype, WH 542, produced a 2S reaction with CI values of 10.14. Genotypes WL 711, HD 2967, UP 2338, PBW 343, and Raj 3765 revealed CI values between 5.1 and 10.0 and were considered moderately susceptible (1S). Genotypes DDK 1009, HD 29, HP 1531, W 485, and DPW 621-50 were considered resistant (2R), with CI values ranging between 0.1 and 5.0 (Table 2). ANOVA revealed highly significant values at $P < 0.001$.

3.2. Virulence test

Considerable variation was found in CIs among the isolates of *T. indica* across the host differentials. The average CI was calculated from the field data. Among the 34 isolates evaluated, the highest CI (14.67) across the host cultivars was recorded in isolate KBH1 from Safido (Haryana), followed by KBRaj1 from Chaksu (Rajasthan), and the lowest severity was observed in KBRaj4 (CI 2.11) collected from Hindon (Rajasthan). Calculated CIs were further transformed into a reaction matrix of isolates on host differentials. A maximum CI (79.42) was obtained with isolate KBR1 (Chaksu) on the susceptible host HD 2009; however, the same MS reaction for this isolate was recorded on another susceptible genotype WL 711. Isolates of KBH1, KBH2, KBH6, KBH7, KBH9, KBH11, KBH13, KBRaj1, KBPB6, KBHP1, KBHP2, and KBUK2 were used for the infection of all 13 host differentials and produced 2R to 2S disease reactions on the host differentials, except for HD 2009, where these isolates produced a 3S reaction (Table 2). On the basis of the reaction matrix (avirulent/virulent) the 34 isolates could be differentiated into 3 groups: a maximum of 18 isolates were included in the pathogenic group of 2R, 12 isolates were assigned to group 1S, and 4 were assigned to group 2S. Most of the isolates in group 2R produced a 1R to 1S reaction, except for KBH8, KBPb1, KBUK1, KBUK2, and KBUK3, which produced a 2S or 3S reaction on the highly susceptible genotype HD 2009. Similarly, the 12 isolates of the 1S reaction and the 4 isolates of the 2S reactions were differentiated by their

Table 1. Details of the *Tilletia indica* isolates collected and used for pathogenic variability.

S. no.	State	Code	District/area	Year of collection
1	Haryana	KBH5	Karnal	2010
2		KBH6	Sonipat	2010
3		KBH2	Bhiwani	2010
4		KBH7	Asandh (Karnal)	2010
5		KBH4	Fatehabad	2011
6		KBH13	Sirsa	2011
7		KBH12	Uklana (Hisar)	2011
8		KBH8	Bhuna (Sirsa)	2011
9		KBH9	Barwala (Hisar)	2011
10		KBH1	Safidon (Jind)	2011
11		KBH10	Ladwa (Yamuna Nagar)	2011
12		KBH11	Sahabad (Kurukshetra)	2011
13		KBH3	Indri (Karnal)	2012
14	Rajasthan	KBRaj1	Chaksu (Jaipur)	2010
15		KBRaj2	Kotputali (Jaipur)	2010
16		KBRaj6	Alwar	2011
17		KBRaj4	Hindon	2011
18		KBRaj5	Jaipur	2011
19		KBRaj7	Jhunjhunu	2011
20		KBRaj3	Dausa	2011
21	Uttarakhand	KBUK2	Dehradoon	2010
22		KBUK3	Haridwar	2011
23		KBUK1	Pantnagar (Haldwani)	2011
24	Himachal Pradesh	KBHP3	Sirmour	2010
25		KBHP1	Dhaulakuan (Sirmour)	2010
26		KBHP2	Tarau (Sirmour)	2010
27	Uttar Pradesh	KBUP1	Hapur	2012
28		KBUP2	Dhanipur (Aligarh)	2012
29	Punjab	KBPb6	Ludhiana	2012
30		KBPb1	Moga	2012
31		KBPb2	Nawansaher	2012
32		KBPb4	Ropar	2012
33		KBPb5	Khanna	2012
34		KBPb3	Ropar Site 2	2012

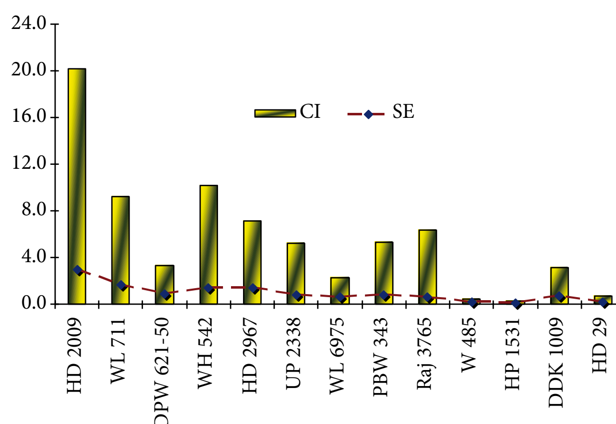

Figure 1. Reactions of 13 host differentials of wheat to isolates of *Tilletia indica* (CI: coefficient of infection, SE: standard error).

