Indian Phytopath. 56 (4) : 392-397 (2003)

Pathogenic and genetic diversity among Indian isolates of *Sclerospora graminicola* from pearl millet

S. SIVARAMAKRISHNAN[1], R.P. THAKUR, SEETHA KANNAN and V.P. RAO
International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru 502 324

ABSTRACT: Pathogenic and genetic diversities among 15 isolates of *Sclerospora graminicola*, the pearl millet downy mildew pathogen, collected from various pearl millet cultivars in India were assessed. Pathogenic variability was assessed by their disease causing potential on 12 pearl millet differential lines in a greenhouse experiment. The dendrogram based on mean disease incidence data classified the 15 isolates into five major virulence groups. Genetic diversity was assessed using amplified fragment length polymorphism (AFLP). Of the 12 primer combinations used in AFLP, five gave highly polymorphic patterns. The dendrogram of the AFLP marker data classified the 15 isolates into five major genetic clusters. Results indicated host-directed virulence selection and genetic changes in some isolates that were maintained on alternative host for multiple asexual generations.

Key words: *Sclerospora graminicola*, pearl millet, downy mildew, virulence, AFLP

Downy mildew, caused by the oomycetous biotrophic fungus *Sclerospora graminicola* (Sacc.) Schröet, is economically the most important disease of pearl millet [(*Pennisetum glaucum* (L.) R. Br.)] in India. The fungus induces systemic infection in pearl millet plants that manifests itself through foliar chlorosis and panicle malformation, also called "green-ear". The fungus is heterothallic (Michelmore *et al.*, 1982) and reproduces both by sexual and asexual means, and is therefore highly variable (Ball and Pike 1984; Thakur *et al.*, 1999). The commercial cultivation of a number of genetically homogeneous single-cross F_1 hybrids of pearl millet in India has led to increased virulence in *S. graminicola* populations and thus shortening the useful life of the hybrid cultivars (Thakur *et al.*, 1999). Monitoring virulence changes in the pathogen population, identifying resistance to specific and multiple pathotypes, and directing breeding program towards strategic utilization and deployment of resistance genes form the basis of a long-term downy mildew management of pearl millet at ICRISAT (Thakur, 1999). Application of molecular marker techniques has been useful for studying the genetic changes in the pathogen populations and the associated virulence changes in several host-pathogen systems. DNA fingerprinting using SSR markers (Sastry *et al.*, 1995) and RAPD (Sivaramkrishnan *et al.*, 1996) have been used to assess the genetic variability among the isolates of *S. graminicola*. Of the several molecular markers, amplified fragment length polymorphism (AFLP) has been found to be a better method for detecting genetic variability among fungal pathogens (O'Neill *et al.*, 1997). In this study, we assessed pathogenic and genetic diversity using AFLP among isolates of *S. graminicola*, and determined the influence of the host genotypes on genetic and virulence changes.

MATERIALS AND METHODS

Sclerospora graminicola isolates

Isolates of *S. graminicola* were collected from different pearl millet cultivars during field surveys in different parts of India. The isolates were established from oospores on the pot grown seedlings in a greenhouse. Asexual inocula of 15 isolates were established from single zoospores

[1] Current Address: Department of Biotechnology, Acharya N.G. Ranga Agricultural University, Rajindernagar, Hyderabad 500 030

derived from the parental isolates. These isolates were maintained through asexual generations either on the seedlings of collection host or on a universally susceptible host (7042S) in greenhouse isolation chambers (Table 1). Three of the 15 isolates (*Sg* 139, *Sg* 150, and *Sg* 151) were simultaneously maintained on two different host genotypes to determine their genetic and virulence changes, if any.

Pathogenic variability

In a greenhouse experiment, pot-grown seedlings of 12 pearl millet genotypes (Table 2) were spray inoculated with sporangial suspensions (1×10^5 sporangia ml^{-1}) of S. *graminicola* isolates. Scoring for downy mildew infection in seedlings was done two weeks after inoculation and incidence percentage was calculated. The experiment was conducted in a completely randomized design in three replications with 40 seedlings in each replicate. The experiment was repeated once.

