Effects of Inoculation and Pollination on Smut Development in Near-Isogenic Lines of Pearl Millet

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ABSTRACT

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Inoculating pearl millet heads during the first 72 hr after emergence with a sporidial suspension of *Tolyposporium penicillariae* in the late afternoon (1600–1700 hours) and covering the heads overnight (until 0800–0900 hours) with prewetted polyethylene bags appeared adequate to assure smut infection in a susceptible host. The *tr* gene that removes plant trichomes, stylar branches, and pleiotropically affects a number of other plant characteristics conferred a high level of smut resistance. The male sterile lines in the absence of pollen had the highest level of smut infection, demonstrating that pollination is a major factor inhibiting smut development. Age of florets at the time of inoculation was a major factor regulating smut development. Timing of pollination of inoculated plants significantly influenced smut development, especially when pollination was delayed more than 72 hr after inoculation. Between the near-isogenic lines there appeared to be genetic differences in smut resistance because dwarf, late, inbred 23D was significantly more resistant than tall, early 23E.

Additional key word: *Pennisetum americanum*.

Pearl millet, *Pennisetum americanum* (L) Leeke, is primarily a forage crop in the southern United States, but it is the staple cereal throughout the hot-arid and semiarid areas of the Indian subcontinent and Africa. During recent years extensive genetic improvement efforts have been under way. However, the new high-yielding hybrids have been more prone to ergot and smut than the land races of pearl millet.

Ergot, caused by *Claviceps fusiformis* Loveless, and smut, caused by *Tolyposporium penicillariae* Bref., are two major diseases of pearl millet in which the pathogens infect the individual florets and the pathogens and host combine to produce fungal masses, sclerotia, and spore balls, respectively, in lieu of seed grains. Ergot has not been reported on pearl millet in the United States, but the smut was reported in 1962 (13).

Since ergot is a potentially dangerous disease because of toxicity to humans and animals, from *C. fusiformis* alkaloids, it has received major attention from researchers. Thakur and Williams (10) and Thakur et al (11,12) have demonstrated that both the time of pollination and time of inoculation with *C. fusiformis* are major factors regulating ergot development. Pollination before or at time of inoculation with *C. fusiformis* reduced ergot from 60–80% to less than 3% of florets infected, and pollination delayed 16 hr after inoculation resulted in an intermediate level of ergot. Most ergot (80% of florets infected) was obtained when heads were inoculated at the time when a maximum number of fresh stigmas were present and was lowest (2% of florets infected) when inoculation was delayed until stigmas had withered. Significant progress has been made in identifying and developing sources of resistance to ergot (7,11).

Bhatt in 1946 (1) showed that *T. penicillariae* sporidia infected individual florets resulting in the production of smut in lieu of seed. He demonstrated that the smut was not carried within the seed but could overseason in the soil, and teliospores produced sporidia that became airborne to initiate infection in the following crop. This was highly dependent on moisture at time of flowering. He also showed that different floral stages affected smut development and that pollination affected smut infection by causing stigmas to wither after fertilization. Thakur et al (8,9) demonstrated that time of inoculation affected smut development. Highest levels of smut development resulted from inoculations in the boot leaf stage with no smut development when inoculation was delayed until anthesis. Male sterile lines were more susceptible than lines producing pollen.

Patel and Desai (4) showed that polyethylene bags were superior to butter paper bags as head covers for smut infection when relative humidity was below 90%. Thakur et al in 1983 (9) found that polyethylene bags were superior to parchment bags in the greenhouse but not in the field. However, field inoculations using the parchment bag technique were highly dependent on the rainy season or sprinkler irrigation for success. At Tifton, GA (Wells, *unpublished*), we have had excellent success using prewetted polyethylene bags to cover the inoculated heads just after inoculation at approximately 1600–1700 hours and removing the bags at approximately 0800–0900 hours the next morning under field, screenhouse, and greenhouse conditions during both rainy and dry periods. The prewetted polyethylene contained a film of moisture inside the bag that resulted in near 100% relative humidity within the bag during the time it was left on the head. These bags have been effective at maximum daily temperatures ranging from 25 to 40 C; however, these temperatures were probably not attained during the period the heads were under the polyethylene bag.

