Mechanisms Associated with the *tr* Allele Contributing to Reduced Smut Susceptibility of Pearl Millet

Jeffrey P. Wilson

Research Plant Pathologist, USDA-ARS Forage and Turf Research Unit, University of Georgia Coastal Plain Experiment Station, Tifton 31793-0748.

Cooperative investigation of the USDA-ARS and the University of Georgia, College of Agricultural and Environmental Sciences, Agricultural Experiment Stations.

I thank Andy Hornbuckle and Charles Markham for their technical assistance.

Accepted for publication 25 May 1995.

ABSTRACT

Wilson, J. P. 1995. Mechanisms associated with the *tr* allele contributing to reduced smut susceptibility of pearl millet. Phytopathology 85:966-969.

The *tr* allele in pearl millet confers trichomeless plant structures. Plants homozygous for the allele have reduced severities of smut, caused by *Moesziomyces penicillariae*, compared to plants with the *Tr* genotype. Experiments were conducted to determine the mechanisms conferring reduced susceptibility in *trtr* inbreds. Flag leaf sheaths of field-grown Tift 23DAS (*trtr*) were wrapped around unemerged panicles more tightly than were sheaths of Tift 23DA (*TrTr*), as measured by reduced infiltration of fluid into the boot, recovery of sporidia of *M. penicillariae* from unopened boots, and smut severity of panicles bagged prior to emergence from the boot. When exposed panicles were inoculated with *M. penicillariae* in the greenhouse, moisture periods required for a given level of smut severity were greater for Tift 23DAS than for Tift 23DA. Severity after 8 h of moisture was greater on Tift 23DA. Increase of disease severity with longer moisture periods was essentially linear and parallel for both inbreds for moisture periods of up to 48 h. Intercepts (severity at 8 h) differed, but slopes did not. The *tr* allele may be useful as an easily identifiable, monogenic marker for smut resistance in pearl millet.

Pearl millet [*Pennisetum glaucum* (L.) R. Br.] is an important crop in the arid areas of several Asian and African countries. Because it reliably produces grain under adverse growing conditions and requires few inputs compared to many other crops, it has potential value as a feed grain crop in low-input and sustainable agricultural systems in the United States.

Smut, caused by *Moesziomyces penicillariae* (Bref.) Vánky, is a major disease of pearl millet (11). The fungus presumably infects stigmas and nonsystemically colonizes individual ovaries (2). A sorus is formed in place of a seed if pollination does not occur within 4 to 8 days from infection (16,20). Field surveys of the pearl millet grain hybrid 'HGM 100' grown at and near Tifton, GA, indicate that most smut infection occurs on the early- and late-flowering panicles, which develop when pollen is least abundant in this cross-pollinating crop. Grain yields from early- and late-emerging panicles can be reduced by 43 and 83%, respectively, of yields from panicles that emerge in the peak flowering period (J. P. Wilson, *unpublished data*).

Although fungicides can control the disease (3,19), host resistance is the only practical means of control. Smut resistance exists in pearl millet, but it appears to be relatively scarce. Of the 21,919 pearl millet accessions maintained by ICRISAT, 161 accessions were classified as smut resistant (7). An evaluation of more than 7,700 pearl millet genotypes revealed 34 sources of resistance (18). Through an intensive breeding and screening effort spanning more than 7 years, agronomically improved breeding lines with smut resistance were developed (18). Complete resistance has not been reported, and resistance is measured quantitatively as a reduction in the proportion of smutted florets on a panicle.

Few studies have examined the inheritance of smut resistance in pearl millet. The male-sterile A_1 cytoplasm has no effect on the expression of resistance, and nuclear genes were the primary determinant of smut resistance in one group of hybrids (23). Resistance has been attributed to both additive and nonadditive genetic effects (10), and significant general and specific combining ability for resistance have been found (5). The quantitative inheritance and expression of smut resistance complicates and reduces the efficiency of breeding for resistance.

