

# Fertility and Mating Type Frequency in Indian Isolates of *Sclerospora graminicola*, the Downy Mildew Pathogen of Pearl Millet

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## ABSTRACT

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*Sclerospora graminicola*, the downy mildew pathogen of pearl millet, is an oomycetous obligate parasite which reproduces by both sexual and asexual means. Fertility and mating type frequencies were studied in 70 single-zoospore isolates (SZIs) obtained from seven representative oospore isolates (Sg 021, Sg 048, Sg 110, Sg 139, Sg 149, Sg 152, and Sg 153) of *S. graminicola* collected from major pearl millet-growing states of India. Of the 70 SZIs tested for fertility according to oospore production potential, 62 were self-sterile and 8 were self-fertile, indicating the low occurrence of homothallism in the *S. graminicola* populations. The sexual mating type test of the 70 SZIs, conducted by pairing each isolate with the two standard mating type tester isolates PT2 (Mat A) and PT 3 (Mat B), revealed 28 (40.0%) isolates of Mat A, 33 (47.14%) of Mat B, 8 (11.43%) of both Mat A and Mat B, and 1 (1.43%) as unknown. The frequencies of Mat A and Mat B were in approximately equal proportions among the isolates tested, except in three parental isolates. Implications of these results in understanding the dynamic genetic structure of *S. graminicola* population and potential for evolution of new virulence in the pathogen are discussed.

Additional keywords: heterothallism, *Pennisetum glaucum*

Downy mildew, caused by *Sclerospora graminicola* (Sacc.) J. Schröt., is widely distributed and an economically significant disease in the major pearl millet (*Pennisetum glaucum* (L.) R. Br)-growing areas of Asia and Africa (6,18,28). The disease is highly destructive, particularly in genetically homogeneous single-cross hybrids, and causes substantial yield loss (23,25). The pathogen *S. graminicola* is an oomycetous fungus and reproduces by both sexual and asexual means and, thus, is genetically highly variable. Michelmore et al. (12) reported heterothallism in *S. graminicola* and assigned the isolates to one of two sexual compatibility types. Idris and Ball (3) demonstrated inter- and intracontinental sexual compatibility among isolates of *S. graminicola* obtained from diverse pearl millet cultivars in India, Burkina Faso, Nigeria, Niger, and Senegal, and identified two compatibility types, G<sub>1</sub> and G<sub>2</sub>, which were found in approximately equal proportions. To identify the

compatible mating types among the isolates of *S. graminicola*, Rao et al. (17) inoculated the seedlings of 7042S with six isolates (PT 1 through PT 6) of *S. graminicola* singly and in all possible combinations. They observed abundant oospores in a paired inoculation with the isolates PT 2 and PT 3. These two isolates were designated as the reference mating type isolates, Mat A and Mat B, respectively, and are being maintained at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India. In recent times, with large-scale cultivation of high-yielding and genetically diverse single-cross pearl millet hybrids in India, there has been a corresponding change in the diversity of *S. graminicola* populations and, currently, there are at least seven major pathotypes known to exist in hybrid-intensive states of India (27). In oomycetes, sexual mating types and their frequency greatly contribute toward developing new recombinants in the pathogen population (10,20,21). In this study, we investigated the fertility status and mating type frequencies of 70 single-zoospore isolates obtained from seven pathotype isolates from major pearl millet-growing areas of India with a view to understanding the mechanism involved in evolution of new virulence in *S. graminicola* populations.

## MATERIALS AND METHODS

**Establishment of isolates from oospore inocula.** Isolates were established from oospores of *S. graminicola* contained in leaf powder samples of pearl millet collected from different cultivars grown in major states of India (Table 1). Plastic pots (15 cm diameter) filled with autoclaved potting mixture were inoculated by mixing 1 g of oospore-leaf powder per pot in the top 5-cm-layer of the mix. Surface-sterilized seed of pearl millet genotype 7042S, highly susceptible to downy mildew, were sown at 25 seed per pot, and three pots were maintained for each inoculum source (isolate). Pots for each isolate were kept separately in a polyacrylic isolation chamber (45 cm × 45 cm × 45 cm) in a greenhouse at 25 ± 2°C to avoid any cross contamination. Pots were watered regularly and seedlings were observed daily for the appearance of downy mildew symptoms. One month after inoculation, seedlings were thinned to remove the healthy seedlings and infected seedlings were maintained. Sporangia from these infected seedlings were used for subsequent inoculation of seedlings for isolate maintenance.

