Quantitative Genetic Analysis of Pathogenicity and Virulence of *Moesziomyces penicillariae*

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ABSTRACT

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Forty-five isolates were selected to determine the genetic basis of pathogenicity and virulence of *Moesziomyces penicillariae*, the smut pathogen of pearl millet. Solopathogenicity of mycelial isolates, sporidial isolates, and sporidial isolates with a low frequency of reversion to the mycelial type were evaluated. The mycelial and revertant isolates expressed the highest levels of solopathogenic ability, as determined by greater numbers of sori formed per panicle ($\bar{x}$=42.3% and 28.0% infected florets, respectively). Sporidial isolates expressed the lowest levels of solopathogenic ability ($\bar{x}$=1.2% infected florets). Twenty-two sporidial isolates were used alone and in all combinations to inoculate 3 inflorescences each of Tift 23DA in the greenhouse and Tift 85DB in the field. In the partial diallel inoculations, specific combining ability effects were significant, indicating the presence of nonadditive genes conferring compatibility types. General combining ability effects were also significant, suggesting that the isolates differed in additive genes conferring virulence. Six mating or compatibility groups were identified among the isolates. Differences in general combining ability effects existed among isolates within mating groups.

Smut, caused by *Moesziomyces penicillariae* (Bref.) Vánky, is one of the major diseases of pearl millet (*Pennisetum glaucum* (L.) R. Br.) (19). The fungus can be found where pearl millet is grown in Pakistan, parts of Africa, and India, and was first observed in the United States in 1962 (27). Yields of smut-infected pearl millet hybrids can be reduced 23 to 30% (4,23). Fungicides can control the disease (4,26); however, host resistance is the only practical means of control. The fungus infects the pistil of individual flowers, rendering seed treatments ineffective and fungicide applications to flowering crops uneconomical.

In the genus *Moesziomyces*, sori are formed in the ovaries of the host. Teliospores with surface ornamentation, agglutinated in spore balls, are borne within the sori (25). Individual teliospores of *M. penicillariae* aggregated in spore balls have been observed germinating to form 4-celled promycelia or, occasionally, 3- to 8-celled promycelia. Sporidia were borne laterally or terminally on the promycelium and could bud to form branched chains or clusters. Sporidia also formed in branched chains without any promycelia. Individual, free teliospores were only observed with 4-celled promycelia (21).

When spore balls were placed on pearl millet stigmas, infection hyphae grew in the stigmatic region and entered the style. Binucleate mycelium surrounded and penetrated the ovary (2). A sorus will form in infected ovaries unless timely pollination occurs, which prevents development of the infection into a sorus (24,28).

In all experimental work reported to date, bulk or composite mixtures of either sporidia or sporidia and teliospores have been used for inoculum. Sporidia are generally obtained by germinating teliospores in water or on agar. Growth of *M. penicillariae* in culture can be mycelial, sporidial (yeastlike), or a combination of both (21). Fast- and slow-growing "teliosporic" cultures with yeastlike colony morphology have been identified, and both types of cultures are pathogenic (5). The term "teliosporic" was not defined, but it is likely that the cultures were sporidial composites derived from cultured sporeballs or individual teliospores. A "single-colony isolate" has been reported to cause infection of pearl millet (15), but again, the described method of selecting the isolate suggests that it may have been a colony derived from an isolated spore ball or teliospore.

Because inoculations with *M. penicillariae* reported in the literature have been made with bulked inoculum of sporidia or teliospores or both, little is known about the genetic variability and requirements of the fungus for infection. In typical smut fungi, teliospores germinate to develop promycelia, which produce haploid basidiospores or sporidia. Plasmogamy occurs between compatible cells, and the resulting dikaryotic mycelium is generally obligately parasitic (1). The only evidence that mating is necessary for infection of pearl millet is that the hyphae colonizing the ovary are binucleate (2).

If we define pathogenicity as the ability of the fungus to cause disease and virulence as a measure of the extent of disease caused by a pathogen, the distinction between pathogenicity and virulence of *M. penicillariae* can be made. An isolate that causes any sori to form on a pearl millet panicle can be considered to be pathogenic. An isolate resulting in more florets becoming infected can be designated as being more virulent than isolates causing fewer sori. Because single-sporidial cultures can be isolated and maintained, controlled matings between isolates can be made. Differences in the level of infection caused by individual isolates or by crosses between isolates permits a quantitative genetic analysis of the inheritance of pathogenicity and virulence. Single-sporidial isolates can be considered as parental genotypes. If mating occurs between sporidia, the pathogenicity and virulence of the dikaryon can be used to determine the performance of parental isolates in hybrid combination. Genetic analyses could then lead to estimation of general combining ability (GCA) and specific combining ability (SCA). The objectives of these experiments were to determine whether mating or compatibility types are necessary for infection of pearl millet, and to apply quantitative genetic analyses to determine variation in pathogenicity and virulence among sporidial isolates of *M. penicillariae*.