Table 2. Pathogenic variability of *Tilletia indica* isolates on differential wheat hosts.

S. no.	Isolate	Host differentials													
		HD 2009	WL 711	DPW 621-50	WH 542	HD 2967	UP 2338	WL 6975	PBW 343	Raj 3765	W485	HP 1531	DDK 1009	HD 29	Average
1	KBH1	35.41^{bcde}	52.58^{a}	17.42^{a}	15.55^{bcde}	18.44^{bc}	16.74^{a}	0^{c}	16.79^{cde}	7.52^{abcde}	0.02^{c}	0.62^{c}	9.4^{c}	0.32^{c}	14.67
2	KBH2	27.24^{defg}	3.55^{cd}	0.41^{b}	5.53^{dc}	7.86^{def}	14.1^{a}	5.65^{abc}	5.25^{de}	6.36^{abcde}	0^{c}	0.03^{b}	0.81^{f}	0.08^{c}	5.91
3	KBH3	8.13^{ij}	9.51^{cd}	7.64^{ab}	10.04^{dc}	4.2^{ef}	11.73^{ab}	0^{c}	3.81^{de}	6.11^{abcde}	0^{c}	0^{c}	0.73^{f}	0^{c}	4.76
4	KBH4	49.3^{b}	12.03^{c}	1.16^{b}	15.7^{bcde}	6.97^{ef}	12.59^{ab}	7.87^{ab}	19.48^{de}	7.65^{abcde}	0^{c}	0^{c}	0.8^{f}	0^{c}	10.27
5	KBH5	30.14^{cdef}	9.81^{cd}	0.9^{b}	18.9^{bcd}	8.66^{def}	7.97^{bcd}	0.1^{c}	2.71^{e}	7.8^{abcde}	0^{c}	0.2^{c}	14.14^{a}	0.033^{c}	7.79
6	KBH6	32.72^{cde}	5.15^{cd}	0^{b}	7.2^{de}	1.2^{f}	8.47^{bc}	0.26^{c}	5.2^{de}	3.64^{cde}	0.16^{c}	0.1^{c}	3.13^{de}	0.033^{c}	5.17
7	KBH7	43.16^{bcd}	20.31^{b}	0.9^{b}	4.94^{dc}	4.1^{ef}	1.38^{ef}	0.1^{c}	2.66^{e}	1.2^{e}	0.13^{c}	0.06^{c}	1.86^{ef}	0.06^{c}	6.22
8	KBH8	10.1^{hij}	2.9^{cd}	0.93^{b}	3.1^{e}	2.8^{ef}	3.77^{cdef}	0^{c}	6.9^{cde}	2.16^{de}	0.1^{c}	0^{c}	0.5^{f}	0^{c}	2.55
9	KBH9	25.7^{efgh}	6.4^{cd}	0.9^{b}	3.1^{e}	10.8^{de}	3.77^{cdef}	0^{c}	2.4^{e}	1.8^{de}	0.1^{c}	1.2^{a}	10.8^{bc}	$0.8b^{c}$	5.21
10	KBH10	6.18^{j}	4.36^{cd}	0^{b}	6.01^{de}	5.17^{ef}	2.3^{cdef}	0.1^{c}	3.86^{de}	3.57^{cde}	0^{c}	0^{c}	0.1^{f}	0^{c}	2.43
11	KBH11	3.1^{j}	2.3^{cd}	0.92^{b}	10.1^{de}	4.76^{ef}	0.92^{f}	0.1^{c}	3.73^{e}	4.54^{bcde}	0.8^{bc}	0.1^{c}	0.9^{f}	0^{c}	2.48
12	KBH12	8.2^{ij}	9.3^{cd}	0.9^{b}	7.74^{de}	7.94^{def}	2.1^{def}	0.9^{c}	1.4^{e}	6.5^{bcde}	2.1^{ab}	0^{c}	0.9^{f}	0.02^{c}	3.69
13	KBH13	33.1^{cde}	22^{b}	2.32^{b}	11.76^{cde}	7.1^{ef}	14.21^{a}	0.9^{c}	10.08^{bcd}	6.54^{abcde}	1.78^{abc}	0.9^{b}	0.3^{f}	2.8^{abc}	8.75
14	KBRaj1	79.42^{a}	8.48^{cd}	10.03^{ab}	24^{abc}	23.1^{b}	7.25^{bcde}	0.7^{c}	2.42^{e}	9.2^{abcde}	0.1^{c}	0.03^{c}	13.85^{ab}	0.33^{bc}	13.76
15	KBRaj2	15.16^{fghi}	3.27^{cd}	0.8^{b}	5.23^{de}	4.1^{ef}	2.1^{def}	2.43^{bc}	2.52^{e}	3.1^{de}	0.2^{c}	0^{c}	2.66^{ef}	0.06^{c}	3.20
16	KBRaj3	6.23^{j}	5.7^{cd}	1.8^{b}	7.2^{de}	2.7^{ef}	0.82^{bc}	6.67^{abc}	4.54^{de}	7.6^{abcde}	0.3^{c}	0^{c}	1.89^{ef}	$0.8b^{c}$	3.55