Husain and Thakur in 1963 (3) and Subba Rao and Thakur in 1983 (6) demonstrated that *T. penicillariae* sporidia grown in culture were superior to spore balls as a source of inoculum for pearl millet.

The purpose of this study was to more clearly define the effects of timing of pollination and timing of inoculation in relation to age of florets on smut development in pearl millet using a number of near-isogenic, male sterile and maintainer lines of Tift 23 pearl millet. A portion of this work has been reported in an abstract (14).

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MATERIALS AND METHODS

Inoculum and inoculation. *T. penicillariae* cultures were obtained from intact smutted florets collected from the field at Tifton, GA, in 1984 and 1985. The smutted florets were surface sterilized by dipping in alcohol, pierced with a sterile needle, and the spore balls dusted onto 20% V-8 agar (V8A) in 9-cm culture dishes. Pure cultures were transferred to other dishes of V8A and serially transferred by spreading over entire dish surface as needed to supply the desired amounts of inoculum. Inoculum was removed from 5- to 10-day-old cultures by scraping with a spatula and comminuted with deionized water in a Waring Blendor (sporidial concentration was about 10^6 per milliliter). The suspension was sprayed onto heads until run-off with a knapsack sprayer at approximately 1600–1700 hours. Heads were immediately covered with polyethylene bags (6.5 × 26 cm) that had been prewetted inside with deionized water. To be sure the polyethylene bags had adequate moisture inside to serve as humidity chambers, the bags were prewetted by opening and immersing in deionized water before use. Bags were taken directly out of the water and stapled over the heads. The polyethylene bags were replaced approximately 0800–0900 hours the following morning with glasine or kraft bags or heads left uncovered depending on treatment regime. After anthesis all heads were covered with kraft bags that had been treated to retard insect infestation.

A study was initiated when adequate plants were available at the heading stage (day 1) to do a test. Heads selected were from one-third to two-thirds out of the boot but had no visible indication of stigma emergence. The flag leaf and sheath were peeled away from the head and the head was covered with a glasine bag to prevent pollination and natural smut infection. Heads were color coded and numbered according to treatment they would receive. All treatments were replicated 10 or more times in a completely randomized experimental design. All heads remained bagged, except during various experimental manipulations, throughout the period of study. Smut ratings were made 21 days after the last inoculation of a test. Disease ratings were scored on an empirical scale of 0 to 5 in order of increasing disease severity (0 = no smutted florets, 1 = < 10, 2 = 10 < 30, 3 = 30 < 70, 4 = 70 < 90, and 5 = 90–100% of florets) infected unless otherwise stated. Data were subjected to statistical analyses using analysis of variance with Fisher's F-test and Duncan's multiple range test, regression, and general linear models procedures.

Screenhouse test 1984. Pearl millet cytoplasmic male sterile (A) early (E) line Tift 23 EA (23 EA) and near isogenic fertile (B) maintainer line Tift 23 EB (23 EB) growing in a screenhouse were inoculated on day 1. No pollen was added to 23 EA (84 heads). Ten heads of 23 EB were pollinated 24 hr after inoculation, and the remainder (80 heads) were allowed to self-pollinate only.

Field test 1984. Male sterile inbred Tift 23DA (23DA) was used in this study. A control consisted of bagging 10 heads on day 1 to determine the natural field level of smut. Effects of timing of pollination on smut development were evaluated by inoculating plants on day 1 and pollinating on day 2, 3, 4, 5, or 6. Pollination was achieved by removing bags on indicated date and allowing cross pollination from adjacent pollen bearing plants. Effects of timing of inoculum application on smut development were evaluated by inoculating on day 1, 2, 3, 4, or 5. Pollen was excluded from the heads receiving inoculum on different days.

