Relationship Between Kernel Infection and Spike Infection of Wheat by *Tilletia indica*, Causal Agent of Karnal Bunt

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ABSTRACT

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Plants of the spring wheat cultivar Olaf were inoculated with pairs of monosporidial lines of *Tilletia indica* (= *Neovossia indica*) by injecting sporidial suspensions into the boot sheath enclosing the spikes or by drenching the exposed spikes that had emerged from the boot with sporidial suspensions. The number of bunted spikes per total number of inoculated spikes was not significantly different between the two inoculation methods. The percentage of bunted kernels per bunted spike for the boot-injection method (74%) was significantly different from that for the spike-drench method (59%). With the latter inoculation technique, more infected kernels occurred in spikes inoculated immediately after spike emergence from the boot than in spikes inoculated immediately prior to or at anthesis. Regression models were applied to the data to examine the relationship between bunted kernels and bunted spikes. There was a significant linear relationship between the percentage of bunted kernels per inoculated spike and the percentage of bunted spikes. Thus, a given incidence (number) of bunted spikes can be used to predict the incidence of kernel infection with artificial inoculation of Olaf wheat in the greenhouse. The regression models may be solved for different cultivars. The regression parameters may be useful indicators of differences in cultivar resistance as well as pathogen virulence across different stages of spike development.

Karnal bunt was first described in India and has since been reported in Iraq and Afghanistan (8). Karnal bunt-diseased wheat has also been intercepted by India in seed lots received from Lebanon, Syria, Turkey, and Sweden (8). Karnal bunt is listed by the European and Mediterranean Plant Protection Organization as a quarantined disease (1). The disease has not been reported in the United States, and there are no wheat (*Triticum aestivum* L. em. Thell) cultivars known to be immune to infection by the causal agent, *Tilletia indica* (Mitra) (= *Neovossia indica* (Mitra) Mundkur)), in the United States.

The interception (2) of Karnal bunt of wheat from Mexico by the Plant Protection and Quarantine division (PPQ) of the Animal and Plant Health Inspection Service (APHIS) of the USDA has encouraged the establishment of Karnal bunt screening nurseries in India and Pakistan. Less than 1% of the wheat and triticale accessions tested in the greenhouse during 1983 at the International Maize and Wheat Improvement Center (CIMMYT) in Mexico showed no signs of infection (E. J. Warham, *personal communication*). If *T. indica* were to become established in the United States, the pathogen would pose a threat to that portion of the U.S. wheat market that exports to countries that quarantine this pathogen.

Wheat varieties traditionally have been evaluated in India for resistance to *T. indica* by counting infected kernels (7,15). The sporadic occurrence of Karnal bunt and the great variability in varietal performance has hampered varietal evaluations. A more reliable method is needed to screen large numbers of wheat varieties for possible resistance to this pathogen.

There is evidence that inoculation of sporidia into the boot sheath surrounding the spike will result in higher levels of kernel infection than would occur with natural infection (4). Natural infection of wheat by sporidia probably occurs before or near the time of anthesis (3); however, because of the lower levels of infection resulting from artificial inoculations performed close to anthesis, larger sample sizes are required to detect small differences in varietal performance. Additionally, the differences in spike maturity and degree of spike emergence from the boot sheath are confounding factors that could mask differences in varietal resistance under artificial conditions. Durán and Cromarty (6) reported greater infection when they performed inoculations by injecting sporidia into the boot sheath than by atomizing sporidia onto exposed spikes. Singh and Krishna (16) found that the "awns emerging" stage was more favorable than earlier stages of spike development for artificial inoculation.

Investigations of the mode of infection of wheat have shown that the pathogen penetrates the epidermal cells of the glumes with subsequent entry into the ovaries (11). Dhaliwal et al (5) proposed that *T. indica* may establish primary infection sites within a particular spike and spread "systematically" to other spikelets within the spike. They also suggested that late infection may occur at any time until the dough stage.

More than 30 yr ago, Bedi et al (3) studied the incidence of Karnal bunt on individual kernels within each spike under natural conditions. The significance of the relationship between plant and kernel infection was not statistically analyzed or otherwise discussed as a breeding tool.