**Inoculum preparation and seedling inoculation.** Infected leaves from individual isolates were collected, excised into pieces, and washed in running tap water using a cotton swab to remove old sporangia from the leaf surface. These leaf pieces were wiped dry with tissue paper and placed with their abaxial surfaces up in plastic-tray humidity chambers lined with moist blotting paper. The humidity chambers were incubated in dark at 20°C for 6 h. The incubator was programmed in such a way that the temperature was reduced to 2°C after 6 h of incubation in order to prevent the release of zoospores from maturing sporangia until the sporangial collection on the next day. Sporangia from sporulated leaves were harvested into ice-cold (4°C) sterilized distilled water, separately for each isolate, using a soft camel-hair brush. The sporangial suspension was filtered through a double-layered muslin cloth to remove sporangiophores and other particles. The concentration of sporangia was measured using a hemacytometer and adjusted to a desired level.

Potted seedlings were spray inoculated with sporangial suspension at the coleop-

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tile to first-leaf stage using a hand sprayer in the inoculation chamber and covered immediately with moist polyethylene sheet to provide the >95% relative humidity necessary for infection. Inoculated seedlings were incubated in the dark at 20°C for 24 h. The pots then were transferred onto a greenhouse bench at 25 ± 2°C.

**Establishment of single-zoospore isolates from parental isolates.** Of the total of 21 isolates that were studied for pathogenic and genetic variability (16), single-zoospore isolates (SZIs) were established from the representative 7 pathotype isolates from seven Indian states (Table 1). Inoculum from each isolate was prepared as described earlier and the spore suspension thus obtained was diluted to a concentration of two to three sporangia per field of microscope (10× by 40×). This diluted suspension was kept at 25°C for about 30 min to allow the release of zoospores from sporangia. A small amount (0.5 ml) of zoospore suspension was spread uniformly over the surface of sterile water agar medium (1%) in a petri plate and the excess suspension was drained off. Single, well-isolated zoospores were marked on water agar using a dummy objective (10×) under the microscope. Single zoospores were picked up with the help of a flat-tipped needle and transferred onto the emerging coleoptile of 3-day-old pearl millet seedlings (cv. 7042S) which had been grown in 5-cm-diameter pots in an incubator at 28°C. Pots then were covered with polyethylene bags and incubated overnight at 20°C. Pots were transferred to isolation chamber (30 cm × 30 cm × 30 cm) on a greenhouse bench. Seedlings were observed daily for the appearance of downy mildew symptoms. Ten days after inoculation, healthy seedlings were thinned out and the infected seedlings were maintained. In all, 70 SZIs (10 SZIs from each isolate) were established and maintained separately in isolation chambers in a greenhouse.

**Assay for sexual compatibility.** The 70 SZIs derived from the seven representative isolates were tested for their oospore formation potential by inoculating the pot-grown seedlings of cv. 7042S with individual isolates in the greenhouse. Based on oospore formation potential, these SZIs were classified broadly into two groups, self-sterile and self-fertile. Of the self-

sterile SZIs, 10 (Sg 139-1 through Sg 139-10) were derived from a highly virulent isolate, Sg 139, and six (Sg 110-1, Sg 110-3, Sg 110-4, Sg 110-5, Sg 110-8, and Sg 110-10) from a weakly virulent isolate, Sg 110 (16). These 16 SZIs were tested for their sexual compatibility by inoculating them individually and in all possible (120 pairwise) combinations on to the seedlings of 7042S. For inoculation, sporangial inocula ( $5 \times 10^5$  sporangia ml<sup>-1</sup>) from both isolates were mixed in equal (1:1) proportion and pairwise inoculations were made among the 16 SZIs. For each combination and single-isolate inoculation, two pots with 50 seedlings/pot were maintained. One month after inoculation, 10 leaf pieces (1 cm<sup>2</sup>) were collected from necrotic leaves of five infected seedlings in each pot and examined microscopically for the presence of oospores as described below. All the isolates and isolate combinations were maintained separately in isolation chambers to avoid any cross contamination.