MATERIALS AND METHODS

Selection of isolates. Several single-colony isolates were selected from a bulk inoculum used at the USDA-ARS Forage and Turf Research Unit at Tifton, Georgia. The bulk inoculum had been produced from composites of sporidia derived from teliospores, taken from infected panicles randomly selected from the field, and germinated on agar. The bulk inoculum consisted of a sporidial composite suspended in 20% glycerin (aqueous solution v/v) and stored at −73 C. Loops of the bulk inoculum were streaked onto 20% V8 juice + 1.5% NaOH (V8) agar and incubated under continuous fluorescent lighting at 24 C. After approximately 24 hr, isolated colonies were selected from the plates and suspended in sterile, deionized water. A loop of the suspension was streaked onto V8 agar and incubated. Two days later, well-isolated colonies were selected for purification by streak-plate dilutions. After six successive single-colony isolations from the streak plates, 22 isolates with a typical sporidial or yeastlike growth were increased on V8 agar for 5 days. Sporidia of these isolates were suspended in 20% glycerin and frozen at −73 C. Isolates numbered 23–30 were predominantly sporidial, yet even though only sporidial colonies were selected from the dilution plates, these cultures always had a low frequency of reversion to mycelial types, averaging 1.2% reversion. Isolates 31–45 had a predominantly mycelial cultural characteristic; however, sporidial colonies consistently formed in all the streak plates (average = 15.3% sporidial types) even though mycelial colonies were always selected for transfer. The two types of unstable isolates were purified for six additional cycles of streak plating with no change in their cultural characteristics. These isolates were increased on V8 agar for 5 days, suspended in 20% glycerin, and stored at −73 C.

Pathogenicity among isolates. The pearl millet inbred Tift 23DA was grown in the greenhouse in 11.5-mm-diameter pots containing equal volumes of coarse building sand, peat moss, and perlite, amended with 8.8 g/L agricultural gypsum and 5.2 g/L Osmocote fertilizer (3–4 mo release, NPK:14-6.1-11.6, Sierra Chemical Co., Milpitas, CA). Greenhouse temperature was maintained at approximately 30 C, and the plants were grown under natural lighting.

Isolates were increased on V8 agar, and inoculum was prepared by suspending sporidia in deionized water at a concentration of 2×10^7 sporidia/ml. A total of eight panicles of Tift 23DA were inoculated when half to fully emerged from the boot. Replications (single panicles) were blocked by time of inoculation. Flag leaves were removed, and panicles were immersed in test tubes containing 20 ml of sporidial suspensions of each of the 45 isolates described above. Each inoculated panicle was covered with a prewetted plastic bag. After 16 hours the plastic bag was removed and the inflorescence was covered with a glassine bag. After 14 days, the percentage of florets with telial sori was estimated.

Percent infection by the individual isolates was transformed to log(percent smut + 1) to reduce the relationship between means and variances. Transformed data were analyzed by least-squares analysis of variance using a model that included replicate and isolate effects.

Sporidial sizes of each of the 45 isolates was determined by staining with 0.1% trypan blue in lactophenol sporidia of 10-day-old cultures grown on V8 agar. Lengths (l) and widths (2r) of 15 random sporidia were measured, and means were calculated. Approximate sporidial volume was calculated by volume = $\pi r^2 l$.

Inheritance of pathogenicity and virulence. Isolate numbers 1–22 were increased on V8 agar and inoculum was prepared by suspending sporidia in deionized water as described above. Isolates were used alone and in all combinations to inoculate three panicles each of Tift 23DA in the greenhouse from April to June, 1989, and of the near-isogenic, alloplasmic cultivar Tift 85DB in the field from August to September, 1989. Both of the pearl millet cultivars are susceptible to *M. penicillariae*. In both experiments, each inflorescence represented a replicate, and inoculations were blocked by time of inoculation.