One potential approach for simplifying breeding for smut resistance in pearl millet is to incorporate the recessive *tr* allele into improved inbreds. The *tr* allele confers trichomeless leaves and unbranched stigmas in pearl millet. When near-isogenic and alloplasmic derivatives of the inbred 'Tift 23' were inoculated in the field and greenhouse, plants homozygous for the *tr* allele were less susceptible to smut than those homozygous for *Tr* (20). After determining that *trtr* pearl millet genotypes expressed smut resistance, Wells et al. (20) hypothesized that reduced stigmatic branching may be responsible for the resistance of *trtr* inbreds since the probability of sporidia adhering to a stigma is reduced. However, reduced stigmatic branching may not have played a role in reducing smut severities because their controlled inoculations were performed prior to stigma emergence, inoculum concentrations were sufficient to not limit the amount of disease, and panicles were protected by bagging after inoculation. Some other mechanism(s) must account for the lower level of smut infection of *trtr* versus *TrTr* plants. The objectives of the present experiments were to determine if other effects associated with the *tr* allele contribute to smut resistance. Specifically, differences between near-isogenic *trtr* and *TrTr* pearl millets were examined for tightness of the flag leaf sheath around the unemerged panicles and for moisture periods required for smut infection.

Corresponding author: Jeffrey P. Wilson; Fax: 912/386-7225.

MATERIALS AND METHODS

Inbreds Tift 23DA (*TrTr*) and Tift 23DAS (*trtr*) were planted on three different dates at Tifton, GA. The first planting was made on 3 August 1993. Two-row plots were 12 m long and spaced 1 m apart. Fertilizer (5-10-15 NPK) was applied in the row at planting at a rate of 280 kg ha^{-1}. In 1994, two-row plots of the inbreds were 25 m long and were planted, as described above, on two dates, 5 May and 23 June. The plantings made in 1994 were located in a field where pearl millet smut occurs regularly and with high severities.

To evaluate the relative tightness of the flag leaf sheath around unemerged panicles, 10 culms of each inbred were taken from the first planting on 21 and 22 September 1993 and from the first 1994 planting on 22 July. At 1700 h, culms having undamaged flag leaves and panicle tips that had emerged no more than 1 cm above the flag leaf ligule were cut off at the base of plants and the cut ends were placed in water. Culms were collected from approximately equidistant intervals along the length of the plots. In the laboratory, culms were cut cleanly with a razor so that three intact leaves remained on the stem, inserted into individual water-filled test tubes, and randomized within a test tube rack. Five drops of red food dye were placed by Pasteur pipet at the base of the flag leaf and in contact with the edge of the panicle tip. After 13 h, the lower circumference and the sides of the flag leaf sheath were cut with a razor and the flag leaf sheath was removed without disturbing the distribution of the dye on the panicle. The distance (cm) of dye penetration into the boot down the length of the panicle was measured. Each panicle was considered a replication for data analysis.

Sporidia in dew and infiltration into boots were measured in two experiments. Dew was collected by Pasteur pipet at 0700 h from flag leaves of Tift 23DA and Tift 23DAS along the length of the plots. Samples were collected on 21 July 1994 from the first 1994 planting described previously, and from the second planting on 24 August 1994. Dew collected from either inbred was combined into a sterile test tube until approximately 10 ml was obtained. In the lab, dew was serially diluted (1:10, 1:100, and 1:10,000) in sterile deionized water, and 1 ml of each of the dilutions was plated onto each of five plates of V8 agar (20% V8 juice, 1.5% 0.1 N NaOH) amended with 500 ppm of chlortetracycline and nalidixic acid. Colonies of *M. penicillariae* from dew dilutions were counted 3 to 4 days after plating. Each plate was considered a replication for data analysis.

On the same mornings that dew was collected, 10 culms of each inbred were collected by cutting at the base. All selected culms had swollen but fully closed boots, with none of the panicle tip emerged. Culms were collected from approximately equidistant intervals along the length of the plots.

Immediately after collection from the field, culms were taken to the laboratory. Flag leaf sheaths were sliced twice lengthwise with a sterile razor to facilitate removal of the unemerged panicle. The 10 panicles of each inbred were placed in a flask containing 1 L sterile deionized water. After agitation for 4 h, the water was suction filtered through a sterile Millipore filter (pore diameter 0.45 μm). The filter was agitated in 10 ml sterile deionized water to resuspend filtered material. One milliliter of the suspension was plated onto each of five plates of amended V8 agar. Colonies of *M. penicillariae* were counted 3 to 4 days after plating. Because filtered material from 10 panicles was resuspended in 10 ml water, colony counts per plate were considered equivalent to colony-forming units (CFUs) per panicle. Each plate was considered a replication for data analysis.

