Comparison of the Virulence of Isolates of *Tilletia indica*, Causal Agent of Karnal Bunt of Wheat, from India, Pakistan, and Mexico

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ABSTRACT

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Four *Tilletia indica* teliospore field populations, two from Mexico and one each from India and Pakistan, were tested for virulence on five Karnal bunt–resistant cultivars, one moderately susceptible, and two Karnal bunt highly susceptible wheat cultivars. The five resistant cultivars represented the most Karnal bunt–resistant germ plasm in the breeding programs at the International Maize and Wheat Improvement Center (Centro Internacional de Mejoramiento de Maiz y Trigo [CIMMYT]), Mexico, and the Department of Plant Breeding, Punjab Agricultural University, Ludhiana, India. Plants at the boot stage were inoculated by injecting into the boot 1 ml of a water suspension containing 10,000 allantoid sporidia per ml, incubated in a mist chamber for 3 days, then maintained until maturity in a greenhouse. All inoculated and control wheat spikes were harvested individually, and percentages of *T. indica*–infected seeds were determined. In addition, infected seeds from 10 randomly selected infected spikes per treatment were examined to estimate the proportion of each infected seed converted to a sorus. On the most resistant wheat cultivar (HD-29), percentage of seeds infected varied from 10 to 30%, depending on pathogen aggressiveness. On the most susceptible cultivar (Bacanora), infection varied from 55 to 84%. Although there were differences in pathogen aggressiveness, there was no evidence of the existence of races among the field populations. Wheat cultivars resistant to the Mexican fungal populations also were resistant to those from Asia, and vice versa; there was a significant correlation ($P \leq 0.05$) between percentage of seeds infected and extent of fungal colonization of infected seeds with all but one pathogen population when comparing resistant versus other wheat cultivars.

Additional keywords: disease resistance, partial bunt, smut

Karnal bunt of wheat (*Triticum aestivum* L.), caused by *Tilletia indica* Mitra (= *Neovossia indica* (Mitra) Mundkur), was discovered in 1930 at the Botanical Research Station at Karnal, Haryana, India (11). The disease has since been found in Pakistan (12), Iraq (5), Mexico (6), and Nepal (14), and intercepted in wheat from Afghanistan (10). On 8 March 1996, the U.S. Department of Agriculture, in conjunction with the Arizona Department of Agriculture, announced the discovery of Karnal bunt in the state of Arizona (Release No. 0115.96, AG NEWS FAX); on 21 March 1996, the Secretary of Agriculture implemented "extraordinary emergency" action to deal with the disease outbreak (Washington Post. 1996. March 22. Page A23).

Although disease loss is considered by some to be minor (4), *T. indica* can have profound effects on international trade of commercial grain and wheat germ plasm (13). Several countries, including the United States, have embargoed wheat from areas reported to have the disease (13).

In the early 1980s, The International Maize and Wheat Improvement Center (CIMMYT) implemented a breeding program for Karnal bunt resistance divided into three phases: (i) identification of sources of resistance, (ii) hybridization in order to incorporate resistance into agronomically suitable cultivars, and (iii) evaluation and selection of plant progenies that could be used in international agricultural programs. Since then, approximately 20,000 lines have been tested for resistance to the disease, including *T. aestivum*, *T. turgidum* L., *Triticosecale* species, and other *Triticum* species and grasses. Fuentes-Davila and Rajaram (7) reported 33 bread wheat lines that consistently showed levels of infection below 5% during 5 years of inoculation in the field. Presently, there are approximately 90 bread wheat lines in this category at CIMMYT (G. Fuentes-Davila, *unpublished*).

The Department of Plant Breeding, Punjab Agricultural University, Ludhiana, India, pursued a parallel program to screen germ plasm and develop wheat cultivars resistant to Karnal bunt. At present, approximately 20,000 lines have been tested at Punjab Agricultural University (S. S. Aujla and G. S. Nanda, *unpublished*). Both research groups artificially inoculated wheat plants at the boot stage (stage 10.0 to 10.1 on the Feekes scale) (9) with a sporidial suspension to assess susceptibility (1,8).