Field test 1985. Near-isogenic pearl millet Tift 23 lines used included: cytoplasmic male sterile (A), 23EA (tall early), 23DA (dwarf late), 23EA *tr* (trichomeless), 23DA *tr* (trichomeless), and their respective fertile maintainer B lines 23EB, 23DB, 23EB *tr* and 23DB *tr*. The *tr* gene suppresses trichomes on the plant and stigmatic branching (5) and has a pleiotropic effect on a number of agronomic characteristics (2). Effects of time of pollinating on smut development were evaluated by inoculating heads on day 1 and pollinating on day 2, 3, 4, 5, or 6. Effects of time of inoculating were evaluated by inoculating heads on day 1, 2, 3, 4, or 5. One set of controls received no pollen or inoculum. The B (maintainer) lines were allowed to self-pollinate at time of anthesis in addition to receiving pollen on indicated dates. Source of pollen applied on all lines was from 23 EB. Pollen was secured by placing glasine bags over heads just beginning anthesis the day before use. Heads were inverted and pollen dusted into bag; the bags were then transferred to target head and placed over head and shaken to distribute pollen onto florets.

Greenhouse test 1985. Tift 23 near-isogenic A lines 23 EA and 23 DA having the normal *Tr* gene pair, and 23 EA *tr* and 23 DA *tr* carrying the *tr* gene pair with pleiotrophic effects, were inoculated with smut on day 1 and not pollinated. Number of heads per entry varied from 37 to 56. Smut infestations were estimated and expressed as percent of total florets infected on each head.

RESULTS

Screenhouse test 1984. The average disease ratings were 3.0, 2.0, and 1.0 for the 23EA, 23EB, and 23EB with supplemental pollen, respectively. These differences were all significant at $P = 0.01$. Percentages of heads rating a 3.0 or higher were 66, 35, and 0% for 23EA, 23EB, and 23EB with supplemental pollen, respectively.

Field test 1984. The natural field level of smut as determined from heads remaining bagged throughout the test averaged 1.7. When plants were inoculated on day of heading average smut scores were 1.3, 2.6, 3.3, 3.7, and 4.5 for pollination on day 2, 3, 4, 5, and 6, respectively. Average smut scores were 4.2, 4.4, 2.9, 2.1, and 1.5 when inoculating with *T. penicillariae* on day 1, 2, 3, 4, or 5, respectively. Differences in means for both time of pollinating and time of inoculating were highly significant ($P = 0.01$).

Field test 1985. The greatest difference of any of the factors studied in 1985 resulted from the evaluation of smut infection in the trichomeless *tr* gene pair with an average smut rating of 0.78 as compared with 2.22 for the normal *Tr* plants. Because the effect of the *tr* gene pair was so great ($P = 0.001$) in suppressing smut and the overall smut level in this *tr* gene type was so low, we excluded lines carrying the *tr* genes from further analysis and means for various treatments are not presented. Further discussion of results will relate only to *Tr* normal plants.

Average smut ratings for the noninoculated and nonpollinated of 23EA and 23DA were 0.3 and 1.3, respectively. This represents the natural smut level at the test sites. Increases in ratings above this level indicate treatment effects. The control heads of 23EA and 23DA receiving inoculations on day 1 and no pollination treatment had an average rating of 4.8 and 4.1, respectively, indicating that the inoculation technique should be of value in separating resistant and susceptible pearl millet phenotypes. Average smut ratings for the A and B lines carrying the *Tr* genes were 2.74 and 1.70, respectively, which were significantly different ($P = 0.01$). Average ratings for 23E and 23D lines were 2.77 and 1.67, respectively, which were also significantly different ($P = 0.01$).

Mean scores for effects of time of inoculating on smut ratings for 23DA and 23DB and 23EA and 23EB are presented in Table 1. Regressions of time of inoculation on smut development are shown in Fig. 1 for 23DA and 23DB and Fig. 2 for 23EA and 23EB, respectively. The regression curves peak when inoculations were conducted not later than day 3. Inbred 23DB (Table 1) was least affected by time of inoculation, never getting much above the background level of smut. The 23DA regression line for smut development declined steadily when inoculations were performed after day 1. Regression lines for smut development for inbred lines 23EB and 23EA showed rapid declines when smut inoculations were delayed past day 2 and 3, respectively.