To evaluate the potential for smut infection to occur by sporidia entering into unopened boots, 10 boots of each inbred at the identical stage of development as those selected for isolation of *M. penicillariae* were bagged on the same mornings. Bagging was performed to prevent additional inoculum from contacting panicles and to prevent pollination by exogenous pollen. Panicles were bagged at approximately equidistant intervals along the length of the plots. Bagged panicles were evaluated for smut infection about 23 days after bagging. Smut severities of individual panicles were estimated visually as percentage of florets infected. Each panicle was considered a replication for data analysis.

Data for dye penetration into panicles, transformed CFUs per panicle, and transformed smut severities were analyzed by analysis of variance. Data of CFUs per panicle were transformed to log(CFUs + 1) and smut severities were transformed to log(% severity + 1) prior to analysis. Data were transformed to reduce associations between means and variances. Sums of squares were partitioned into experiment, inbred, and experiment × inbred interaction. Inbred means were separated by *t* tests.

Effect of moisture period following inoculation on smut infection of exposed panicles of near-isogenic inbreds Tift 23DA and Tift 23DAS was evaluated in the greenhouse in three experiments. In each experiment, 50 pots of each inbred were planted at 7-day intervals for three to five plantings depending upon the experiment. Stands were thinned to two plants per pot. Plants were selected for inoculation when panicles were about half-emerged from the boot. Prior to inoculation, the flag leaf and sheath were removed to completely expose the panicle.

Inoculum was prepared by growing *M. penicillariae* isolates MP-1 and MP-6, which are highly virulent in combination (22), individually on V8 agar under continuous fluorescent light at 24°C. Sporidia of the cultures were recovered from storage in 20% glycerin at –80°C. After the first subculture, sporidia from 3- to 7-day-old cultures were mixed in approximately equal quantities for a final inoculum concentration of 1.4×10^7 sporidia/ml.

Plants were inoculated from 10 to 21 April 1992 for the first experiment, 8 to 16 July 1992 for the second, and 7 April to 11 May 1993 for the third. Seven, fourteen, and twenty-three replications were evaluated in the first, second, and third experiments, respectively. The number of replications in each experiment was determined by the availability of an adequate number of plants at the correct growth stage on a given day when inoculations were performed. In each replication, six plants each of Tift 23DA and Tift 23DAS were inoculated at 1500 h by immersing panicles for approximately 15 s into a test tube containing inoculum. Immediately after inoculation, panicles were covered with prewetted plastic bags. The plastic bag from one plant of each inbred was removed at 8, 16, 24, 32, 40, and 48 h after inoculation and covered with a white paper selfing bag to prevent fertilization by exogenous pollen. Smut severities, determined as percentage of florets infected, were visually estimated about 4 weeks after inoculation.

In the analysis of variance, sums of squares were partitioned into effects of experiment, replication within experiment, inbred, moisture period, and inbred × moisture period interaction. Severity means (6) at each moisture period for each inbred from the

TABLE 1. Variables measured to differentiate tightness of the flag leaf sheath around boots of pearl millet inbreds Tift 23DA (*TrTr*) and Tift 23DAS (*trtr*)

	Inbred	
Variable	Tift 23DA	Tift 23DAS
Dye penetration (cm)[a]	13.0	5.4**[b]
Colony-forming units/panicle[c]	2220.0	206.0**
Smut severity (%)[d]	28.6	6.3**

[a] Dye penetration after 13 h into boots with panicles just beginning to emerge.

[b] ** = significantly different ($P = 0.01$) from Tift 23DA as determined by *t* tests.

[c] Colony-forming units of *Moesziomyces penicillariae* isolated from unopened boots. Log-transformed data were analyzed, untransformed means are presented.

[d] Smut severity of panicles bagged while fully enclosed in the boot. Log-transformed data were analyzed, untransformed means are presented.

first and third experiments were analyzed with the general linear model procedure of SAS (12) to evaluate severity as a linear function of moisture period. Because infection resulting from moisture periods less than 8 h was not evaluated, 8 h was subtracted from all moisture periods prior to regression to estimate the intercept (percent infection) at 8 h. Intercepts and slopes of the regression lines were compared for differences by *t* tests.

