Archives Of Phytopathology And Plant Protection

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454295

Characterisation of pathogenic and molecular diversity in Sclerospora graminicola, the causal agent of pearl millet downy mildew

R. Sharma [a]; V. P. Rao [a]; R. K. Varshney [a]; V. P. Prasanth [a]; S. Kannan [a]; R. P. Thakur [a]
[a] International Crops Research Institute for the Semi-Arid Tropics, Patancheru, Andhra Pradesh, India

First published on: 10 November 2009

To cite this Article Sharma, R., Rao, V. P., Varshney, R. K., Prasanth, V. P., Kannan, S. and Thakur, R. P.(2010) 'Characterisation of pathogenic and molecular diversity in **Sclerospora graminicola**, the causal agent of pearl millet downy mildew', Archives Of Phytopathology And Plant Protection, 43: 6, 538 — 551, First published on: 10 November 2009 (iFirst)

To link to this Article: DOI: 10.1080/03235400801939896

URL: http://dx.doi.org/10.1080/03235400801939896

Archives of Phytopathology and Plant Protection
Vol. 43, No. 6, 10 April 2010, 538–551

Taylor & Francis
Taylor & Francis Group

Characterisation of pathogenic and molecular diversity in *Sclerospora graminicola*, the causal agent of pearl millet downy mildew

R. Sharma*, V.P. Rao, R.K. Varshney, V.P. Prasanth, S. Kannan and R.P. Thakur

International Crops Research Institute for the Semi-Arid Tropics, Patancheru, Andhra Pradesh, India

(*Received 21 December 2007; final version received 4 January 2008*)

Genotypic diversity among 46 isolates of *Sclerospora graminicola* collected from seven states in India during 1992–2005 was determined through pathotyping and AFLP analysis. A high level of variation was observed among the isolates for downy mildew incidence, latent period and virulence index. Based on the reaction on a set of nine pearl millet lines, 46 isolates were classified in 21 pathotypes. Quantitative differences in virulence levels of the test isolates were assessed by calculating the virulence index (disease incidence $\times$ latent period $^{-1}$). A dendrogram generated by the average linkage cluster analysis of virulence index clustered the 46 isolates into eight groups. Region-specific grouping of five isolates from Gujarat and six from Rajasthan was observed within two distinct groups. Temporal variation was also observed among the isolates collected from the same location and same host over the years. A total of 297 bands were scored following selective amplification with three primer combinations E-TT/M-CAG, E-AT/M-CAG and E-TG/M-CAT and all of them were polymorphic. Cluster analysis of AFLP data clustered the test isolates into seven groups. Analysis of molecular variance indicated that variation in the *S. graminicola* populations was largely due to differences among the isolates within the states.

Keywords: AFLP; downy mildew; pathotype; virulence

Introduction

Sclerospora graminicola, the causal agent of downy mildew, is a serious pathogen of pearl millet [*Pennisetum glaucum* (L.) R. Br.]. The disease is highly destructive and widespread in major pearl millet growing areas of the world (Williams 1984; Jeger et al. 1998). *S. graminicola* is an obligate pathogenic oomycete and reproduces both asexually by producing sporangia and sexually by means of oospores. The fungus is largely heterothallic but homothalism has also been reported (Michelmore et al. 1982; Idris and Ball 1984). The existence of mating types and their frequency greatly contribute towards the development of new recombinants in the pathogen populations (Pushpavathi et al. 2006a). These characteristics of the fungus make it highly variable.

Evolution of host-specific virulences in pearl millet downy mildew is well documented (Thakur et al. 1992; Sastry et al. 2001; Pushpavathi et al. 2006b). As a result of evolution of host specific virulences, resistant genotypes lose their effective resistance within a short period which leads to the development of new pathotypes/races in the pathogen

*Corresponding author. Email: r.sharma@cgiar.org

ISSN 0323-5408 print/ISSN 1477-2906 online

DOI: 10.1080/03235400801939896
http://www.informaworld.com

populations (Kolmer et al. 2006). Therefore, pathogen populations in the major crop growing areas need to be periodically monitored and characterised to identify new pathotypes in the target area. Populations across the regions are compared to determine genetic similarity among them and prevalence of different pathotypes/races across the regions. The occurrence of new virulence and virulence shift in *S. graminicola* is also monitored through on-farm surveys and a multilocational virulence nursery (Thakur et al. 2003).

Pathogenic variation in *S. graminicola* populations from different parts of the world has been studied by their reaction type on a set of differential hosts (Ball and Pike 1984; Thakur and Shetty 1993; Thakur and Rao 1997). It is important to study genetic variation in relation to virulence shift in the pathogen population to understand the changes in the genetic structure of the pathogen. In this context, molecular markers offer numerous genetic features to characterise diverse pathogen populations. DNA markers such as RFLP (Sastry et al. 1995), RAPD (Sastry et al. 2001) and AFLP (Singru et al. 2003; Sivaramakrishnan et al. 2003; Pushpavathi et al. 2006b) have been used successfully to study the genetic variation in the *S. graminicola* populations. Apart from elucidation of genetic diversity, molecular markers can also be used to study evolution and monitoring movement/shift of pathogen populations over time and space. Comprehensive study on the genetic change in *S. graminicola* populations on temporal and spatial levels in different geographic regions is lacking. Therefore, the present investigation was undertaken to study the temporal and spatial diversity among *S. graminicola* populations from the different pearl millet growing states of India.