17	KBRaj4	4.36^{j}	2.1^{cd}	0.9^{b}	4.76^{de}	2.78^{ef}	1.34^{ef}	1^{c}	3.67^{e}	3.18^{de}	0^{c}	2.1^{b}	1.18^{f}	0.1^{c}	2.11
18	KBRaj5	7.86^{ij}	7.7^{cd}	2.1^{b}	9.8^{de}	2.78^{bc}	3.42^{cdef}	1^{c}	3.67e	3.18^{de}	0^{c}	2.1^{b}	1.18^{f}	0.1^{c}	3.45
19	KBRaj6	13.76^{ghij}	22.29^{b}	1.2^{b}	10.33^{de}	5.2^{ef}	4.26^{cdef}	0.4^{c}	11.38^{bc}	3.2^{de}	0.1^{c}	0^{c}	8.1^{cd}	0^{c}	6.17
20	KBRaj7	16.18^{fghi}	5.77^{cd}	2.8^{b}	8.61^{de}	4.7^{ef}	2.1^{def}	7.91^{ab}	2.86^{e}	3.01^{de}	0.32^{c}	0^{c}	0^{c}	0^{c}	4.17
21	KBUP1	13.22^{ghi}	3.71^{cd}	17.76^{a}	3.69^{e}	5.8^{ef}	1.42^{ef}	7.85^{ab}	2.46^{e}	15.64^{a}	0^{c}	0^{c}	2.06^{ef}	1.54^{abc}	5.78
22	KBUP2	3.38^{j}	4.64^{cd}	0.06^{b}	2.79^{c}	8.05^{def}	1.44^{ef}	7.85^{ab}	0.83^{e}	11.01^{abcd}	0^{c}	0^{c}	0.74^{f}	0^{c}	3.13
23	KBPb1	10.44^{hij}	8.41^{cd}	1.56^{b}	$8.76d^{e}$	3.31^{ef}	4.96^{cdef}	0^{c}	6.45^{cde}	14^{ab}	0^{c}	0^{c}	2.44^{ef}	1.04^{bc}	4.72
24	KBPb2	5.24^{j}	4.44^{cd}	1.5^{b}	3.22^{e}	1.2^{f}	5.65^{cdef}	0^{c}	4.24^{cde}	9.2^{abcde}	0^{c}	0.2^{c}	1.86^{ef}	0.98^{bc}	2.90
25	KBPb3	6.92^{ij}	4.8^{cd}	15.2^{a}	15.67^{bcde}	4.2^{ef}	12.88^{ab}	2.39^{bc}	6.85^{cde}	2.7^{de}	2.35^{ab}	0^{c}	0.13^{f}	1.14^{bc}	5.78
26	KBPb4	13.06^{ghij}	9.14^{cd}	8.2^{ab}	28.26^{ab}	14.88^{cd}	7.99^{bcd}	1.36^{bc}	3.1^{e}	11.29^{abcd}	0.1^{c}	0^{c}	2.06^{ef}	4.93^{a}	8.02
27	KBPb5	6.11^{j}	3.84^{cd}	0^{b}	3.94^{e}	3.0^{ef}	4.2^{cdef}	0^{c}	3.4^{e}	13.03^{abc}	0^{c}	0^{c}	0.1^{f}	2.9^{abc}	3.11
28	KBPb6	22.92^{efghi}	8.3^{cd}	1.5^{b}	24.2^{abc}	9.12^{def}	3.62^{cdef}	12.15^{a}	4.33^{de}	6.98^{abcde}	0.92^{bc}	0.9^{b}	7.89^{cd}	0.1^{c}	7.91
29	KBHP1	44.69^{bc}	10.16^{cd}	0.8^{b}	8.66^{de}	1.9^{f}	0.63^{f}	0.2^{c}	2.67^{e}	3.1^{de}	0.1^{c}	0.065^{c}	2.62^{ef}	0.033^{c}	5.81
30	KBHP2	36.7^{bcde}	23^{b}	2.8^{b}	35.36^{a}	42^{a}	1.2^{ef}	0.8^{c}	14.23^{b}	9.7^{abcde}	0.066^{c}	0.2^{c}	5.33^{de}	0.33^{bc}	13.20
31	KBHP3	9.3^{ij}	6.04^{cd}	0.1^{b}	3.78^{e}	1.1^{f}	2.63^{cdef}	0^{c}	1.35^{e}	2.4^{de}	0^{c}	0.06^{c}	0.93^{f}	0^{c}	2.13
32	KBUK1	14.96^{fghi}	2.04^{cd}	0.82^{b}	5.55^{dc}	4.89^{ef}	2.38^{cdef}	6.16^{abc}	3.13^{e}	9.4^{bcde}	2.89^{a}	0^{c}	2.17^{ef}	3.93^{ab}	4.48
33	KBUK2	31.92^{cde}	1.67^{d}	0.3^{b}	5.94^{de}	1.2^{f}	2.36^{cdef}	0.3^{c}	6.94^{cde}	4.93^{bcde}	0.2^{c}	0.2^{c}	2.8^{ef}	0.2^{c}	4.53
34	KBUK3	10.31^{hij}	7.89^{cd}	7.6^{ab}	5.65^{de}	6.73^{ef}	5.3^{cdef}	3.12^{bc}	4.17^{de}	5.76^{bcde}	2.8^{a}	0.3^{c}	2.1^{ef}	1.09^{bc}	4.83
Mean		20.14	9.22	3.30	10.15	7.14	5.24	2.30	5.28	6.38	0.46	0.28	3.13	0.69	5.67