Genetic variability

Spore collection: For collection of spores of *S. graminicola*, Mira cloth (Calbiochem, USA) pieces (2×5 cm^2) were wetted in sterile distilled water, placed over the sporulating pearl millet leaves and wiped gently. These Mira cloth pieces were transferred to sterilized small glass vials and capped. The samples were stored at 4°C until the DNA extraction was carried out.

DNA extraction: For each isolate, four pieces of Mira cloth containing spores from the infected plants in the greenhouse were soaked in 5 ml sterile distilled water and kept at 4°C overnight. The water-suspended spores were collected in a 2 ml tube by centrifugation at 12,000-x g for 10 min. Genomic DNA was extracted from each isolate using the phenol-chloroform method (Sastry *et al.,* 1995).

AFLP analysis: AFLP analysis was carried out using the commercial kit (Life Technologies, USA) following the manufacturer's protocols with slight modifications. Primary template DNA was prepared in a one-step restriction-ligation reaction. Fungal genomic DNA (400 ng) was digested with *Eco*RI and *Mse*I at 37° C for 2 h and heated at 70° C for 15 min to inactivate the enzyme. The DNA fragments were ligated to *Eco*RI and *Mse*I adapters at 20° C for 2 h. After terminating the reaction, the ligation mixture was diluted 10-fold with TE and the fragments were preamplified in a thermal cycler (MJR, USA) using a temperature cycle of 94°C for 30 s, 56°C for 60 s, and 72°C for 60 s in a total of 30 cycles.

Table 1. Isolates of *Sclerospora graminicola* (*Sg*) used in the study

Isolate (Year)	Collection genotype	Location	Maintenance genotype
Sg 008 (1992)	NHB 3	Patancheru, Andhra Pradesh	NHB 3
Sg 013 (1992)	7042S	Patancheru, Andhra Pradesh	7042S
Sg 021 (1993)	MLBH 104	Ghari, Maharashtra	7042S
Sg 048 (1994)	7042S/HB3	Mysore, Karnataka	852B
Sg 139 (1997)	*Nokha* local	Jodhpur, Rajasthan	*Nokha* local
Sg 139 (1997)	*Nokha* local	Jodhpur, Rajasthan	7042S
Sg 140 (1997)	70425/HB 3	Jamnagar, Gujarat	7042S
Sg 149 (1997)	*Kushwadara* local	Gwalior, Madhya Pradesh	7042S
Sg 150 (1997)	MBH 110	Jalna, Maharashtra	834B
Sg 150 (1997)	MBH 110	Jalna, Maharashtra	MBH 110
Sg 151 (1997)	81A	Durgapura, Rajasthan	*Nokha* local
Sg 151 (1997)	81A	Durgapura, Rajasthan	7042S
Sg 153 (1997)	7042S/NHB 3	Patancheru, Andhra Pradesh	7042S
Sg 200 (1998)	ICMH 451	Ramgarh, Gujarat	ICMP 451
Sg 212 (1998)	PG 5822	Pura ki Dhani, Rajasthan	ICMP 451

Table 2. Phenotypic reactions (R and S)[a] and downy mildew incidence (%) on 12 pearl millet host differentials to 15 isolates of *Sclerospora graminicola* in two tests in greenhouse, 2000-2001