Panicles of Tift 23DA were inoculated as previously described. To reduce the effect of exogenous inoculum in the field experiment, and to insure that pollination did not confound the level of infection, inflorescences of Tift 85DB that had the tip of the panicle just beginning to emerge from the boot were chosen for inoculation. Approximately 3 ml of sporidial suspensions were injected into the encased inflorescence, which was then covered with a glassine bag. Panicles were harvested after 3 wk, and the percentage of florets infected was estimated. In the field experiment, we were able to collect data for only two replicates for 13 of the crosses, because some of the bags were blown off during a storm.

Diallel analysis III of Gardner and Eberhart (12) was performed to estimate the general (GCA) and specific (SCA) combining ability effects for pathogenicity and virulence associated with the crosses of isolates. Variance contributions due to GCA and SCA components were estimated according to Falconer (11), and these values were used to estimate the contributions of additive and nonadditive genetic variances (13). In these analyses, we assumed that isolates included in this study were a random sample from a population of smut isolates.

RESULTS

Pathogenicity among isolates. The 45 isolates differed in their ability to cause disease (Table 1). Severities resulting from inoculation with individual isolates ranged from 0.0 to 63.5% diseased florets. In general, mycelial isolates caused the greatest smut severities, and the sporidial isolates resulted in the lowest

TABLE 1. Analysis of variance of percent smut infection of pearl millet inbred 23DA by isolates of *Moesziomyces penicillariae*

Source	df	Mean square	Expected mean square
Isolate[a]	44	3.182**[b]	$\sigma^2_e + 45\sigma^2_i$
Replicate	7	1.045**	$\sigma^2_e + 8\sigma^2_r$
Error	308	0.200	σ^2_e

[a]Smut severities transformed to log (%smut + 1) prior to analysis.
[b]**Indicates significance at $P = 0.01$.

smut severities (Fig 1). Percent smut infection was correlated with sporidium volume ($r = 0.56$, $P = 0.0001$). The data suggested that sporidial isolates were haploid and required mating to cause infection. These isolates were used in the diallel crossing design.

Inheritance of pathogenicity and virulence. Differences in smut severities among crosses between sporidial isolates in both the greenhouse and field experiments were highly significant (Table 2). Averaged across experiments, severities for the crosses ranged from 0.1 to 68.3% diseased florets. The cross sums of squares was further partitioned into GCA and SCA sums of squares. Effects due to SCA and isolate vs. cross, which are both attributable to nonadditive genetic effects, were highly significant. GCA, attributable to additive genetic effects, was also highly significant and accounted for 27.1% of the total genetic variance. The population genotypic variance was partitioned by the expected mean squares of the combining abilities (Table 2). Assuming a 2-locus model, the expectation of $2\sigma^2_{GCA} = \sigma^2_A + 0.5\sigma^2_{AA}$ and the expectation of $\sigma^2_{SCA} = \sigma^2_D + 0.5\sigma^2_{AA} + \sigma^2_{AD} + \sigma^2_{DD}$ (13), where A and D represent additive and dominance effects, respectively. The ratio of the contributions to the population genotypic variance of additive to the nonadditive genetic variances was approximately 1:2.7.

Differences among isolates was a significant source of variation in the greenhouse but not in the field experiment (Table 2). In the greenhouse experiment, sporidial isolates tended to be either nonpathogenic or solopathogenic, but the distinction between nonpathogens and solopathogens was not as clear under the conditions of the field experiment, in which many of the isolates were weakly virulent. This could be the result of the use of cytoplasmic-sterile plants in the greenhouse and fertile plants in the field, or the different inoculation technique used in the different experiments. Mean infection by isolates was not clearly related to their GCA effects (Table 3), indicating that nonadditive gene effects were significant in the expression of virulence in crosses. Pooled GCA effects and percent disease were significantly correlated ($r = 0.48$, $P = 0.02$); however, the slope of the regression line was nearly horizontal. The significant correlation coefficient was the result of the extreme smut severities caused by isolates 12, 13, 18, and, to some extent, 8. Upon reexamination of these isolates in culture, they all expressed a very low frequency of reversion (average of 0.1% reversion) to mycelial types, and are classified as such in Figure 1.