CD at 5% is highly significant for all the treatments. Means in columns followed by the same letters are not significantly different ($P < 0.01$; Tukey test 1%).

reaction to all the host differentials. Out of the 13 wheat genotypes used for pathogenic reaction of *T. indica* isolates, even the resistant genotypes, such as DDK 1009 and DPW 621-50, were distinguished on the basis of their reaction to individual isolates. ANOVA showed significant differences in disease severity among the wheat genotypes, isolates, and their interactions. The mean square for genotypes was lower (13,112.13) than that for isolates (35,887.70). The mean square for the genotype × isolate interaction was very high (46,771.67) (Table 3).

3.3. Cluster analysis

A dendrogram based on UPGMA analysis clusters the 34 isolates of *T. indica* into 3 major groups. Cluster I contains 10 isolates, including the highly virulent isolates of KBH1 and KBRaj1. Cluster I is further subdivided into 2 clusters comprising 5 isolates each. In subcluster 1, 3 isolates (KBPb4, KBH5, and KBH9) produced disease in all genotypes except 1. In subcluster 2, all 5 isolates revealed CIs from 5.0 to 15. Cluster II contains 13 isolates divided into 3 subclusters. Isolates of KBH3 and KBPb3 were found to be more virulent to DPW 621-50 and WH 542 as compared to HD 2009. Two isolates from UP were grouped in same node in Cluster III, and both isolates produced resistant reaction. Most isolates in the lower group of Cluster III were less aggressive (Figure 2).

3.4. Development of Ms lines

Isolation of all possible monosporidial lines from a single teliospore is difficult because of the production of a large number (>180) of primary sporidia and their tendency to desiccate quickly during isolation. Furthermore, it may not be practically possible to observe compatible interactions of such a large number of Ms lines in all possible combinations. Thus, a random sample of Ms lines from single teliospores was used for self-paired combinations and was crossed from Ms lines originating from different states. Out of 75 lines developed in this study, only 64 were found to be true Ms lines, as these 64 could not individually produce infection on highly susceptible wheat cultivar HD 2009. The successful lines were developed from 19 isolates in 6 states within the disease-prone region of India (Table 4).