Field studies in Mexico and India have identified lines with high Karnal bunt resistance. The goal of our research reported here was to determine whether the resistance found at CIMMYT in Mexico was effective against the Karnal bunt pathogen in Asia, and conversely to determine if resistance found at Punjab Agricultural University in India was effective against the pathogen in Mexico. Comparisons were made side-by-side under nearly identical conditions in the plant disease containment facility in Frederick, Maryland. This report compares reactions of five resistant, one moderately susceptible, and two highly susceptible wheat cultivars to field collections of *T. indica* from India, Pakistan, and Mexico. The information presented here may prove valuable in initiating a breeding program to develop Karnal bunt–resistant cultivars to be grown in the United States should the disease eradication effort fail.

MATERIALS AND METHODS

Pathogen isolates. *T. indica* teliospores were obtained from infected seed from four separate field collections of teliospores. One was collected in 1991 from

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Pantnagar, India (IND91); a second in 1987 from Pakistan (PAK87); a third in 1981 from Sonora, Mexico (MX81); and the last in 1991 from Sonora, Mexico (MX91).

Wheat cultivars and lines. Five wheat lines previously identified in field studies as having high levels of resistance to Karnal bunt, one moderately susceptible wheat line, and two highly susceptible cultivars were used in this study. Three lines, HD-29, PBW-159, and WL-6975, had been identified by researchers at Punjab Agricultural University in India as having high levels of resistance to Karnal bunt (1; S. S. Aujla and G. S. Nanda, *unpublished*). Two lines, PF-71131 and Shanghi-8-48B-04, were identified by CIMMYT researchers in Mexico as highly resistant (15), while MRNG/BUC"S"/BIO"S"/PSN"S"-CM69191-5Y-1M-2Y-1M-1Y-OM (designated here as M3) was found to be moderately susceptible (G. Fuentes-Davila, *unpublished*). WL-711 was a popular cultivar grown in India but was found to be highly susceptible to Karnal bunt. Bacanora is a cultivar grown in northwest Mexico that is highly susceptible to Karnal bunt.

We will refer to all wheat accessions tested as cultivars. We planted six seeds in each of 25 10-cm-diameter clay pots for each wheat cultivar every 3 days for 9 days (75 pots per cultivar) to insure an adequate number of wheat spikes at the appropriate stage for inoculation for each experiment. This planting was repeated two additional times. Plants were fertilized every 2 weeks with an N-P-K 20-20-20 general purpose liquid fertilizer.

Preparation of inocula. For each teliospore field collection, 20 to 30 infected seeds were placed in a 15-ml conical centrifuge tube with 10 ml of water containing one drop of Tween 20 (sodium monolaurate) per 100 ml. The tube was vortexed for 2 min to dislodge and suspend the teliospores, then poured through a 100-µm mesh screen to remove seeds and large debris. The spore suspension was centrifuged 2 min at 250 × *g* (Beckman GPR, horizontal rotor, Beckman Inst., Fullerton, CA). The supernatant was decanted, the pellet was resuspended in 0.5% sodium hypochlorite solution to surface sterilize the teliospores, the suspension was immediately centrifuged for 1 min at 900 × *g*, the supernatant was decanted aseptically, and the pellet was resuspended in 12 ml of sterile distilled water. Teliospores were rinsed twice in sterile distilled water, and the final pellet was resuspended in 10 ml of sterile distilled water.

One ml of teliospore suspension was used to seed each of 10 2% water agar (Difco Bacto-Agar, Difco Laboratories, Detroit, Michigan) 100 × 15 mm plates. This procedure was repeated for each field collection. Petri plates with teliospores were incubated at 18°C for 12 days, after which teliospore germination and the presence of sporidia were evaluated. Five ml of sterile water was added to a plate, and the agar surface was gently scraped with a spatula to suspend the germination products. This suspension was used to inoculate five potato-dextrose agar (PDA) plates, which were incubated at 18°C for 5 to 14 days. This process was repeated with fresh teliospores every 2 weeks for each pathogen collection.