This investigation was undertaken to study the relationship between frequency of spike and kernel infection, the influence of spike maturity (between heading and anthesis) on susceptibility, and the possible use of these statistical relationships as selection criteria for plant breeders when inoculations are performed at the boot and heading stages.

MATERIALS AND METHODS

All experiments were performed in the containment laboratory and greenhouse facilities of the USDA Plant Disease Research Laboratory at Ft. Detrick, Frederick, MD. Seed infected with *T. indica* collected from the Yaqui and Mayo valleys, Sonora, Mexico, in 1981–1984 was obtained from J. M. Prescott, CYMMYT, and USDA-APHIS. Infected seed from Sangrur, Amritsar, and Patiala, India, collected in 1983 was obtained from L. M. Joshi, IARI (Indian Agricultural Research Institute, New Delhi). All infected seed samples were sent under permits issued by APHIS.

Six kernels of the spring wheat cultivar Olaf (*T. aestivum*) were planted in 12-cm-diameter clay pots in a soil mix containing silty clay loam, sand, perlite, and peat (2:1:1:1) amended with 6.4 g of 10:10:10 fertilizer per liter and 25.7 g of lime per liter. Plants were grown in a glasshouse at 15–21 C until most of the tillers in a pot had reached growth stages

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10–10.5.1 (9). All tillers in a pot that were between growth stages 10 and 10.5.1 were selected for inoculation and labeled. The date, inoculum density, inoculum concentration, pot number, plant number, growth stage (10 = boot, 10.1–10.5.1 = heading) (9), percentage of dehisced anthers in each spike, and distance from the bottom of the spike to the ligule of the flag leaf were recorded. The heading stage used here included the emergence of spikes to the beginning of flowering.

Monosporidial lines were established on potato-dextrose agar from single primary sporidia isolated from germinated teliospores. Inoculum was prepared by the method of Royer and Rytter (13) by transferring 1-cm^2 blocks of agar from cultures of the monosporidial lines to the lids of petri plates, then inverting the lids over 2% water agar and incubating the plates at 20 C. Sporidia that were released onto the surface of the agar were harvested, and inocula were quantified by flooding the agar surface with distilled water after 10–15 days, then dislodging the primary and secondary sporidia with a rubber policeman. Inoculum from individual monosporidial lines was quantified to 10^3–10^5 sporidia per milliliter of distilled water with a hemacytometer, mixed in pairs, and inoculated onto wheat. Thirty to 60 spikes of Olaf wheat were inoculated with a particular pair of monosporidial lines. Sixty pairs of monosporidial lines were used as inocula over a period of 6 mo in 1984.

Half of the sporidial inoculum was injected into the boot as described previously (13). Spikes that had already emerged from the boot were drenched with the other half of the inoculum by dripping the sporidial suspension onto the florets until runoff. Inoculated plants were placed in a misting tent for 3–4 days at 15–21 C to provide constant free moisture. The plants were then removed to glasshouse benches and maintained at 15–21 C for 3–5 wk until they reached maturity (growth stages 11.3–11.4) (9), and bunted kernels were counted.

Because compatibility cannot be determined by in vitro pairing and fusion of monosporidial lines as with certain other smut fungi (6), the ability of paired monosporidial lines to infect the plant and produce symptoms was interpreted as an indication of compatibility as well as pathogenicity (13). Homothallism does not occur in this organism (6), and *T. indica* was shown to have multiple alleles controlling bipolar compatibility. Different pairs of compatible lines may differ in virulence; however, the data were analyzed over different inoculation dates and crosses with compatible monosporidial lines to include as much variation as would be encountered in greenhouse screening of germ plasm for resistance to *T. indica*.

Regression analysis was applied to the data from each of the two inoculation methods to determine 1) the relationship between the percentage of bunted kernels per bunted spike and the percentage of bunted spikes and 2) the relationship between the percentage of bunted kernels per inoculated spike and the percentage of bunted spikes. The relationship between the percentage of each spike that had emerged from the enclosing boot sheath and the percentage of bunted kernels was also analyzed by regression. The number of bunted spikes resulting from the boot-injection and spike-drench inoculations was compared statistically using a 2 × 2 table to calculate the chi-square statistic.