3.5. Compatibility among Ms lines and identification of base mater

To determine the virulence and compatibility of *T. indica* Ms lines, a total of 18 Ms lines were obtained from teliospores of isolates that were identified and maintained in the first crop season (2010–2011): 2 from Chaksu (KBRaj1-Rajasthan), 3 from Sirmour (KBHP3-Himachal Pradesh), 4 from Karnal (KBH5-Haryana) and Ludhiana (KBPb6-Punjab), and 5 from Dehradoon (KBUK2-Uttarakhand). In the second year (2011–2012), 14 of 18 Ms lines identified were used to check the compatibility among cross combinations involving isolates from different states. Simultaneously, more Ms lines were obtained from different isolates and their Ms identity was checked. Four Ms lines from Dehradoon were crossed in all possible combinations and all were found to be incompatible. In the preliminary pathogenicity test, only 2 mating types were detected. However, as lines from a single teliospores were paired with isolated lines, additional mating types were detected. To identify the base maters, Ms lines representing 5 states were crossed in all combinations from other states and 3 base maters were identified. These lines were assigned types A1, A2, and A3 from Karnal (H25), Chaksu (R16), and Dehradoon (UK6), respectively. An additional base mater designated as A4 was also used from Dehradoon (UK7) to check their pathogenic potential, as Ms lines from Dehradoon revealed incompatible reactions in self-paired as well as cross-paired combinations.

3.6. Determination of pathogenic/mating alleles in Northwestern Plains Zone of India

Out of 64 Ms lines identified, 46 were used to check the heterothallic alleles, including 4 base maters. A total of 184 combinations were made using 46 Ms lines. Among the 46 Ms lines, 7 were incompatible with all 4 alleles, and a total of 22, 19, 26, and 15 were compatible with

Table 3. Analysis of variance for Karnal bunt reaction of host differential to 34 isolates of *Tilletia indica.*

Source of variation	Pathogenic variability				
	S.S.	D.F.	M. S.	F	P
Replications	221.39221	2	110.6961	5.637	0.0037
Isolates (I)	13,112.138	33	397.3375	20.233	0.0000
Host genotypes (H)	35,887.7064	12	2990.6422	152.294	0.0000
I × H	47,771.673	396	118.110	6.01461	0.0000
Residual	17,320.0108	882	19.63		
Total	113,312.92164	1325			

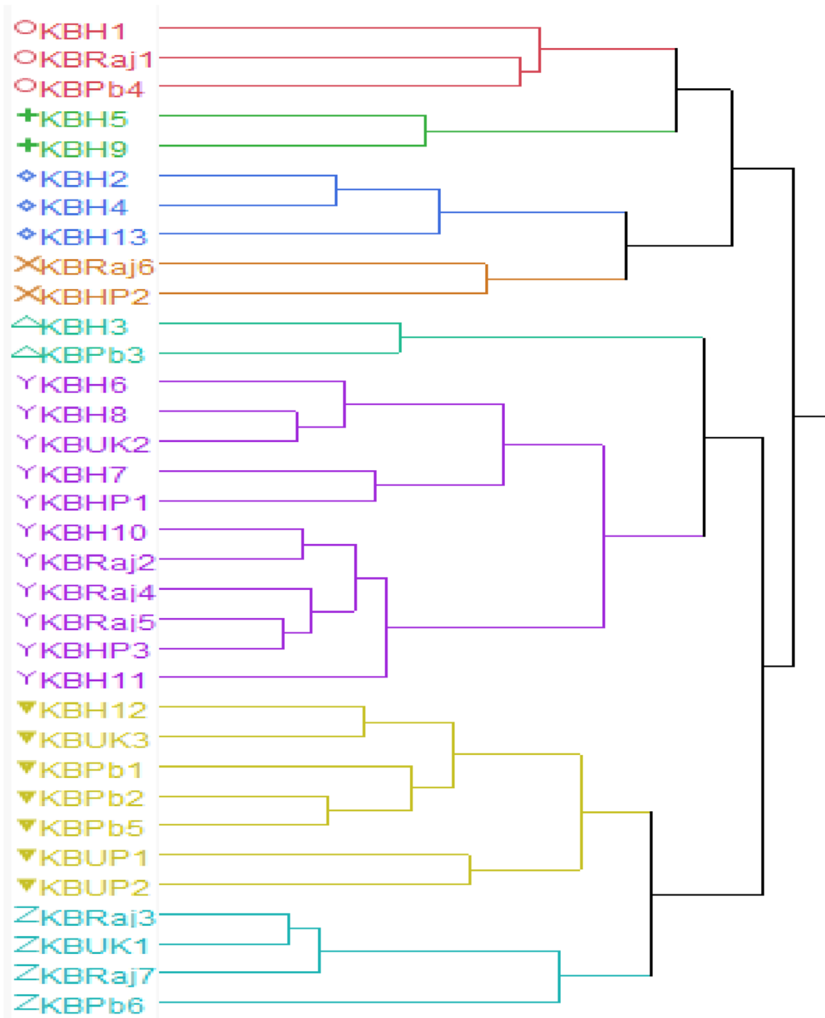

Figure 2. Clustering of *Tilletia indica* isolates based on pathogenicity to host differentials.

Table 4. Details of the *Tilletia indica* monosporidial lines confirmed (2010–2012) and used in the present study.