Isolate	Host differentials											
	IP 18292	IP 18293	700651	P310-17	P 7-4	MBH110	852B	HB3	834B	ICMP 451	843B	7042S
Sg 008-NHB 3	R (0)	R (1)	R (17)	R (6)	R (9)	R (1)	R (11)	S (97)	R (<1)	R (6)	R (2)	S (90)
Sg 013-7042S	R (0)	R (0)	R (12)	R (14)	R (7)	R (1)	R (7)	S (93)	R (2)	R (1)	R (3)	S (96)
Sg 021-7042S	R (0)	R (0)	R (2)	R (3)	R (3)	R (3)	R (8)	S (96)	R (3)	S (51)	S (62)	S (94)
Sg 048-852B	S (85)	S (55)	S (51)	S (38)	S (50)	S (28)	S (77)	S (83)	R (6)	S (77)	S (80)	S (96)
Sg 139-*Nokha*	S (80)	S (28)	R (4)	R (7)	R (5)	R (7)	R (5)	S (94)	R (2)	S (47)	S (64)	S (94)
Sg 139-7042S	S (71)	S (23)	R (7)	R (3)	R (4)	R (4)	R (6)	S (99)	R (2)	S (62)	S (73)	S (97)
Sg 140-7042S	R (0)	R (0)	R (12)	R (4)	R (3)	R (2)	R (6)	S (94)	R (1)	R (12)	S (71)	S (95)
Sg 149-7042S	R (0)	R (2)	R (5)	R (3)	R (3)	R (4)	R (7)	S (96)	R (1)	S (64)	S (66)	S (93)
Sg 150-MBH 110	R (0)	R (9)	S (21)	R (6)	R (17)	S (93)	S (34)	R (1)	S (94)	R (3)	R (0)	S (93)
Sg 150-834B	R (0)	S (24)	R (14)	R (13)	R (16)	S (92)	S (27)	R (0)	S (89)	R (8)	R (4)	S (66)
Sg 151-*Nokha*	S (86)	S (27)	S (47)	S (34)	S (49)	R (4)	S (60)	S (57)	R (2)	S (70)	S (78)	S (86)
Sg 151-7042S	S (63)	R (18)	R (4)	R (8)	R (13)	R (8)	R (13)	S (97)	R (<1)	S (63)	S (76)	S (96)
Sg 153-7042S	R (0)	R (6)	R (8)	R (9)	R (11)	R (2)	R (9)	S (99)	R (3)	S (81)	S (84)	S (97)
Sg 200-ICMP 451	R (0)	R (8)	R (3)	R (3)	R (5)	R (3)	R (11)	S (97)	R (2)	S (61)	S (82)	S (98)
Sg 212-ICMP 451	R (0)	R (2)	R (4)	R (2)	R (4)	R (4)	R (10)	S (97)	R (1)	S (55)	S (61)	S (97)

[a]R < 20% DM incidence and S > 20% DM incidence.

Four *Eco*RI (+2) primers and six *Mse*I (+3) primers were used in 12 combinations for amplification. Selective primers provided in the kit were used and the amplification was carried out according to the manufacturer's protocol. The *Eco*RI primer was labeled with [Y-^{32}P]-ATP (3000 Ci/mmol) and the PCR products in 5.0 µl sub-samples were separated by electrophoresis on 6% denaturing polyacrylamide DNA sequencing gel containing 7.5 M urea. Autoradiograms were obtained using Kodak X-Omat film. Each experiment was repeated at least twice to establish the consistency of the bands.

Statistical analysis

Analysis of variance of downy mildew incidence data was done on both original (%) and arcsin-transformed data. Since the error mean squares were similar, the mean incidence (%) data were presented. The analysis was done using GENSTAT statistical package (Rothamsted Experiment Station, Herpenden, Herts AL52JQ, UK). Mean downy mildew incidence data were subjected to an average linkage cluster analysis using Euclidian distance as dissimilarity measure to classify the isolates into different virulence groups. The presence or absence of each band in the AFLP gel or autoradiogram was scored as 1 or 0, respectively. Cluster analysis of the AFLP and virulence data was based on similarity indices between pathogen isolates using the Clustan Graphics software package (Clustan Limited, UK).

RESULTS AND DISCUSSION

Pathogenic variability

Downy mildew incidence of 15 *S. graminicola* isolates on 12 pearl millet genotypes showed high variability ranging from 0 to 99% (Table 2). All host genotypes, except 7042S provided differential reactions for the isolates. Considering 20% incidence level for the cut-point for resistance (R) and susceptibility (S), isolate *Sg* 048 was most virulent (S reaction on 11 of the 12 pearl millet genotypes), followed by *Sg* 151 (S reaction on 10 genotypes), *Sg* 139 (S reaction on 6 genotypes), and the least virulent isolates were *Sg* 008 and *Sg* 013 with S reaction in only 2 genotypes (Table 2). The two Isolates of *Sg* 151 maintained on two different host genotypes showed differential virulence reaction; the one maintained on *Nokha* local was more virulent (S reaction on 10 genotypes) than that maintained on 7042S (S reaction on 5 genotypes). Cluster analysis of the virulence data classified the 15 isolates into five major groups. Group I consisted of Sg 008 and Sg 013; group II of Sg 021, Sg 149, Sg 153, Sg 200, Sg 212, and Sg 140; group III of Sg 139 (*Nokha* local and 7042S) and Sg 151 (7042S); group IV of Sg 048 and Sg 151 (*Nokha* local) and group V of Sg 150 (MBH 110 and 834B).