RESULTS

Twenty-one of 60 paired inoculations with monosporidial lines were pathogenic. The numbers of bunted spikes per total numbers of spikes inoculated with compatible pairs of monosporidial lines were 142/627 and 153/596 for boot-injection and spike-drench inoculations, respectively. The numbers of bunted spikes were not significantly different between the two inoculation methods ($\chi^2 = 1.36$, $P = 0.01$).

The average percentages of bunted kernels per bunted spike were 74 ($s = 29$) and 59 ($s = 30$) for boot-injection and spike-drench inoculations, respectively. The percentages of bunted kernels per bunted spike were significantly different between the two inoculation methods ($t = 4.33$, $P = 0.001$).

The regressions of the percentage of bunted kernels per inoculated spike (y) on the percentage of bunted spikes per

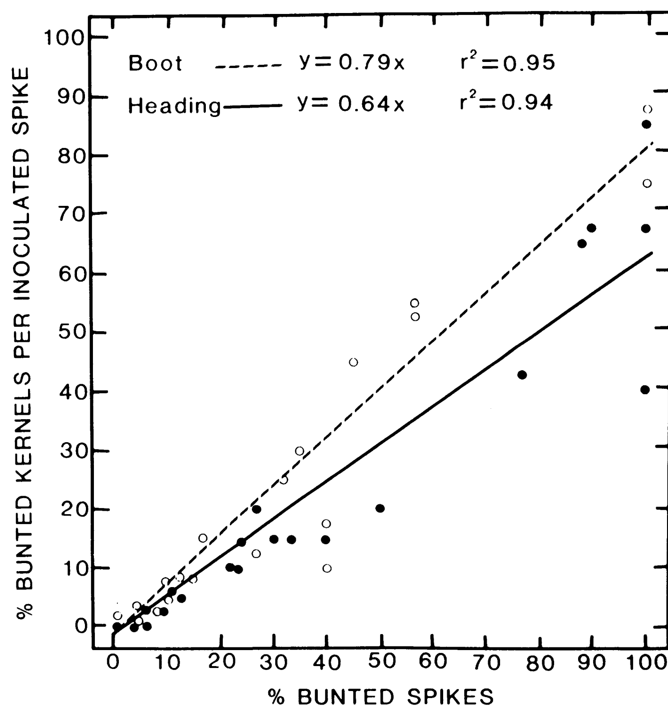

Fig. 1. Regression relationship between the percentage of bunted kernels per inoculated spike and the percentage of bunted spikes over all inoculated spikes. Inoculations of Olaf spring wheat were performed in the boot stage and at different heading stages with sporidia of *Tilletia indica*. Coefficients of determination (r^2) were significant at $P = 0.01$; regression was performed through the origin.

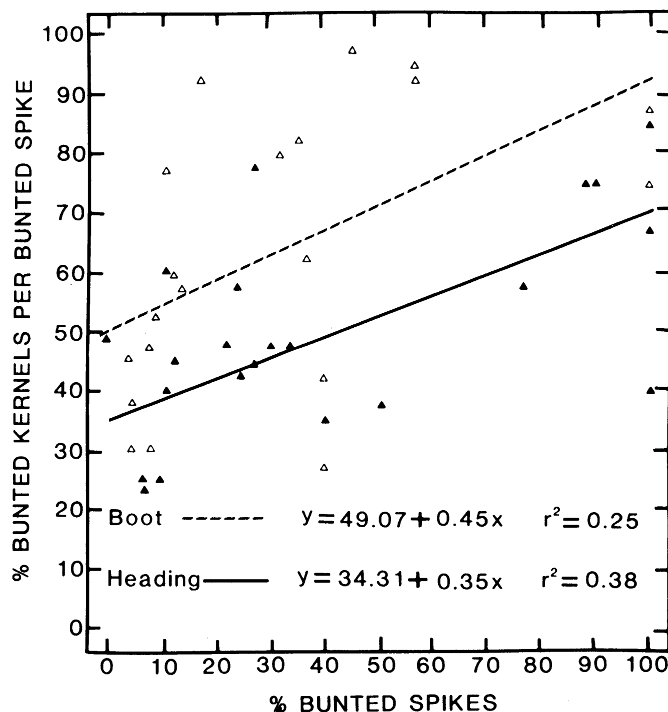

Fig. 2. Regression relationship between the percentage of bunted kernels per bunted spike and the percentage of bunted spikes over all inoculated spikes. Inoculations of Olaf spring wheat were performed in the boot stage and at different heading stages with sporidia of *Tilletia indica*. Coefficients of determination (r^2) were significant at $P = 0.01$.