State	Location	Ms lines	State	Location	Ms. lines
Haryana	Karnal	4	Rajasthan	Jhunjhunu	5
	Fatehabad	1		Haridwar	5
	Sirsa	5	Uttarakhand	Dehradoon	5
	Bhuna	2		Tarau	2
	Barwala (Hisar)	7	Himachal Pradesh	Sirmour	3
	Sahabad (Kurukshetra)	4		Dhaulakuan	1
	Safido (Jind)	2		Ludhiana	4
Rajasthan	Chaksu (Jaipur)	2	Punjab	Moga	1
	Alwar	3	Uttar Pradesh	Dhanipur	2
	Jaipur	6			

base alleles A1, A2, A3, and A4, respectively. Depending on pathogenic potential, the most virulent compatible combination was A1 × A2, followed by A3 × Pb1 and A1 × HP5, which produced CI values of 74.91%, 26.92%, and 25.89%, respectively. The A4 base mater, which was initially incompatible in all possible combinations, produced disease when crossed with others. For the analysis of virulence, a paired combination with base mater average CI of all the compatible combinations was calculated from the field data and was further transformed into the reaction matrix of paired Ms lines on highly susceptible genotype HD 2009. After 3 years of study, a total of 31 incompatible pair combinations (including self-paired) using the A4 allele, followed by A2, A1, and A3, were recorded (Table 5). A total of 15 mating alleles are postulated based on the results of the pathogenicity test. Maximum A5 (total of 8) alleles were recorded, followed by A9 (7) and A6 (5). Maximum mating alleles (9) were observed in the state of Haryana, followed by Rajasthan, Uttarakhand, Himachal Pradesh, and Punjab. Among the 9 mating alleles identified in Haryana, 8 were common to the other states, while only one allele, A14, did not occur in the other states. Similarly, allele A15 occurred only in Rajasthan. Mating allele A5 was common in samples originating from Uttarakhand, Rajasthan, and Haryana, while mating allele A9 was common in Rajasthan, Uttar Pradesh, and Haryana.

4. Discussion

The selection of wheat germplasm resistant to *T. indica* is essential for the successful management of KB disease. Screening of wheat varieties against *T. indica* in the field is difficult due to experimental, agricultural, and natural constraints. In the present study, considerable differential reactions of different *T. indica* isolates were observed on a set of host genotypes. Two years of evaluation of these genotypes showed that irrespective of environmental conditions, host pathogen reactions were consistent. Researchers have speculated about the existence of pathogenic variability in this fungus based on teliospore size, shape, number of primary and secondary sporidia, and host differential reactions (Mitra, 1935; Bansal et al., 1984; Aujla et al., 1987; Bonde et al., 1996; Singh et al., 1996). The present study confirms the earlier studies about the existence of an aggressive type of *T. indica*. Existence of races in *T. indica* is controversial (Bonde et al., 1997). Based on the reaction of various *T. indica* isolates on a set of 17 host differentials, 4 pathotypes (K1, K2, K3, and K4) have been reported (Aujla et al., 1987). Although the race concept cannot necessarily be applied to *T. indica* because of heterothallism, it is appropriate to acknowledge a variation among isolate attributes, particularly aggressiveness (Nagarajan et al., 1997). Thirumalaisamy et al. (2006) identified 3 aggressive groups on the basis of disease response in different hosts and confirmed that most of the aggressive types exist in western Punjab and eastern and central Uttar Pradesh. Aujla et al. (1989) reported that isolates from Himachal Pradesh and KB-prone areas of Punjab are the most virulent. In the present study, isolates from Chaksu (Rajasthan) and Safido (Haryana) were found to be the most virulent types. We conclude that the virulence test gave no clear evidence for the existence of different pathotypes or different races in *T. indica*. Differences in virulence may be due to the different host plant genotypes used in different studies or to teliospores originating from different locations, and additionally because nuclear fusion takes place between secondary sporidia in each cycle of the pathogen and new variants are continuously developed. A recently released wheat genotype, HD 2967, largely considered to have a 1S reaction, was found to be 3S against a particularly virulent isolate (KBRaj1). HD 2967 is a desirable variety and is cultivated commercially on a large scale in many regions of India. However, in India, wheat cultivars are evaluated under field conditions for resistance to KB in a multiple-hotspot plant nursery known as the Karnal Bunt Screening Nursery (KBSN) before their release to farmers. Wheat cultivars grown in KB-affected areas of India are released only if they maintain a CI of <5 in the KBSN prior to their release, but the desired level of KB resistance is not found in field cultivation. In general, the pathogen cultures used for evaluating disease resistance constitute a mixture of heterothallic collections, possibly representing a limited pathogenic pool. It is difficult to screen genotypes with broad spectrum approaches, as the information on the genetic variability of *T. indica* is currently fairly limited (Kumar et al., 2009). In India, genetic resistant varieties of wheat (for example, HD 29, W 485, HP 1531, and WL 6975) have been widely used for resistance breeding for KB (Shoran et al., 2006). In this study, however, the host genotype WL 6975 produced a 1S reaction against the KBH4 isolate, indicating the possible limitations of currently existing KB-resistant strains. This study supports the findings of Thirumalaisamy et al. (2012) regarding the existence of particularly aggressive isolates and the movement of these isolates from one area to another in the KB prevailing in northern and northwestern India. Detailed virulence studies, using a well-defined set of differential wheat cultivars and a screening of all possible isolates from disease prone areas, are required to conclusively demonstrate the existence of different races of the pathogen. The virulent isolates identified in this study may be used for the screening of resistance genotypes.