RESULTS

Penetration of dye into boots with panicle tips just beginning to emerge was reduced for Tift 23DAS as compared to Tift 23DA (Table 1). Experiment, inbred, and experiment × inbred effects were significant in the analysis of variance. Dye penetration was greater for Tift 23DAS in the first two experiments than in the third.

CFUs of *M. penicillariae* were abundant in dew, with approximately 1.42×10^5 and 6.4×10^3 CFUs ml^{-1} on 21 July and 24 August, respectively. The numbers of CFUs isolated from panicles and smut severities for both inbreds were greater when sporidial concentration in dew was greater. Approximately 10 times more CFUs were isolated from unopened boots of Tift 23DA than from Tift 23DAS (Table 1). Experiment and inbred were significant sources of variation, with more CFUs obtained from panicles from the first experiment. Average number of CFUs isolated from Tift 23DA on 21 July and 24 August were 4,068 and 376 CFUs per panicle, respectively, compared to 372 and 40 CFUs per panicle for Tift 23DAS on the two dates.

Panicles bagged prior to opening of the boots were infected by smut. Severities were about five times greater for Tift 23DA than for Tift 23DAS when panicles were bagged prior to emergence from the boot (Table 1). Experiment and inbred were significant sources of variation. Smut severities were greater in the first experiment. Average smut severity of Tift 23DA bagged on 21 July and 24 August was 42.7 and 14.4%, respectively, compared to 8.8 and 3.7% for Tift 23DAS on the two dates.

In the analysis of variance of smut severity as a function of moisture period, all sources of variation except inbred × moisture period interaction were significant ($P < 0.01$). Results obtained in the second experiment differed from the first and third. In the second experiment, levels of infection on Tift 23DA and Tift 23DAS were similar at all moisture periods, and averaged 90.7 and 56.9%, respectively. Slopes of both regression equations from experiment 2 did not differ from zero ($P > 0.05$). Unidentified conditions in this experiment were highly favorable for infection of both inbreds with 8 h of moisture.

Smut severity increased linearly on both Tift 23DA and Tift 23DAS with increased moisture periods (Fig. 1) in experiments 1 and 3. Regression equations differed between inbreds for intercept ($P < 0.05$), indicating that smut infection on Tift 23DA was greater after an 8-h moisture period. Slopes of the regression equations did not differ between inbreds.

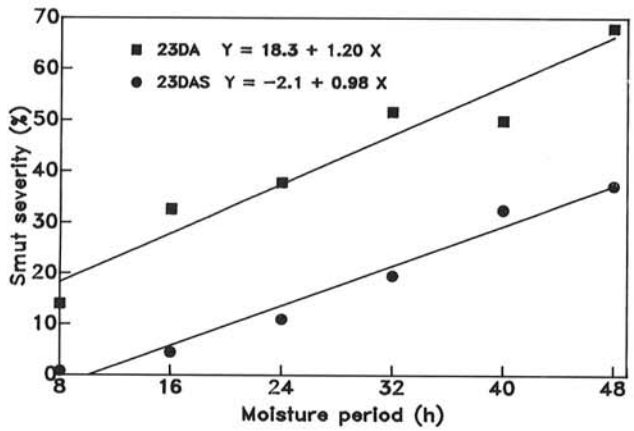

Fig. 1. Influence of moisture period on smut severity of pearl millet inbreds Tift 23DA (*TrTr*) and Tift 23DAS (*trtr*) inoculated with *Moesziomyces penicillariae* in the greenhouse. Data are means of two experiments. r^2 for Tift 23DA = 0.51 ($P < 0.01$) and for Tift 23DAS = 0.81 ($P < 0.01$).

DISCUSSION

Two effects associated with the *tr* allele, a tighter flag leaf sheath and longer moisture requirements for infection, are associated with reduced smut severity on *trtr* pearl millet.

Flag leaf sheaths are wrapped more tightly around the boots of Tift 23DAS than of Tift 23DA. The tighter flag leaf sheath reduced infiltration of fluid into the boot, as measured by dye penetration. A tighter leaf sheath is hypothesized to reduce smut severity by reducing infiltration of inoculum into the boot. Panicles are most susceptible prior to stigma emergence and levels of infection decrease as stigmas emerge (2). Inoculations resulting in optimum infection for pathogenicity tests and resistance screening are usually performed by injecting sporidia into the boot (1,4,9,17). In the present experiments, infection of panicles bagged prior to opening of the boot must have been the result of inoculum infiltrating into the boot, suggesting that natural infection may occur very early as the panicle is emerging from the boot.