Ten days prior to the estimated time for inoculation of test plants, 5 ml of sterile water was added to one or two PDA plates. A mycelial and sporidial suspension was made by scraping the surface of the mycelial mat with a flat spatula. The suspension was used to seed 10 to 15 2% water agar plates. These plates were incubated at 18°C for 10 days, at which time they were examined for the presence of allantoid sporidia.

One hour before inoculating wheat spikes, a sporidial suspension was made by scraping the water agar plates, and the suspension was filtered through a 60- to 100-µm mesh screen. Inocula were prepared by diluting suspensions to 10,000 allantoid sporidia per ml, as determined with a hemacytometer. All sporidial suspensions were kept on ice until required for inoculations.

Inoculation of wheat spikes. For each wheat line–pathogen collection (treatment), 18 spikes were inoculated with 1 ml of sporidial suspension at the time of awn emergence (Feekes growth stage 10.0 to 10.1) (9) by inserting the 20-gauge needle of a 2-cm^3 Cornwall syringe down into the boot cavity. Control plants were injected with distilled water. All plants were placed into an 18 to 21°C greenhouse with a 70% sunscreen and controlled misting system (Mist-o-matic, E. C. Geiger Inc., Harleysville, Pennsylvania) to maintain free moisture on the surface of plants and 87 to 97% RH. After 3 days, plants were placed on a greenhouse bench at a mean room temperature of 24°C (range of 18 to 32°C) for disease development. This experiment was repeated two additional times. Plants were randomized on greenhouse benches within each experiment, and spikes were harvested individually at maturity.

Data collection and analyses. Data collected for each spike included numbers of infected and healthy seeds and were analyzed using the General Linear Models procedure of SAS (Version 6.08, SAS Institute, Cary, NC) to compare percent seeds infected within each of 18 spikes per treatment for the three experiments. The Bonferroni (Dunn) *t* test was used to determine if significant differences existed among wheat cultivars or among pathogen collections.

Data also were analyzed using the General Linear Models Procedure of SAS to compare the extent of colonization of individual infected seeds within 10 randomly selected infected spikes per treatment. Each seed was categorized into one of four infection grades according to a modification of the method of Aujla et al. (3). The grades were assigned numerical values of 0.25, 0.50, 0.75, and 1.00, each reflecting that proportion of the seed replaced by the fungal sorus. Point infections, in which infection was restricted to one tip of the seed, were included in the 0.25 grade. A seed entirely converted to a sorus, yet with the pericarp generally intact, was given the rating 1.00. The number of seeds in each grade was multiplied by the numerical value, and a gross total and coefficient of infection was calculated as illustrated in Table 1.

The coefficients of infection were used as responses in an analysis of variance to determine effects of wheat cultivar, pathogen isolate, and wheat cultivar–pathogen isolate interaction on responses. Since we determined that there was an interaction between wheat cultivar and pathogen isolate (Table 2), further analyses involved separate isolates to determine their respective effects on seed colonization. Contrasts

Table 1. Example of calculation of the coefficient of infection for a sample of wheat

Grade of Infection	1	2	3	4
Numerical value	0.25	0.50	0.75	1.00
Number of seeds	15	8	5	9
Values after multiplication	15 × 0.25 = 3.75	8 × 0.50 = 4.00	5 × 0.75 = 3.75	9 × 1.00 = 9.00
Gross total	3.75 + 4.00 + 3.75 + 9.00 = 20.50			
Total seeds	37			
Coefficient of infection	20.50 × 100/37 = 55.41			

Table 2. Analysis of variance[a] for coefficients of infection (measuring fungal colonization of infected seeds) of eight wheat cultivars infected with four isolates of *Tilletia indica*

Source	df	Sum of squares	Mean square	*F* value	*P* value
Wheat cultivar	7	13,155.53	1,879.36	6.65	0.0001
Isolate	3	685.48	228.49	0.81	0.4902
Isolate cultivar wheat	21	12,883.80	613.51	2.17	0.0026

[a] Dependent variable was coefficient of infection (described in Table 1) and independent variables were wheat cultivar and pathogen isolate.

were constructed and used to compare susceptible wheat cultivars (WL-711 and Bacanora) with the other six cultivars. Contrasts also were used to compare resistant wheat cultivars (HD-29, PBW-159, WL-6975, PF-71131, and Shanghi-8-48B-04) with the other three wheat cultivars. In each instance, M3 was included in the "other" class since, under greenhouse conditions, it was moderately susceptible to susceptible.