**Preparation of leaf pieces for microscopy.** Necrotic leaf pieces collected from five seedlings infected by each isolate were dried under shade in brown paper bags and stored at room temperature (approximately 25°C) until observation. Leaf pieces (1 cm<sup>2</sup>) were surface sterilized with NaOCl (2%) and washed thoroughly with sterilized distilled water. These leaf pieces were cleared by incubating them at 40°C in NaOH (5%) for 12 to 16 h. Cleared leaf pieces were rinsed in distilled water and observed under a microscope using a 10× objective for the presence of oospores. In each replication for each host genotype, 10 leaf pieces were observed. Oospore production was scored on a 1-to-4 scale, where 1 = no oospores, 2 = 1 to 100 oospores/cm<sup>2</sup>, 3 = 101 to 1,000 oospores/cm<sup>2</sup>, and 4 > 1,000 (numerous) oospores/cm<sup>2</sup> of leaf area (26).

**Assay for mating types.** The 70 SZIs, irrespective of their fertility, were cross inoculated with PT2 (mating type Mat A) and PT3 (mating type Mat B), the two known standard tester mating type isolates of *S. graminicola* (17). Sporangial inoculum ( $5 \times 10^5$  sporangia ml<sup>-1</sup>) from two isolates of a combination were mixed in 1:1 proportion and inoculated onto seedlings of 7042S. For each combination, two pots with 50 seedlings per pot were main-

tained and kept separately in isolation chambers under greenhouse conditions. Leaf samples were collected as above and examined microscopically for the presence of oospores as described earlier.

**Data analysis.** The data from mating type tests were subjected to  $\chi^2$  analysis (14) to determine the ratio of goodness-of-fit.

## RESULTS

**Fertility in *S. graminicola*.** Of the 70 SZIs, when inoculated alone, no oospores were produced in 62 isolates and oospores were produced in 8 isolates in fewer numbers (13 to 46 oospores/cm<sup>2</sup> leaf area, mean of 20 leaf pieces of 1 cm<sup>2</sup>; data not presented). Isolates in which oospore production was not observed were referred to as self-sterile isolates and those supporting oospore production were referred to as self-fertile.

**Mating types in *S. graminicola*.** The 16 self-sterile SZIs used to determine mating types fell into two groups when inoculated in all possible paired combinations. Seven isolates (Sg 139-1, Sg 139-3, Sg 139-5, Sg 139-6, Sg 139-7, Sg 139-10, and Sg 110-9) belonged to one group and eight (Sg 139-2, Sg 139-4, Sg 139-8, Sg 139-9, Sg 110-1, Sg 110-4, Sg 110-7, and Sg 110-10) to another group, while one (Sg 110-2) remained unique (Table 2). Oospores were produced in abundance (>1,000/cm<sup>2</sup> leaf piece) when any one of the isolates of one group was paired with any one of the isolates of the other group and vice versa. Inoculations with isolates of the same group in all paired combinations did not result in the production of oospores. The isolate Sg 110-2 failed to produce oospores with any one of the isolates tested; its behavior was unique of its kind. The two mating type groups identified were designated as Mat A and Mat B.

**Frequency and distribution of mating types.** Of the 70 SZIs analyzed for oospore production by pairing with the two reference mating type tester isolates, 28 (40.0%) were classified as Mat A (PT2 type), 33 (47.1%) as Mat B (PT3 type), and 8 (11.4%) behaved as both Mat A and Mat B, while 1 (Sg 110-2) was incompatible with any of the 15 isolates and did not produce oospores with either of the mating type tester isolates (Table 3). Oospore production was abundant (>1,000/cm<sup>2</sup> leaf piece) in mating involving self-sterile isolates, whereas it was sparse (varied from 126 to 584/cm<sup>2</sup> leaf piece) in mating involving self-fertile isolates. The 28 isolates that produced oospores only with PT3 and the 33 that produced oospores only with PT2 were self-sterile, whereas the 8 isolates that produced oospores with both these isolates were self-fertile. Among the eight self-fertile isolates, five produced more oospores (441 to

**Table 1.** Selected representative isolates of *Sclerospora graminicola* from major pearl millet-growing states of India used in this study

Isolate designation	Collection host genotype	Year of collection	Site of collection (location, district, state)
Sg 021	MLBH 104	1993	Ghari, Ahmadnagar, Maharashtra
Sg 048	7042S and HB 3	1994	University of Mysore, Mysore, Karnataka
Sg 110	CO-3	1996	Illupanatham, Kovai, Tamil Nadu
Sg 139	<i>Nokha</i> local	1997	Jodhpur, Jodhpur, Rajasthan
Sg 149	Local	1997	Gwalior, Madhya, Pradesh
Sg 152	Local	1997	Durgapura, Jaipur, Rajasthan
Sg 153	7042S and NHB 3	1997	Patancheru, Medak, Andhra Pradesh