In pearl millet, as in many species of the Gramineae family, the flag leaf is extended at an angle and the base of the leaf is cupped similar to a funnel over the tip of the boot just prior to inflorescence emergence. Water from dew or rainfall can be retained at the base of the leaf where it can seep into the boot. Aerial populations of *M. penicillariae* sporidia are greatest with high relative humidity and light rainfall (8). Because inoculum is present in dew on the flag leaf, a tighter flag leaf sheath that prevents infiltration of fluid into the boot will allow less inoculum to come in contact with unemerged panicles.

Differences between Tift 23DA and Tift 23DAS for penetration of dye, recovery of sporidia of *M. penicillariae*, and smut severities support the hypothesis that the tightness of the flag leaf sheath around the boot affects smut severity.

Moisture period requirements for infection differ between Tift 23DA and Tift 23DAS, indicating another mechanism for smut resistance associated with the *tr* allele. Not only are fewer sporidia able to infiltrate the boots of Tift 23DAS compared to Tift 23DA, but those sporidia also are less likely to produce a successful infection.

Although smut infection does occur on Tift 23DAS, longer moisture periods are required for infection to reach a particular level of severity compared to Tift 23DA, which suggests that fungal growth may be slower in *trtr* stigmas. It is not known if the presumed slower growth is the result of physical constraints such as stigmatic constriction (21), nutritional deficiencies, or defense mechanisms imposed by the host.

Pearl millet smut and ergot, caused by *Claviceps fusiformis* Loveless, are similar diseases in many respects. Both diseases are expressed on the panicle, and pollination reduces severity of smut (16,20) and ergot (14). This has probably led to the conclusion that factors affecting the resistance to *M. penicillariae* and *C. fusiformis* would be similar.

Differences between expression of resistance do differ, however. Thakur et al. (15) determined that the protogeny period, time between full protogeny and anthesis initiation, and stigma length were significantly shorter in ergot-resistant and intermediate resistant cultivars than in ergot-susceptible cultivars. In comparisons of smut-resistant and susceptible lines, Thakur (13) found no significant correlations between smut infection and time from boot to stigma emergence, from boot to anther emergence, or from stigma emergence to anther emergence. In addition, Thakur and Williams (14) demonstrated that ergot is more severe when plants are inoculated when the maximum number of fresh stigmas

are available. This contrasts with the results of inoculations performed by Bhatt (2), who reported that maximum infection of pearl millet by *M. penicillariae* occurs when inoculations are performed when inflorescences are young and without emerged stigmas. Smut infection decreased when inoculations were made on emerged or mature stigmas. A tighter flag leaf sheath and increased moisture requirements for infection are the first documented mechanisms that would confer smut resistance to pearl millet.

The *tr* allele may be useful for conferring smut resistance in grain hybrids of pearl millet. Although genetic differences in the ability to cause disease were detected previously among *M. penicillariae* isolates (22), the mechanisms of resistance observed in the present studies, particularly the tight flag leaf sheath which reduces inoculum in the boot, may not be overcome by changes in the pathogen population.

Whether smut resistance is conferred by the *tr* allele or tightly linked genes, selecting for the trichomeless character will give a high probability of selecting for smut resistance in improved inbreds. Tift 23DB and Tift 23DBS, the maintainer inbreds of Tift 23DA and Tift 23DAS, are essentially isogenic with at least seven backcrosses made in the transfer of the *tr* allele into Tift 23DB. No selection for smut resistance was practiced in developing Tift 23DBS or Tift 23DAS. Because the trichomeless character is easily identified in adult plants, labor-intensive inoculations may not be required for identifying smut resistance in breeding lines. The *tr* allele may be a useful marker for smut resistance because it is monogenic and when homozygous, can be identified by plant phenotype.