Mean scores for effects of time of pollination on smut ratings are presented in Table 2. The results for 23DB (Table 2 and Fig. 3) indicated that self-pollination was about as effective as any treatment on 23DB, but 23EB smut levels (Table 2 and Fig. 4) were significantly reduced by the use of supplemental pollen, especially on day 3. On the A lines that depended entirely on donated pollen, pollinating on day 2 was not as effective in reducing smut. Pollinating on day 3 was most effective in reducing smut, and smut control declined rapidly when pollinating was delayed after day 4. It appears that pollination may be delayed for up to 72 hr and still prevent smut development in heads inoculated with *T.*

TABLE 1. Means for effects of day of pollination on smut development in near-isogenic pearl millet inbred lines[x]

Treatment	Pearl millet inbred lines			
	23DA	23DB	23EA	23EB
Noninoculated nonpollinated control	1.3bc[y]	0.6ab	0.3a	0.2a
Inoculated[z] nonpollinated	4.1hij	1.5bcd	4.5ij	4.8j
Inoculated pollinated on day 2	3.7ghi	1.1abc	4.8j	3.3fgh
Inoculated pollinated on day 3	2.4def	0.8ab	2.5ef	1.1abc
Inoculated pollinated on day 4	1.9cde	1.0abc	2.3de	3.5gh
Inoculated pollinated on day 5	2.9efg	1.0abc	4.8j	3.6gh
Inoculated pollinated on day 6	3.8ghi	1.0abc	4.9j	3.7ghi

[x] Means for disease ratings for 10 heads per entry. Disease ratings were scored on a scale of 0 to 5 in order of increasing disease severity (0 = no smutted florets, 1 = <10, 2 = 10 <30, 3 = 30 <70, 4 = 70 <90 and 5 = 90–100% of florets infected).

[y] Means followed by the same letter are not significantly different ($P=0.05$) according to Duncan's multiple range test.

[z] Inoculations with *Tolyposporium penicillariae* were all made on day of heading.

TABLE 2. Means for effects of day of inoculation with *Tolyposporium penicillariae* on smut development in near-isogenic pearl millet inbred lines[y]

Treatment	Pearl millet inbred lines			
	23DA	23DB	23EA	23EB
Noninoculated	1.3abc[z]	0.6a	0.3a	0.2a
Inoculated on day 1	4.1fg	1.1ab	4.5fg	4.8fg
Inoculated on day 2	3.6ef	1.1ab	5.0g	4.9g
Inoculated on day 3	1.3abc	1.0a	4.5fg	2.4cde
Inoculated on day 4	1.0a	1.0a	2.3bcd	0.6a
Inoculated on day 5	1.2abc	0.8a	0.9a	0.5a
Inoculated on day 6	1.1ab	0.8a	0.8a	0.4a

[y] Means for disease ratings for 10 heads per entry. Disease ratings were scored on a scale of 0 to 5 in order of increasing disease severity (0 = no smutted florets, 1 = <10, 2 = 10 <30, 3 = 30 <70, 4 = 70 <90 and 5 = 90-100% of florets infected).

[z] Means followed by the same letter are not significantly different ($P=0.05$) according to Duncan's multiple range test.

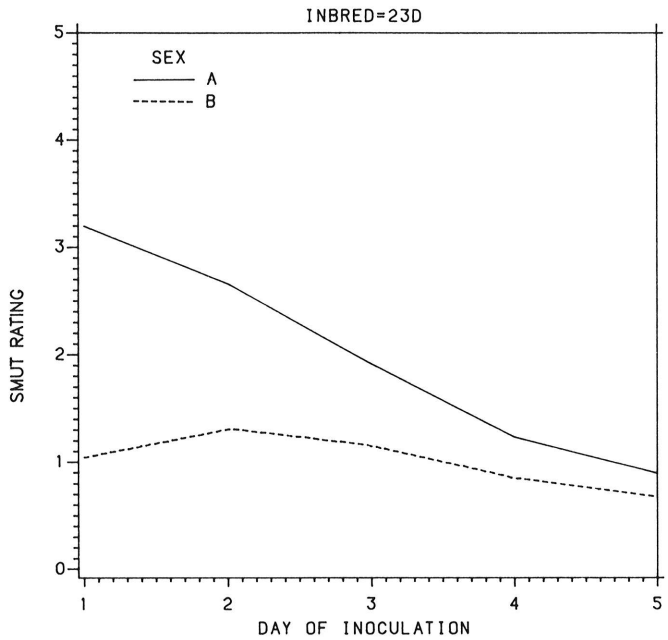

Fig. 1. Regression for day of inoculation on smut development on pearl millet inbred line 23DA and maintainer line 23DB.

penicillariae.