RESULTS AND DISCUSSION

Percent seed infected. Data for mean percent seed infected for each treatment are presented in Figure 1. Analysis shows that significant differences existed among several pathogen isolates (Fig. 2) and also among wheat cultivars (Fig. 3). There were no treatment interactions. *T. indica* MX81 overall caused the most infection and PAK87 the least (Fig. 2). IND91 and MX81 were intermediate in overall disease incidence (aggressiveness). Wheat cultivars WL-711, Bacanora, and M3 were the most susceptible, and HD-29, PBW-159, WL-6975, PF-71131, and Shanghi-8-48B-04 were the most resistant (Fig. 3).

When examining individual pathogen isolates on specific wheat cultivars, MX81 caused the highest percentages of infection on wheat cultivars WL-711, Bacanora, HD-29, PBW-159, WL-6975, and PF-71131, and the second highest on the two remaining cultivars (Fig. 1). In contrast, *T. indica* PAK87 caused the least infection on all but WL-6975, on which it was second to lowest (Fig. 1). Isolates MX91 and IND91 were approximately equal and almost always intermediate to isolates MX81 and PAK87 (Fig. 1). "Virulence" frequently refers to differential pathogenicity of pathogen isolates on a set of cultivars (i.e., the presence of an isolate × wheat cultivar interaction), and "aggressiveness" refers to an overall measure of disease level caused by one isolate versus others. The similarities of disease incidence rankings on the cultivars for the respective isolates suggest that either differences in virulence do not exist among the pathogen isolates we tested or we lack the resistance genes to detect specific virulence (i.e., races). In India, however, Aujla et al. (2) detected races among 21 *T. indica* field collections obtained from different agroclimatic regions of Punjab and Himachal Pradesh.

Seed colonization. There was a significant interaction ($P \leq 0.05$) between wheat cultivars and pathogen isolates (Table 2) when coefficients of seed colonization were compared in an analysis of variance. Consequently, further analyses involved separate pathogen isolates. To determine if there was a direct correlation between the percent seeds infected and the amount of seed colonization, we constructed contrasts to compare susceptible cultivars WL-711 and Bacanora with the other cultivars, and to compare resistant cultivars (HD-29, PBW-159, WL-6975, PF-71131, and Shanghi-8-48B-04) with WL-711, Bacanora, and M3. Results of the contrasts are presented in Table 3 and show a significant difference between susceptible versus all other cultivars for isolates IND91 and MX81, and between resistant cultivars versus all other cultivars for each pathogen isolate except PAK87. Thus, the data show a direct relationship in five out of eight comparisons between percent seeds infected (disease incidence) and extent of colonization per seed for those seeds that were infected (disease severity). Wheat cultivars HD-29, PBW-159, WL-6975, PF-71131, and Shanghi-8-48B-04 had both the lowest percentages of seeds infected (Fig. 3) and the lowest colonization ratings (means of coefficients of infection ranged 65 to 76). Cultivars WL711 and Bacanora had both the greatest percentages of seeds infected (Fig. 3) and the highest colonization ratings (means 81 to 83). These differ-