In the present study, mating types included different alleles for compatibility. Incompatibility was identified in a few Ms lines representing the small area prone to

Table 5. Compatibility/incompatibility assay among *T. indica* monosporidial lines and postulated mating alleles.

S. no.	Location	Base maters	A1	A2	A3	A4	Postulated alleles following results of pathogenicity test
			Coefficient of infection				
1.	Haridwar	UK1	0^{g}	0^{e}	0^{f}	4.0^{ef}	A10
2		UK2	11.7^{cdef}	0^{e}	10.3^{cdef}	9.10^{ef}	A2
3		UK3	20.8^{bc}	13.6^{bc}	29.9^{a}	0^{f}	A4
4		UK4	0^{g}	0^{e}	0^{f}	0^{f}	A5
5	Dehradoon	UK5	6.07^{efg}	9.94^{bcde}	7.35^{def}	0^{f}	A4
6		UK6 (A3)	0^{g}	0.56^{e}	0^{f}	0^{f}	A8
7		UK7 (A4)	0^{g}	0^{e}	0^{f}	0^{f}	A5
8	Ludhiana	Pb1	6.94^{efg}	0^{e}	26.92^{ab}	20.34^{b}	A2
9		Pb2	0^{g}	1.38^{e}	7.72^{def}	15.76^{c}	A1
10		Pb3	2.93^{fg}	0^{e}	7.85^{def}	14.46^{d}	A2
11	Moga	Pb4	11.11^{cdef}	4.61^{de}	21.01^{abcd}	11.34^{de}	A6
12	Dhaulakuan	HP1	12.5^{cde}	4.34^{de}	4.59^{ef}	1.89^{f}	A6
13		HP2	6.01^{efg}	0^{e}	0^{f}	0^{f}	A7
14	Sirmour	HP3	3.92^{fg}	3.02^{de}	0^{f}	0^{f}	A11
15		HP4	12.65^{cde}	4.60^{de}	15.94^{bcde}	19.4^{bc}	A6
16	Tarau	HP5	25.89^{b}	2.93^{de}	4.93^{ef}	0.36^{f}	A6
17		HP6	6.25^{efg}	4.53^{de}	2.5^{ef}	0^{f}	A4
18	Jhunjhunu	Raj1	0^{g}	0^{e}	8.84^{def}	0^{f}	A9
19		Raj2	3.27^{fg}	16.62^{b}	0^{f}	0^{f}	A11
20		Raj3	0^{g}	0^{e}	0^{f}	0^{f}	A5
21		Raj4	0^{g}	7.34^{cde}	0^{f}	2.91^{ef}	A15
22	Jaipur	Raj5	0^{g}	0^{e}	0^{f}	0^{f}	A5
23		Raj6	0^{g}	1.90^{e}	0.98^{f}	0^{f}	A12
24		Raj7	4.18^{fg}	0^{e}	3.56^{ef}	0^{f}	A11
25	Alwar	Raj8	3.88^{fg}	3.70^{de}	0^{f}	0^{f}	A11
26		Raj9	0^{g}	13.18^{bc}	2.29^{f}	0^{f}	A12
27	Chaksu (A2)	Raj16	70.86^{a}	0^{e}	0.56^{f}	0^{f}	A13
28	Karnal	H1	0^{g}	0^{e}	0^{f}	0^{f}	A5
29		H2	0^{g}	7.39^{cde}	4.27^{f}	0^{f}	A12
30		H3	11.01^{cdef}	12.69^{cd}	24.09^{ab}	24.46^{a}	A6
31	Sirsa	H4	0^{g}	2.60^{e}	0^{f}	4.01^{ef}	A13
32		H5	8.32^{def}	0^{e}	3.64^{f}	0^{f}	A13
33	Bhuna	H7	0^{g}	0^{e}	4.30^{f}	0^{f}	A9
34	Safidon	H8	0^{g}	0^{e}	7.21^{fe}	0^{f}	A9
35		H9	0^{g}	0^{e}	7.64^{ef}	2.77^{ef}	A9
36	Barwala	H10	0^{g}	0^{e}	0^{f}	0^{f}	A5
37		H11	0^{g}	0^{e}	0^{f}	0^{f}	A5
38		H12	0^{g}	0^{e}	8.8^{def}	0^{f}	A9
39		H13	0^{g}	0^{e}	14.98^{cdef}	0^{f}	A9
40	Sahabad	H14	0^{g}	0^{e}	0^{f}	3.87^{ef}	A10
41		H15	3.83^{fg}	0^{e}	0^{f}	0^{f}	A7
42		H16	0^{g}	0^{e}	0^{f}	0^{f}	A5
43	Faetehabad	H17	3.25^{fg}	0^{e}	0^{f}	3.36^{ef}	A14
44	Karnal (A1)	H25	0^{g}	70.86^{a}	0.89^{f}	0^{f}	A12
45	Dhanipur	UP1	0^{g}	0^{e}	1.86^{f}	0^{f}	A9
46		UP2	4.1^{fg}	0^{e}	0^{f}	0^{f}	A7