Two isolates *Sg* 048 (852B) and *Sg* 151 (*Nokha* local) showed high virulence against all the host genotypes, except 834 B and MBH 110, and included in a single cluster (group IV). The isolate *Sg* 139 maintained either on its original host, *Nokha* local or 7042S exhibited virulence reactions similar to that of *Sg* 151 maintained on 7042S and were clustered together in group III . Of the six isolates in group II, four (*Sg* 021, *Sg* 140, *Sg* 149, and *Sg* 153) were maintained on 7042S and two (*Sg* 200 and *Sg* 212) on ICMP 451. The isolates *Sg* 008 and *Sg* 013 though collected and maintained on two different host genotypes showed almost identical virulence reaction and were clustered together in Group I. Isolate Sg 150 maintained either on MBH 110 or 834B showed identical virulence and classified in group V.

Genetic variability

A total of 50 to 70 AFLP markers were scored for each of the 12 primer combinations and the percentage polymorphism (number of polymorphic bands /total number of bands scored) varied from 25 to 60 among the isolates. In AFLP analysis, of the 12 primer combinations five (E-AT, E-TA, M-CTG, M-CAG and M-CTA) showed high levels of polymorphism among the three *S. graminicola* isolates (Sg 139, Sg 150, and Sg 151) that were maintained on different host genotypes (Table 3). The numbers of polymorphic bands recorded for isolates Sg 139 (7042S), Sg 151 (7042S), and Sg 150 (MBH 110) were higher than those on their other host genotypes. The isolate, *Sg* 139 although multiplied on 7042S (Table 1), could be distinguished from the same isolate maintained for several generations on *Nokha* local by 1-6 polymorphic AFLP markers in different primer combinations (Table 3).

The dendrogram constructed, based on the similarity index data of AFLP markers classified the 15 *S. graminicola* isolates into five major

Table 3. Level of polymorphism for the same pathogen isolate of *S. graminicola* maintained on different host genotypes of pearl millet

Primer combination used		Number of polymorphic bands observed					
		Sg 139 maintained on		Sg 151 maintained on		Sg 150 maintained on	
Eco RI primer	Mse I primer	7042S	*Nokha* local	7042S	*Nokha* local	834B	MBH 110
E- AT	M- CTG	3	1	9	2	2	10
E- AT	M- CAG	6	1	6	5	1	8
E- AT	M- CTA	1	2	7	3	0	10
E- TA	M- CTG	1	2	6	4	0	12
E- TA	M- CAG	1	0	2	1	1	1

genotypic groups (Fig. 1). The AFLP patterns of the same isolate maintained on different host genotypes for varying number of asexual generations showed significant differences. In contrast, *Sg* 151 collected from 81A, but maintained on *Nokha* local and 7042S, were placed into two different clusters (groups II and III). In these groupings, the number of polymorphic AFLP markers varied between 3 and 11 with various primer combinations. Similarly, *Sg* 150 maintained on 834B and MBH 110 was identified in two separate groups (II and IV). Three isolates Sg 048, Sg 150 and Sg 151 collected from different locations and different hosts were clustered together in group III. Two isolates (Sg 139 and Sg 151) from Rajasthan and other two (Sg 200 and Sg 140) from Gujarat were clusterd together in group IV, although these had different collection hosts and maintenance hosts. Similarly, isolates Sg 021 and Sg 150 from Maharshtra were identified into the same genetic group (V) with different collection and maintenance hosts.