LITERATURE CITED

1. Andrews, D. J., Sing, S. B., Witcome, J. R., Singh, S. D., Rai, K. N., Thakur, R. P., Talukdar, B. S., Chavan, S. B., and Singh, P. 1985. Breeding for disease resistance and yield in pearl millet. Field Crops Res. 11:241-258.
2. Bhatt, R. S. 1946. Studies in the Ustilaginales. I. The mode of infection of the bajra plant (*Pennisetum typhoides* Stapf.) by the smut *Tolyposporium penicillariae* Bref. J. Indian Bot. Soc. 25:163-186.
3. Chahal, S. S. 1986. Yield loss due to smut of pearl millet. Indian Phytopathol. 39:292-293.
4. Chahal, S. S., Kumar, K., and Gill, G. S. 1986. Identity and variation in two isolates of *Moesziomyces penicillariae* the causal organism of pearl millet smut. Plant Dis. Res. 1:59-64.
5. Chavan, S. B., Thakur, R. P., and Rao, K. P. 1988. Inheritance of smut resistance in pearl millet. Plant Dis. Res. 3:192-297.
6. Gomez, K. A., and Gomez, A. A. 1984. Statistical Procedures for Agricultural Research. 2nd ed. John Wiley & Sons, New York. 680 pp.
7. Innes, N. L. 1992. Gene banks and their contribution to the breeding of disease resistant cultivars. Euphytica 63:23-31.
8. Kousik, C. S., Thakur, R. P., and Subbarao, K. V. 1988. Influence of environmental factors on production and dispersal of *Tolyposporium penicillariae* sporidia. Indian J. Aerobiol. 1:85-91.
9. Pathak, V. N., and Sharma, R. K. 1976. Method of inoculation of *Pennisetum typhoides* with *Tolyposporium penicillarie* (sic) and evaluation of germplasm for smut resistance. Indian J. Mycol. Plant Pathol. 6:102.
10. Prakash, O., Singh, S., and Khairwal, I. S. 1988. A study of combining ability for grain yield and smut severity in pearl millet. Indian J. Genet. Plant Breed. 48:13-17.
11. Rachie, K. O., and Majmudar, J. V. 1980. Pearl Millet. Pennsylvania State University Press, University Park. 307 pp.
12. SAS Institute, Inc. 1982. SAS User's Guide: Statistics. SAS Institute, Cary, NC. 584 pp.
13. Thakur, R. P. 1989. Flowering events in relation to smut susceptibility in pearl millet. Plant Pathol. 38:557-563.
14. Thakur, R. P., and Williams, R. J. 1980. Pollination effects on pearl millet ergot. Phytopathology 70:80-84.
15. Thakur, R. P., Rao, V. P., and King, S. B. 1992. Factors associated with ergot resistance in pearl millet. Indian Phytopathol. 45:326-330.
16. Thakur, R. P., Subba Rao, K. V., and Williams, R. J. 1983. Effects of pollination on smut development in pearl millet. Plant Pathol. 32:141-144.
17. Thakur, R. P., Subba Rao, K. V., and Williams, R. J. 1983. Evaluation of a new field screening technique for smut resistance in pearl millet. Phytopathology 73:1255-1258.
18. Thakur, R. P., Subba Rao, K. V., Williams, R. J., Gupta, S. C., Thakur, D. P., Nafade, S. D., Sundaram, N. V., Frowd, J. A., and Guthrie, J. E. 1986. Identification of stable resistance to smut in pearl millet. Plant Dis. 70:38-41.
19. Wells, H. D. 1967. Effectiveness of two 1,4-oxathiin derivatives for control of Tolyposporium smut of pearl millet. Plant Dis. Rep. 51:468-469.
20. Wells, H. D., Hanna, W. W., and Burton, G. W. 1987. Effects of inoculation and pollination on smut development in near-isogenic lines of pearl millet. Phytopathology 77:293-296.
21. Willingale, J., and Mantle, P. G. 1985. Stigma constriction in pearl millet, a factor influencing reproduction and disease. Ann. Bot. (London) 56:109-115.
22. Wilson, J. P., and Bondari, K. 1990. Quantitative genetic analysis of pathogenicity and virulence of *Moesziomyces penicillariae*. Phytopathology 80:1173-1177.
23. Yadav, O. P. 1994. Effect of A_1 cytoplasm on the combining ability for smut severity in pearl millet. Theor. Appl. Genet. 88:28-29.