inoculated spike (x) included no intercept terms and were significant ($P = 0.01$) for both boot-injection ($F = 407.5$, error df = 20) and spike-drench inoculations ($F = 294.8$, error df = 20), respectively (Fig. 1). The regressions of the two methods of inoculation were significantly different ($P = 0.01$, $F = 8.1$, error df = 1, 40) according to the full- versus reduced-model approach (12) of testing for homogeneity of regression. The adjusted means of the percentage of bunted kernels for the two inoculation methods were significantly different ($F = 6.1$, $P = 0.02$), but the slopes were not significantly different ($F = 3.8$, $P = 0.05$).

The regressions of the percentage of bunted kernels per bunted spike (y) on the percentage of bunted spikes per inoculated spikes (x) were significant ($P = 0.01$) for both boot-injection ($F = 7.3$, error df = 19) and spike-drench inoculations ($F = 13.2$, error df = 19), respectively (Fig. 2). The regressions of the two methods of inoculation were significantly different ($P = 0.01$, $F = 5.1$, error df = 2, 38) according to the full- versus reduced-model approach (7) of testing for homogeneity of regression. The intercepts were significantly different ($F = 9.9$, $P = 0.01$), but the slopes were not significantly different ($F = 0.3$, $P = 0.05$). Angular and log transformations did not significantly improve the fit of the data to linear regression models.

The regression (over all experiments and crosses with compatible lines) of the percentage of bunted kernels per bunted spike (y) on the percentage of each spike that had emerged from the boot sheath by inoculation (x) was significant because of the large number of degrees of freedom in the error term ($y = 76.38 - 0.22x$, $P = 0.05$, $F = 25.5$, $r^2 = 0.08$, error df = 293). Angular and log transformations did not significantly improve the fit of the data to linear regression models.

DISCUSSION

The significant linear relationships between the percentage of bunted kernels per inoculated spike and the percentage of bunted spikes indicate that a given incidence (number) of bunted spikes can be used to predict the incidence of kernel infection with artificial inoculation of Olaf wheat. The slopes of the above relationships were less than one, indicating that less than 1% increase in bunted kernels per spike can be expected for each percentage increase in bunted spikes under these inoculation conditions.

The regression of the percentage of bunted kernels per bunted spike on the percentage that each spike had emerged from the boot by inoculation explained very little of the variation in the data. However, the negative slope ($B_1 = -0.22$) is an indication of the decrease in infection with spike maturation. This may reflect the success of the different inoculation methods as well as physical and physiological resistance of those spikes that had emerged from the boot sheaths. Singh and Krishna (16) found that fewer bunted kernels and bunted spikes resulted from inoculations at the heading and flowering stages than at the "awns emerging" stage.

The percentage of the anthers that had dehisced by the time of inoculation and the distance from the bottom of the spikes to the ligules of the flag leaves were not significantly correlated with the percentage of bunted kernels or bunted spikes. Thus, there was less of a difference in disease development during flowering and stem elongation compared with the overall trend of less disease development from spike emergence onward to maturity.

The mean percentage of bunted kernels was greater for the boot-injection than the spike-drench method of inoculation. This may have been due to the longer time that the pathogen had to infect the glumes, the susceptible and succulent tissue within the boot, and the greater number of sporidia that could come in contact with the glumes within the boot sheath and not run off the glumes as with the spike-drench method.