Both pathogenic and genetic diversity were exhibited among 15 isolates of *S. graminicola* collected from different pearl millet cultivars. Leonard (1987) stated that emergence of a new virulent pathotype in an asexual population need not be due to genetic recombination alone, and host cultivar-directed selection plays a major role

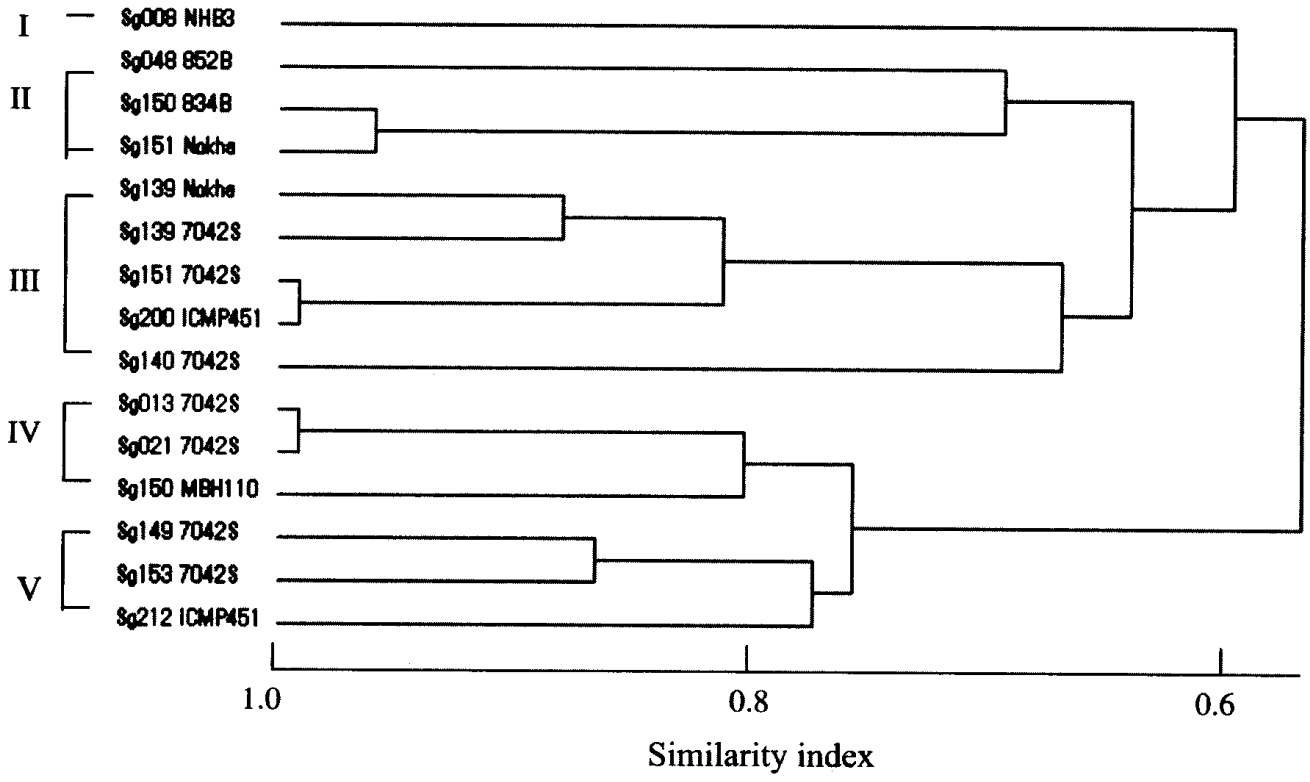

Fig.1. Dendrogram showing the grouping of the 15 isolates of *S. graminicola* based on AFLP markers

in evolution of specific virulence in a variable pathogen population. Thakur *et al.* (1992) showed the effect of host-directed virulence selection in a field population of *S. graminicola* through asexual generations. Evolution of specific virulence may be the outcome of a number of genetic interactions between the plant and the pathogen.