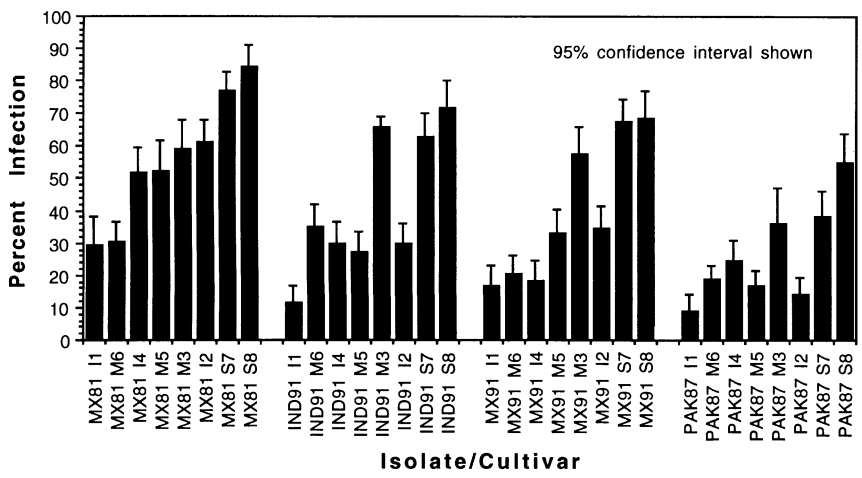

Fig. 1. Percent seeds infected by *Tilletia indica* in 18 wheat heads in each of three experiments per teliospore isolate–wheat cultivar combination. Confidence limits (95%) indicated. HD-29 = I1, PBW-159 = I2, WL-6975 = I4, MRNG/BUC"S"/BLO"S"/PSN"S"-CM69191-5Y-1M-2Y-1M-1Y-OM = M3, PF-71131 = M5, Shanghi-8-48B-04 = M6, WL-711 = S7, and Bacanora = S8.

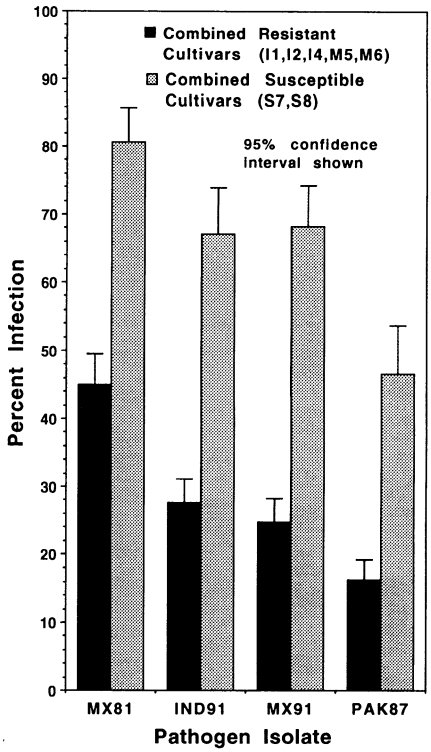

Fig. 2. Comparison of the percent seeds infected by each pathogen isolate, respectively, for combined resistant lines I1, I2, I4, M5, and M6 (data pooled) versus susceptible varieties S7 and S8 (data pooled). HD-29 = I1, PBW-159 = I2, WL-6975 = I4, PF-71131 = M5, Shanghi-8-48B-04 = M6, WL-711 = S7, and Bacanora = S8.

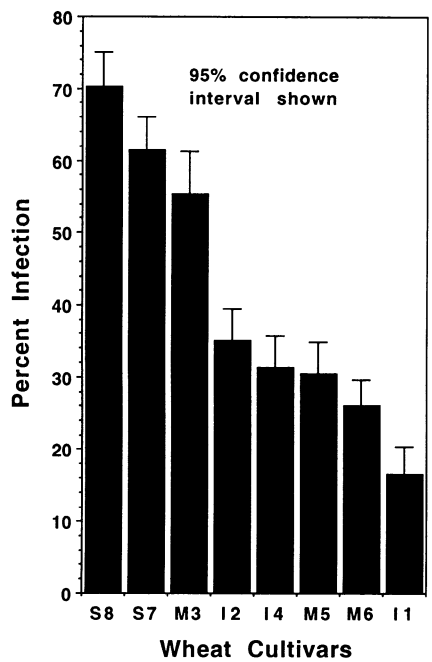

Fig. 3. Mean percent seeds infected for the wheat cultivars. Data were pooled for the teliospore isolates. HD-29 = I1, PBW-159 = I2, WL-6975 = I4, MRNG/BUC"S"/BLO"S"/PSN"S"-CM69191-5Y-1M-2Y-1M-1Y-OM = M3, PF-71131 = M5, Shanghi-8-48B-04 = M6, WL-711 = S7, and Bacanora = S8.