584/cm<sup>2</sup> leaf piece) with the isolate PT 3 than with the isolate PT 2, two isolates produced more oospores (432 to 522/cm<sup>2</sup> leaf piece) with isolate PT 2 than with PT 3, and one isolate produced oospores equally (432/cm<sup>2</sup> leaf piece) with each of the two isolates. The ratio of 28:33 Mat A:Mat B does not differ significantly from a 1:1 ratio ( $\chi^2$  1:1 = 0.40,  $P > 0.01$ ) and the results suggest the occurrence of Mat A and Mat B mating types in equal frequencies among the seven selected parental isolates of *S. graminicola*.

## DISCUSSION

Of the 70 SZIs evaluated, 62 were self-sterile and 8 were self-fertile, indicating that isolates derived from single zoospores in populations of *S. graminicola* are predominantly self-sterile, but some proportion is self-fertile. Self-fertile isolates detected in this study produced relatively small numbers of oospores, which likely indicates reduced fitness associated with self-fertility. Presumably, the lower fitness of self-fertile isolates will prevent heterothallism from being selected out of the pathogen population. Though the overall frequencies of both mating types were found in equal proportions, their distribution within the individual parental isolates was not uniform, especially in isolates Sg 149 (6:3 Mat A: Mat B), Sg 110 (4:1 Mat A:Mat B), and Sg 152 (2:8

Mat A:Mat B). Other than the genetic factors, this could be due to the smaller population size of SZIs. The pattern of production of oospores by pairwise inoculations of 16 self-sterile isolates of *S. graminicola* suggested the existence of two distinct mating type groups, Mat A and Mat B. Crosses within each group were sterile, whereas those between them were fertile. These results are similar to those reported by Michelmore et al. (12) with a limited number of isolates from a single location and confirm the heterothallic nature of *S. graminicola*. In addition, the results show that the frequency of the mating types Mat A and Mat B in populations of *S. graminicola* was approximately equal, thus providing greater chances of producing progenies with new recombinations of alleles. In other heterothallic oomycetes also, generally only two sexual compatibility types have been identified in an approximate 1:1 ratio (1,10,15,20). Because both mating types were found in approximately equal proportion in India, it indicates that sexual reproduction plays an important part in the evolution of new genetic recombinants and, thus, development of new virulent pathotypes. This also helps in the long-term survival of *S. graminicola* in traditional pearl millet fields.

*S. graminicola* also is known to have cross-compatibility between isolates from

geographically diverse locations in West Africa and India (3). This outbreeding capacity of *S. graminicola* is indicative of the potential for evolution of new races and high adaptability to different ecosystems (9). Under these circumstances, if oospores were inadvertently transported from one continent to the other, the pearl millet crop would be at risk. Pathogen isolates previously absent might proliferate rapidly in the absence of appropriate resistance factors in the host and may cause complete devastation of the crop.

Though the fungus was found to be primarily heterothallic in nature, the sparse oospore production by eight of the SZIs when inoculated alone suggests the rare occurrence of self-fertility in populations of *S. graminicola*. Michelmore and Ingram (11) demonstrated self-fertility in *Bremia lactucae* as a form of secondary homothallism and not as a mixture of heterothallic isolates of opposite compatibility types. Such self-fertile isolates have been reported for other heterothallic members of the peronosporales *Phytophthora drechsleri* (13), *Pythium sylvaticum* (15), and *Peronospora parasitica* (22). However, the most detailed studies have been made with *Phytophthora drechsleri* (13,19) and *B. lactucae* (11), where genetic and cytological experiments have indicated that the self-fertile isolates are trisomic for the determinants of compatibility type follow-