Greenhouse test 1985. Near-isogenic lines carrying the *tr* gene averaged only 5.6% of kernels infected which was less than 10% of that of the *Tr* isogenic lines, which were 54.6 and 74.6% for 23 DA and 23 EA, respectively.

DISCUSSION

The procedures used in inoculating pearl millet heads and covering them overnight (1600–1700 to 0800–0900) with prewetted polyethylene bags gave repeatable results that were consistent in the greenhouse, screenhouse, and field as shown by data presented in this paper. The prewetted polyethylene bags contained a film of moisture inside the bag that appeared adequate to assure near 100% relative humidity during the entire period that these bags

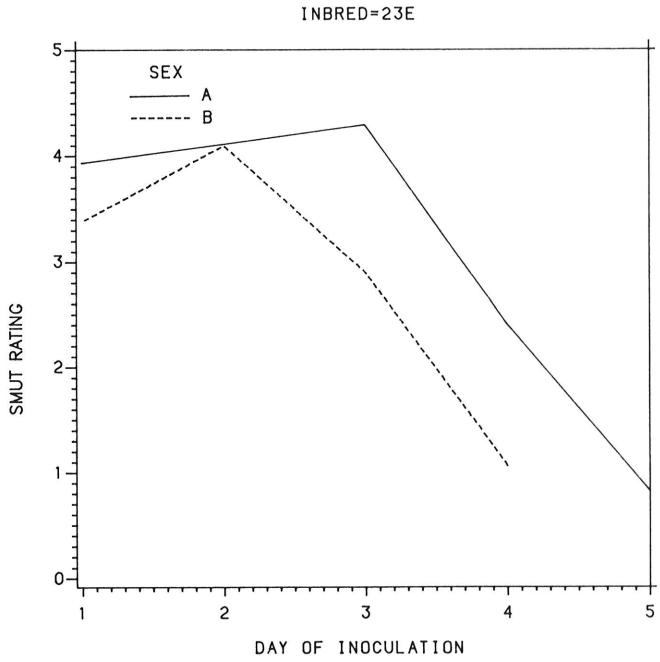

Fig. 2. Regression for day of inoculation on smut development on pearl millet inbred line 23EA and maintainer line 23EB.

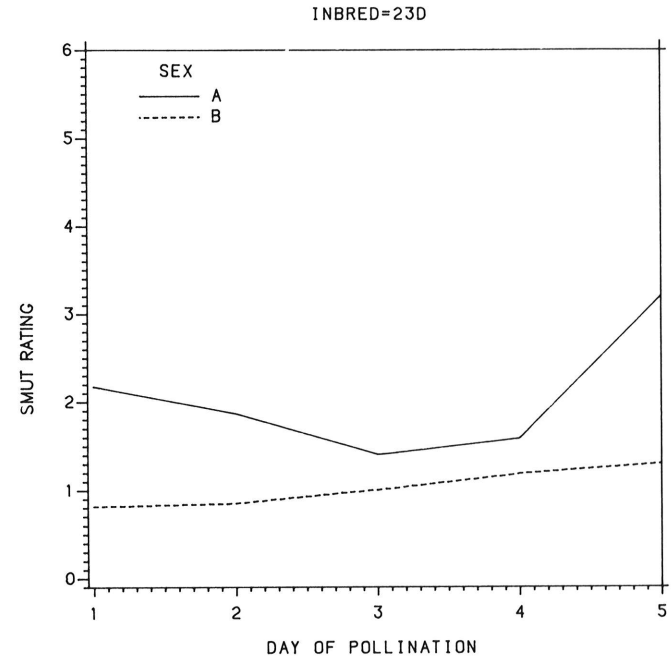

Fig. 3. Regression for day of pollination on smut development on pearl millet inbred line 23DA and maintainer line 23DB. Smut inoculations were made on day 1.