Table 3. Analysis of variance[a] of specific contrasts (described in Materials and Methods) comparing coefficients of infection of susceptible versus all other wheat genotypes and resistant versus all other cultivars

	Contrast			
Contrast	**df**	**Sum of squares**	***F* value**	***P* value[b]**
Suscep. (s) vs. others (o)	1	6,003.46	21.23	0.0001
S. vs. o:IND91	1	1,127.30	3.99	0.0468
S. vs. o:MX81	1	9,726.71	34.40	0.0001
S. vs. o:MX91	1	0.11	0.00	0.9846
S. vs. o:PAK87	1	503.55	1.78	0.1831
Resist. (r) vs. others (o)	1	9,820.32	34.73	0.0001
R. vs. o:IND91	1	2,866.62	10.14	0.0016
R. vs. o:MX81	1	7,599.21	26.88	0.0001
R. vs. o:MX91	1	1,380.79	4.88	0.0279
R. vs. o:PAK87	1	412.98	1.46	0.2278

[a] Dependent variable was coefficient of infection (measure of seed colonization by pathogen) and independent variable was specific contrast using individual pathogen isolate.

[b] Significant differences ($P \leq 0.05$) exist between contrasts comparing susceptible wheat cultivars WL-711 and Bacanora versus all others for isolates IND91 and MX81, and for resistant cultivars HD-29, PBW-159, WL-6975, PF-71131, and Shanghi-8-48B-04 versus the other three cultivars, except when using the pathogen isolate PAK87.

ences in colonization, however, are small and suggest that seed colonization alone would not be reliable as a measure of Karnal bunt resistance.

The significant differences in overall disease levels incited by the specific isolates, irrespective of host cultivar, suggest differences in isolate aggressiveness. MX81, the oldest isolate, produced the highest overall level of disease and therefore was most aggressive. MX81 was collected in Mexico before a breeding program for resistance to Karnal bunt was begun. Presumably, these spores are representative of the pathogen at that time in the field.

Our study demonstrates that the gene(s) for resistance to Karnal bunt found in CIMMYT's screening program in Mexico is also effective against the Karnal bunt pathogen from India and Pakistan, and that the resistance found by scientists at Punjab Agricultural University in India is effective against Mexican isolates. This suggests that the resistance may be effective wherever Karnal bunt is present. In addition, we found that *T. indica* teliospore collections differ in aggressiveness. A teliospore collection of low aggressiveness, such as PAK87, might produce too little disease for effective Karnal bunt screening in the field when disease pressure is low. Conversely, MX81 might produce too much disease for effective Karnal bunt screening in the greenhouse.

Our method of inoculation was designed to screen for resistance in wheat cultivars other than that imposed by morphological barriers preventing the pathogen from gaining entry to the infection court. Resistance due to morphological barriers is more difficult to detect when testing large numbers of lines because of the necessity of simulating natural inoculation, the difficulty in getting consistent infection, and the necessity of maintaining specific environmental conditions over long periods.

The levels of disease we obtained in resistant wheat cultivars in the greenhouse under highly conducive environmental conditions for Karnal bunt were considerably greater than obtained using the same inoculation techniques in the field in Mexico (7) or India (S. S. Aujla and G. S. Nanda, *unpublished*). In field studies, 5% or higher infection was designated as susceptible and less than 5% as resistant. The lines we tested from India when tested at Punjab Agricultural University in the field over a 10-year period gave little or no infection; only eight of 20,000 lines tested were in this "rare" category (S. S. Aujla and G. S. Nanda, *unpublished*). Although the infection levels we obtained were high, statistically significant differences were detected among wheat cultivars. The data suggest that the inoculation and incubation methodologies used partially overcame resistance. Further testing under controlled greenhouse conditions should be less severe and could be useful in screening advanced lines for the highest levels of resistance. It is imperative that breeding for Karnal bunt resistance continue because of the possible development of more virulent races or aggressive forms of the pathogen.

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