Base mater codes: H- Hayrana, HP- Himachal Pradesh, Pb- Punjab, Raj- Rajasthan, UK- Uttarakhand, UP- Uttar Pradesh.

KB disease. The representative Ms lines, collected in 2010 with confirmed alleles, were further crossed with those of other untested Ms lines in 6 states of India. Duran and Cromarty (1977) first reported heterothallism in *T. indica*. A number of mating types ranging from 4 to 16 have since been reported (Krishna and Singh, 1983; Fuentes-Dávila, 1984; Chahal et al., 2003; Kumar et al., 2009). The isolate from Dehradoon is capable of causing infection, but the Ms lines developed from this isolate could not produce a compatible reaction in self- and cross-combinations in the preliminary experiments. Instead, they produced disease when crossed with other Ms. lines, providing further evidence of the effect of heterothallism on the pathogenicity of *T. indica*. Virulence in this isolate was possibly based on the specificity of chances of allelic pairs of the Ms lines participating in mating. A total of 15 mating types were identified in the northwestern zone of India. Mating type A6 also corresponds to Himachal Pradesh and Haryana. Four mating types from Haryana, bearing mating alleles A5, A9, A12, and A13, are similar to mating alleles from Rajasthan. This indicates that 4 alleles are common in Haryana and Rajasthan, of which 1 mating type (A5) is also common in Uttarakhand. Similarly, allele A2 was common in Uttarakhand, Punjab, and Rajasthan. As mentioned in Table 5, the 4 mating types were not comparable to any of the alleles present in any other states. The incompatible types prevail in Dehradoon (A3 and A8), Jhunjhunu (A15), and Fatehabad (A14). Being compatible on pairing with base maters and as compared with other compatible pairs, these mating types revealed alleles with unique behavior. The mating types of Uttarakhand did not resemble the mating types of Rajasthan and Haryana, except for alleles A2 and A5 for Rajasthan and A5 and A10 for Haryana. Similar observations were made about the mating alleles of other states. This indicates that breeding stocks screened with *T. indica* cultures isolated from Punjab and Uttarakhand may not impart protection in all other disease-prone regions for KB. The present study indicates the heterothallic nature of the pathogen and also provides evidence of multiple alleles, but does not provide evidence regarding the number of loci controlling the compatibility system in *T. indica*. However, bipolar and tetrapolar compatibility has been reported (Duran and Cromarty, 1977; Royer and Rytter, 1985; Aujla and Sharma, 1990), yet the understanding of the compatibility system of this species is still not fully resolved. A more elaborate pathogenic and molecular study is required to further elucidate *T. indica* allele compatibility. In the present investigation, the most aggressive pair observed was R16 × H25. Therefore, a field Ms line must be identified, if not feasible in totality then to the maximum extent possible during monitoring of pathotypes in a given area. Furthermore, Ms lines representing a maximum number of mating alleles must be preserved and inoculated as a homogeneous mixture on breeding materials that should be evaluated for KB resistance. Further investigations are needed to resolve whether Ms lines of the same teliosporic lineage yield more than 2 compatible alleles that may result in a complex aggregation of pathotypes in nature.

Acknowledgment
The first author is thankful to the Department of Science and Technology, Government of India, for providing financial support for this research under the Women Scientists Scheme (WOS-A).

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