The genetic changes revealed by the polymorphic AFLP patterns among the different isolates of *S. graminicola* suggest that an isolate collected from a specific genotype may adapt to a new host genotype through several cycles of asexual growth on that host. The two isolates, *Sg* 008 and *Sg* 013, collected and maintained on two host genotypes NHB 3 and 7042S, respectively, for several asexual generations showed different AFLP profiles but similar virulence reaction suggesting genetic changes in the regions other than those involved in virulence. In contrast, *Sg* 151, maintained on *Nokha* local and 7042S showed different genetic and virulence groupings suggesting a significant influence of the host genotype on the pathogen virulence. The number of cycles required for the genetic change in the pathogen isolate will depend on the nature of the host-pathogen interaction. This is illustrated in the case of isolate *Sg* 151 that was grouped differently in both virulence reaction- and AFLP –based clusterings. Some of the changes in the virulence reactions and AFLP patterns strongly suggest the influence of host genotype on the pathogen, but all these need not be translated into virulence shift as is evident from the virulence data.

Genetic differences between any two isolates were little affected by their maintenance on the same host. For instance, isolates *Sg* 200 and *Sg* 212, maintained on the same host ICMP 451 showed 12 and 52 polymorphic bands differences. This suggests that *Sg* 212 (from Rajasthan), was genetically different from Sg 200 (from Gujarat) because they were originally collected from different pearl millet genotypes. In contrast, *Sg 200*, collected and maintained on ICMP 451, and *Sg* 151, collected from 81A and maintained on 7042S, grouped together, suggesting genetic similarity between the isolates. Again, the two isolates, *Sg* 139 (from *Nokha* local) and Sg151 (from 81A) and maintained on *Nokha* local were placed into two different groups. Another significant observation is that the field isolate from Patancheru, Sg 153, and the one purified from it, *Sg* 013, were in different subgroups under the major group I, suggesting the occurrence of genetic heterogeneity in the pathogen population from the same field.

ACKNOWLEDGEMENT

We acknowledge the funding from the USAID under the IARC linkage program.

REFERENCES

Ball, S.L. and **Pike, D. J.** (1984). Intercontinental variation in *Sclerospora graminicola*. *Ann. of Appl. Biol.* **104**: 41-51.

Leonard, K.J. (1987). The host population as a selective factor. In: *Populations of Plant Pathogens: Their Dynamics and Genetics,* (Eds., Wolfe, M.S. and Caten Blackwell, C.E.), pp. 163-179. Scientific Publications, Oxford.

Michelmore, R.W., Pawar, M.N. and **Williams, R.J.** (1982). Heterothallism in *Sclerospora graminicola*. *Phytopathology* **72**: 1368-1372.

O'Neill, N.R., van Berkum, P., Lin, J.J., Kwo, J., Ude, G.N., Kenworthy, W. and **Saunders, J.A.** (1997). Application of amplified restriction fragment length polymorphism for genetic characterization of *Colletotrichum* pathogens of alfalfa. *Phytopathology* **87**: 745-750.

Sastry, J.G., Ramakrishna, W., Sivaramakrishnan, S., Thakur, R.P., Gupta, V.S. and **Ranjekar, P.K.** (1995). DNA fingerprinting detects genetic variability in the pearl millet downy mildew pathogen (*Sclerospora graminicola*). *Theor. Appl. Genet.* **91**: 856-861.

Sivaramakrishnan, S., Harikrishnan, R., Thakur, R. P. and **Rao, V. P.** (1996). Use of RAPD markers for the detection of genetic variability in pearl millet downy mildew pathogen. In: *Proceedings of 2nd Asia –Pacific Conference on Agricultural Biotechnology, Madras,* (Eds., Sharma, R.P., Chopra, V.L. and Swaminathan, M.S.). pp. 51-56, Oxford & IBH Publishing Co., New Delhi.

Thakur, R.P. (1999). Pathogen diversity and plant disease management. *Indian Phytopath.* **52**: 1-9.

Thakur, R.P., Shetty, K.G. and **King, S.B.** (1992). Selection for host-specific virulence in asexual populations of *Sclerospora graminicola*. *Plant Pathol.* **41**: 626-632.

Thakur, R.P., Rao, V.P., Sastry, J.G., Sivaramakrishnan, S., Amrutesh, K.N. and **Barbind, L.D.** (1999). Evidence for a new virulence pathotype of *Sclerospora graminicola* on pearl millet. *J. Mycol. Plant Pathol.* **29**: 61-69.

Received for publication April 25, 2003