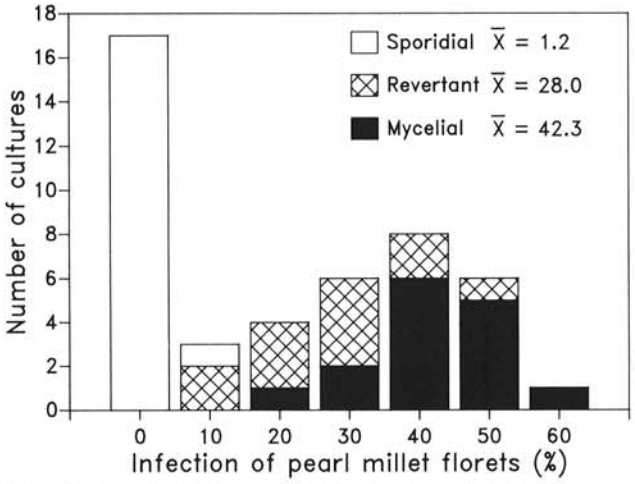

Fig. 1. Infection of pearl millet Tift 23DA in the greenhouse with isolates of *M. penicillariae* that have different cultural characteristics. Values are the means of eight replicates.

Mating or compatibility groups could be identified among the isolates. Often, two isolates caused little or no disease when combined; however, disease would occur when either isolate was combined with a third. Results from both experiments indicated that at least six compatibility groups were present among the 22 isolates used in this study. These groups, represented by isolates 1, 3, 6, 7, 15, and 22, resulted in distinct levels of disease when crossed in all combinations (Table 4). Isolates 3 and 22 were unique in their compatibility patterns. Isolates 1 and 10 were similar in mating behavior, as were isolates 4, 6, and 9; isolates 7, 16, 19, and 20; and isolates 2, 11, 15, and 17. Compatibility or mating behavior of the remaining isolates was somewhat ambiguous and could not be classified into any of these groups. Although groups could be defined on the basis of mating behavior, differences in GCA effects (Table 3) among isolates within a group indicate that isolates within compatibility groups can differ in additive genetic effects controlling virulence to pearl millet. The SCA effects of the isolate combinations representing different mating groups correspond to the level of disease obtained (Table 4). Specific combining ability effects among all crosses ranged from −34.1 for 7 × 20 to 53.1 for 21 × 22.

DISCUSSION

Considerable variation for pathogenicity and virulence of isolates of *M. penicillariae* to pearl millet exists. A few isolates were entirely nonpathogenic under the conditions of these experiments. The remainder of the isolates differed in virulence and caused various amounts of disease, but sporidial isolates were the least virulent of the three types of isolates identified. Solopathogenic isolates of *Ustilago zeae* (Beckm.) Unger, *Tilletia caries* (DC.) Tul. & C. Tul., and *Sphacelotheca* spp. are apparently either diploid or dikaryotic (14). Dikaryotic hyphae are regularly formed when teliospores of smut fungi are cultured (9), and observation of teliospore germination in *M. penicillariae* (21) suggests that ample opportunity exists for fusion between compatible cells, resulting in infectious dikaryotic mycelium. The nuclear status of isolates in this study is not known; however, sporidium size and virulence were related. Polyploid individuals within many species tend to be larger and more vigorous than individuals with lower ploidy rank (8,20), and therefore it is possible that the highly virulent isolates were either diploid or dikaryotic.

The relatively large contribution of dominance and epistatic variances to the total genetic variance reveals the importance of nonadditive genetic variance in infection. Mating types in fungi are usually conferred by a pair of alleles at a single locus. Because the infection phenotype (in this case, percent of diseased florets) resulting from a combination between compatible mating types differs from the phenotype arising from a combination between incompatible mating types, the interaction of mating type alleles

TABLE 2. Analyses of variance of smut infection of pearl millet inoculated with 22 isolates of *Moesziomyces penicillariae* alone and in all combinations

Source	Greenhouse df	Greenhouse MS	Field df	Field MS	Pooled df	Pooled MS	Expectation of MS
Isolate	21	746.7 **[a]	21	120.2	21	609.3	
Isolate vs. cross	1	14,930.4 **	1	10,701.1 **	1	25,651.5 **	
Crosses	230	1,445.5 **	230	901.3 **	230	1,728.1 **	
GCA	21	5,476.7 **	21	2,344.1 **	21	5,016.4 **	$\sigma^2_E + \sigma^2_{SCA} + 20\sigma^2_{GCA}$
SCA	209	1,040.4 **	209	749.9 **	209	1,394.3 **	$\sigma^2_E + \sigma^2_{SCA}$
Error	506	427.8	493	309.3	1252	422.4	σ^2_E

[a] * and ** indicate significance at $P = 0.05$ and 0.01, respectively.