Singh and Krishna (16) reported that the incidence of bunted kernels was greater than that of bunted spikes in cultivar HD 2009 for various artificial inoculation methods. Bedi et al (3) reported that the incidence of bunted kernels was less than that of bunted spikes under natural conditions of infection for eight cultivars. The differences reported in this relationship between kernel and spike infection may be due to variations in inoculation technique, natural versus artificial inoculation, environmental conditions, pathogen cultures, cultivar susceptibility, and developmental stage of the spike at inoculation. The mere existence of this variability offers new problems for the development of analytical tools in breeding programs.

The slopes presented in Figure 2 may reflect the probability of primary or secondary sporidia landing on particular spikelets as well as the "systematic" proliferation of infectious hyphae from initial infection sites to other spikelets within the spike (5). The slopes may also give a relative indication of the speed of infection and would provide an additional analytical tool for the plant breeder. If the slopes are a measure of the secondary spread of infection, then regression analyses may provide a less intensive method of measuring the amount of secondary infection of spikelets than by examining the position and severity of bunted kernels within the spike (5).

The variances of the mean percentage of bunted kernels per bunted spike may be used to calculate the minimum number of spikes that have to be inoculated to determine the differences in susceptibility among varieties under the conditions specified. This information would be useful for researchers who screen wheat varieties for small degrees of resistance to *T. indica* on the basis of the percentage of bunted kernels in a sample. For example, varieties have been screened in India on the basis of 0, 0–1, 1–5, and >5% infection (7).

Assume that the mean percentage of bunted kernels per bunted spike is independent of the size and variance of the sample (the sample reported herein was 21 experiments with compatible monosporidial lines) and that other wheat accessions have similar variation in kernel infection. The variances obtained from the boot-injection and spike-drench inoculations were not significantly different ($P = 0.05$, $F = 1.05$, error df = 152, 141) and a pooled variance estimate was close to the sample variances, 9.0.

The required size of the experiment can be estimated with the assumption that the sample variance is the best estimate of the population variance, the size of the difference between treatments is chosen, and types I and II error rates are chosen (17).

For example, a screening experiment may consist of 100 varieties, with at least two spikes (usually more than 20) per variety. If the experiment is a completely randomized design, the level of significance is set at $P = 0.05$, and the probability of detecting a difference is set at 90%, then the number of spikes required to detect any differences in percentages of bunted kernels among varieties can be estimated (Fig. 3).

When more than 1% (10) to 5% (14) of the kernels are bunted in newly harvested wheat, the grain is considered severely infected and is not acceptable for human consumption. Therefore, to detect a minimum difference between 0 and 3% (at $P = 0.05$), more than 21 spikes per variety should be tested. The number of spikes needed to declare significance at $P = 0.05$ increases to 189 for 1% and 757 for

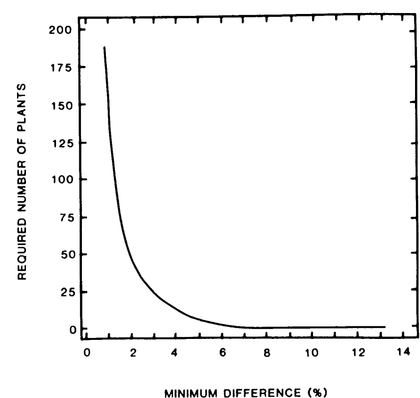

Fig. 3. Number of plants required to declare significance between minimum differences in the percentage of bunted kernels per bunted spike for 100 hypothetical wheat varieties. The probability of detecting a difference = 90% and the level of significance = 95%.

0.5% bunted kernels. The sample size must be further increased by the appropriate fraction if fewer than 100% of all spikes inoculated are expected to become infected. Many plants are therefore needed to effectively screen wheat varieties for resistance to Karnal bunt if tested under the artificial conditions specified herein. Larger Karnal bunt field screening nurseries may be required than those predicted under artificial conditions because of the variability of the environment and less than optimal conditions for infection of every spike.

The information and methods reported in this paper may be useful to plant breeders for detecting small degrees of resistance to the Karnal bunt pathogen. This may be especially relevant since there are no currently grown wheat cultivars that are known to be immune to infection by *T. indica*. Current research is in progress to evaluate the interaction of temperature, dew period, geographical source of the pathogen, and host on the infection processes of *T. indica.*

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