**Table 2.** Mating type behavior of 16 single-zoospore isolates of *Sclerospora graminicola*

	Sg 110-10	Sg 110-9	Sg 110-7	Sg 110-4	Sg 110-2	Sg 110-1	Sg 139-10	Sg 139-9	Sg 139-8	Sg 139-7	Sg 139-6	Sg 139-5	Sg 139-4	Sg 139-3	Sg 139-2	Sg 139-1
Sg 139-1	+	-	+	+	-	+	-	+	+	-	-	-	+	-	+	-
Sg 139-2	-	+	-	-	-	-	-	-	-	+	-	+	-	+	-	...
Sg 139-3	+	-	+	+	-	+	-	+	+	-	-	-	+	-	...	...
Sg 139-4	-	+	-	-	-	-	+	-	-	+	+	+	-	...	...	...
Sg 139-5	+	-	+	+	-	+	-	+	+	-	-	-	...	...	...	...
Sg 139-6	+	-	+	+	-	+	-	+	+	-	-	-	...	...	...	...
Sg 139-7	+	-	+	+	-	+	-	+	+	-	...	...	...	...	...	...
Sg 139-8	-	+	-	-	-	-	+	-	-	...	...	...	...	...	...	...
Sg 139-9	-	+	-	-	-	-	+	-	...	...	...	...	...	...	...	...
Sg 139-10	+	-	+	+	-	+	-	...	...	...	...	...	...	...	...	...
Sg 110-1	-	+	-	-	-	-	...	...	...	...	...	...	...	...	...	...
Sg 110-2	-	-	-	-	-	...	...	...	...	...	...	...	...	...	...	...
Sg 110-4	-	+	-	-	...	...	...	...	...	...	...	...	...	...	...	...
Sg 110-7	-	+	-	...	...	...	...	...	...	...	...	...	...	...	...	...
Sg 110-9	+	-	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Sg 110-10	-	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...

<sup>a</sup> Symbols: + = abundant oospore production (>1,000 oospore/cm<sup>2</sup> of leaf area, mean of 20 leaf pieces of 1 cm<sup>2</sup>) and - = no oospore production.

**Table 3.** Frequency and distribution of mating types within and between single-zoospore isolates (SZIs) of *Sclerospora graminicola*<sup>a</sup>

Parental isolates	SZI	Mat A	Mat B	Mat A > Mat B	Mat B > Mat A	Mat A = Mat B	None of these
Sg 148	10	4	6	0	0	0	0
Sg 149	10	6	3	1	0	0	0
Sg 021	10	3	4	1	2	0	0
Sg 110	10	4	1	3	0	1	1
Sg 153	10	5	5	0	0	0	0
Sg 139	10	4	6	0	0	0	0
Sg 152	10	2	8	0	0	0	0
Total	70	28	33	5	2	1	1

<sup>a</sup> Mat A: produced oospores only with PT3, self-sterile; Mat B: produced oospores only with PT2, self-sterile; Mat A > Mat B: produced more oospores with the PT3 than with PT2, self-fertile; Mat B > Mat A: produced more oospores with PT2 than with PT3, self-fertile; and Mat A = Mat B: produced oospores equally with both the isolates, self-fertile.

ing numerical nondisjunction at meiosis. Therefore, the self-fertility observed in the present investigation in some populations of *S. graminicola* may be a similar form of secondary homothallism as described in *B. lactucae* (11).

Variation in plant-pathogenic fungi arises largely through sexual recombination, heterozygosity and somatic recombination, mutation, and hybridization. Large shifts in pathogenicity occur due to changes in host cultivar and environment. In general, highly variable populations are better adapted than those with little variation. Pathogenicity or virulence has been used as the genetic marker in all the studies where variability has been assessed through virulence surveys, using host differentials having different resistance genes (29). While working with genetics of virulence in Californian populations of *B. lactucae*, Ilott et al. (4) found evidence that the sexual cycle of *B. lactucae* had not functioned in California even though both mating types were present whereas, in northern Europe, the sexual cycle was an important part of the life cycle of *B. lactucae*. Heterothallism also has been reported for other oomycetes fungi, such as *Peronospora effusa* (5), *P. parasitica* (7,22), and *P. infestans* (2,8,21,24) on their respective hosts.

With a larger area being planted to genetically uniform single-cross pearl millet hybrid cultivars in India, there is greater host-directed selection pressure on *S. graminicola* populations. This, added to high cross compatibility and the existence of equal mating type frequencies among the isolates, strongly favors the evolution of new virulence through genetic recombinants in the pathogen population. Therefore, it would be highly critical that the virulence change in the pathogen population be monitored frequently through downy mildew field surveys, and new virulence characterized and utilized in resistance breeding program for effective and strategic management of downy mildew in pearl millet.

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