TABLE 3. Mean infection of pearl millet by individual isolates of *Moesziomyces penicillariae* and their general combining abilities (GCA) from diallel crosses in the greenhouse and field

Isolate	Infected florets (%)	GCA
1	3.6	9.4 **[a]
2	2.1	−3.9 *
3	1.7	1.6
4	0.8	−2.6
5	8.7	−0.2
6	0.6	0.9
7	0.7	9.0 **
8	13.8	3.6 *
9	0.6	−3.1 +
10	6.2	3.6 +
11	0.1	−4.2 *
12	25.0	−4.9 **
13	28.0	10.2 **
14	5.9	−9.1 **
15	3.3	−3.9 *
16	2.5	−9.6 **
17	0.3	−6.2 **
18	36.3	14.0 **
19	0.9	−1.7
20	0.5	4.2 *
21	3.0	−8.5 **
22	2.4	1.1
SE	5.2	1.8

[a]+, *, and ** indicate a difference from 0 at $P = 0.10$, 0.05, and 0.01, respectively.

TABLE 4. Percent infection of pearl millet and specific combining abilities of six isolates of *M. penicillariae* representing different mating or compatibility groups

	Percent infection and specific combining ability[a] when combined with isolate					
Isolate	1	3	6	7	15	22
1	3.6	4.8	68.3	58.3	1.3	10.3
	...	−27.6 **[b]	36.6 **	18.6 *	−25.5 **	−21.5 **
3	(−)[c]	1.7	0.8	56.8	42.2	2.2
		...	−23.2 **	24.9 **	23.1 **	−21.9 **
6	(+)	(−)	0.6	57.2	4.0	57.3
			...	25.9 **	−14.4 +	34.0 **
7	(+)	(+)	(+)	0.7	47.2	5.7
				...	20.8 **	−25.7 **
15	(−)	(+)	(−)	(+)	3.3	51.0
					...	32.5 **
22	(−)	(−)	(+)	(−)	(+)	2.4
						...

[a]Top value is percent infection, bottom value is SCA.
[b]+, *, and ** indicate difference from 0 at $P = 0.10$, 0.05, and 0.01, respectively.
[c](−) and (+) indicate incompatible and compatible combinations of isolates, respectively.

can be considered epistatic. The significant SCA variance component observed in this study suggests that alleles conferring mating or compatibility types are present in this random sample of isolates.

Because six compatibility groups were observed among these *M. penicillariae* isolates, it is possible that a simple bipolar mating system exists that is confounded by other loci controlling host infection, teliospore development, or some other phase of host colonization. For example, five compatibility groups in *Ustilago cynodontis* (Henn.) Henn., the smut pathogen of Bermuda grass (*Cynodon dactylon* (L.) Pers.), have been identified (10). Comparisons of hyphal pairing compatibility in vitro with the ability to form teliospores in vivo suggested that a single locus with two alleles actually controlled mating type, and additional loci conferring in vivo development of the fungus were present. Our data suggest that a similar genetic mechanism might be occurring in the compatibility groups in *M. penicillariae*. Our data tentatively suggest that isolates 7 and 22 possess an allele for mating type that is compatible with an allele in isolates 6 and 15. There appears to be some additional genetic mechanism conferring compatibility with isolates 1 and 3 that further differentiates isolate 7 from 22, and 6 from 15.

Differences in GCA effect among isolates within some compatibility groups indicate that genes with additive effects for virulence are present. In *Ustilago hordei* (Pers.) Lagerh., genes with additive effects conferring virulence (16,17) and genes affecting noncultivar-specific pathogenicity (7,18) to barley (*Hordeum vulgare* L.) have been detected. It is likely that quantitatively inherited virulence will be found in all plant pathogens and thus should be considered when conducting studies of inheritance of pathogenicity and virulence of fungi.

To date, no major genes for immunity to *M. penicillariae* infection have been identified in pearl millet, and the mechanism of resistance in resistant cultivars is not well understood. Timely pollination decreases smut infection to some extent (24). If pearl millet is pollinated within 72 hr of infection, fertilization will occur and a normal seed will develop. If pollination occurs after this period, the fungus will proceed to colonize the ovary and will develop teliospores (28). However, there is no clear correlation between the timing of flowering events and smut resistance (22). Resistance to *M. penicillariae* has been determined to be conferred by a few dominant genes in some cultivars (6). A single gene that holds potential as a source of smut resistance is the recessive *tr* allele, which results in trichomeless plant structures and unbranched stigmas (3). Plants homozygous for the *tr* allele are less susceptible to smut (28); however, limited infection does occur on plants homozygous for the *tr* allele. Because additive genetic variation for virulence exists among *M. penicillariae* isolates and sexual recombination occurs, it is possible that sufficient genetic variation exists that will allow the fungus to become more virulent on *trtr* plants.

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