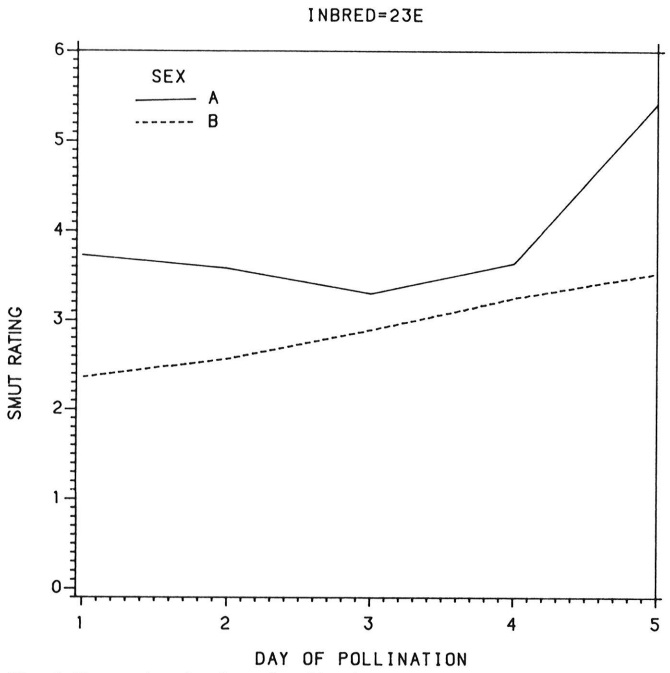

Fig. 4. Regression for day of pollination on smut development on pearl millet inbred line 23EA and maintainer line 23EB. Smut inoculations were made on day 1.

were left in place. This technique should be valuable for screening for smut resistance in that it is not weather-dependent (9). Maximum and minimum daily temperatures during infection period in greenhouse, field, and screenhouse were 40 and 25, 35 and 25, and 25 and 20 C, respectively, on days in which inoculations were conducted. The higher temperatures were probably not in effect for much of the time during which the heads were covered with polyethylene bags. It may be important to make sure that polyethylene bags are not left on too long, especially in some of the hotter climates, where the polyethylene bag could serve as a heat-trap and interfere with pollination.

The *tr* gene for trichomelessness that suppresses stigmatic branching, affects transpiration, forage quality, leaf wettness, and rust development (2) reduced smut development by a magnitude of 30–90% in both field and greenhouse studies. Our previous field observations had led us to believe that the *tr* gene conferred some degree of smut resistance. Lack of stigmatic branching may have conferred resistance by reducing the number of sporidia that remained attached to the stigma. This gene, however, increases degree of rusting (2) and will have to be used with caution in any breeding program.

Stage of inflorescence development is very important in pearl millet inoculations (Table 1 and 2). Therefore, in breeding work it is critical that age of head or stage of development at time of inoculation be kept uniform and clearly defined. Because stigmas had not withered during the period of this test and the A lines received no pollen on heads (represented in Table 1 and 2), factors relating to flower aging were directly related to smut infection or development. This could possibly be associated with stigma constriction as discussed by Willingale and Mantle (15).

All tests confirmed that pollination and time of pollination significantly affected smut development (8). The relatively low level of smut suppression for pollination treatments on A lines on day 2 was related directly to the lack of stigma development on day 2.

The 23D lines were significantly more resistant than the E lines. Additional studies will be required to determine the nature of this resistance in that it was observed in both the A and B lines. The greenhouse 1985 study in which the A lines were compared in the absence of pollen, gave the approximate same difference as observed in the overall field tests with the 23E and 23D lines. The similar patterns of field and greenhouse studies in 1985 indicate that 23D is considerably more resistant than 23E to smut. Neither line, however, contains a satisfactory level of resistance.

The major impact of time of inoculation and time of pollination on the incidence of smut will make it difficult to separate out the various components of resistance that may be valuable to the plant breeder (for example effects of time of pollen availability as compared with morphological or physiological resistance within the female portion of the floret).

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