Materials and methods

Isolates of *S. graminicola* were collected from different pearl millet cultivars during the field surveys in major pearl millet growing states of India. The isolates were established from oospores on the pot-grown seedling in the greenhouse and maintained through asexual progenies derived from single zoospores. Isolates were maintained either on the same host from which they were collected or on a universally susceptible genotype 7042S. Forty-six isolates collected from seven states in India during 1992–2005 were selected for the study (Table 1). Of the 46 isolates, 16 were from Andhra Pradesh, 13 from Rajasthan, 11 from Gujarat, two each from Maharashtra and Haryana and one each from Karnataka and Delhi.

Inoculum preparation and inoculation

Infected leaves were collected, cut into pieces and washed under running tap water. The leaves were wiped with tissue paper to remove old sporangia and placed with their abaxial surface up in humidity chambers lined with moist blotting paper. The humidity chambers were incubated in the dark at 20°C for 6 h. Sporangia from sporulating leaves were harvested in sterilised distilled ice cold water using camel hair brush, and filtered through double-layered muslin cloth. Spore concentration was adjusted to 5×10^5/ml. Pot-grown seedlings of the nine differential lines (P 7-4, P310-17, 700651, 7042R, 852B, IP 18292, IP 18293, ICMP 451 and 7042S) were spray-inoculated at coleoptile stage using atomizer and covered immediately with a polyethylene sheet to provide >95% relative humidity. Seedlings were incubated at 20°C for 24 h and then pots were transferred to greenhouse benches at 25 ± 2°C and >95% RH for disease development during the next two weeks.

Table 1. Sources of *Sclerospora graminicola* isolates collected from different pearl millet growing states of India.

Isolate No.	Identity	Isolate collection		
		Year	Cultivar	Location
A1	Sg 008	1992	NHB3	ICRISAT, Patancheru/Andhra Pradesh
A2	Sg 009	1992	BJ 104	ICRISAT, Patancheru/Andhra Pradesh
A3	Sg 010	1992	MBH110	ICRISAT, Patancheru/Andhra Pradesh
A4	Sg 011	1992	852B	ICRISAT, Patancheru/Andhra Pradesh
A5	Sg 012	1992	700651	ICRISAT, Patancheru/Andhra Pradesh
A6	Sg 013	1992	7042S	ICRISAT, Patancheru/Andhra Pradesh
A7	Sg 018	1992	7042S	ICRISAT, Patancheru/Andhra Pradesh
A8	Sg 019	1992	7042S	ICRISAT, Patancheru/Andhra Pradesh
A9	Sg 153	1997	7042S + NHB3	ICRISAT, Patancheru/Andhra Pradesh
A10	Sg 409	2004	PMB 11571–2	ICRISAT, Patancheru/Andhra Pradesh
A11	K-1977	1977	7042S	ICRISAT, Patancheru/Andhra Pradesh
A12	K-1987	1987	7042S	ICRISAT, Patancheru/Andhra Pradesh
A13	K-1997	1997	7042S	ICRISAT, Patancheru/Andhra Pradesh
A14	K-1999	1999	7042S	ICRISAT, Patancheru/Andhra Pradesh
A15	K-2001	2001	7042S	ICRISAT, Patancheru/Andhra Pradesh
A16	K-2004	2004	7042S	ICRISAT, Patancheru/Andhra Pradesh
M1	Sg 021	1993	MLBH 104	Ghari/Maharashtra
M2	Sg 332	2001	MLBH 267	Aurangabad/ Maharashtra
K1	Sg 048	1994	7042S + HB3	Mysore/Karnataka
G1	Sg 200	1998	ICMH 451	Jamnagar/Gujarat
G2	Sg 348	2001	ICMH 451	Anand/Gujarat
G3	Sg 432	2005	–	Kheda/Gujarat
G4	Sg 435	2005	–	Anand/Gujarat
G5	Sg 437	2005	7042S + HB3	Jamnagar/Gujarat
G6	Sg 438	2005	GHB 577	Banaskantha/Gujarat
G7	Sg 439	2005	GHB 558	Banaskantha/Gujarat
G8	Sg 440	2005	Nandi 3	Banaskantha/Gujarat
G9	Sg 441	2005	–	Mehasana/Gujarat
G10	Sg 442	2005	–	Gandhinagar/Gujarat
G11	Sg 445	2005	AHT-503	Banaskantha/Gujarat
R1	Sg 138	1997	–	Jodhpur/Rajasthan
R2	Sg 139	1997	IP 18292	Jodhpur/Rajasthan
R3	Sg 144	1997	81A	Jodhpur/Rajasthan
R4	Sg 145	1997	HB 3	Jodhpur/Rajasthan
R5	Sg 148	1997	–	Mandor/Jodhpur/Rajasthan
R6	Sg 151	1997	81A	Durgapura/Rajasthan
R7	Sg 212	1998	PG 5522	Durgapura/Rajasthan
R8	Sg 381	2003	OPY 97	Jodhpur/Rajasthan
R9	Sg 382	2003	Local	Barmer/Rajasthan
R10	Sg 383	2003	ICMH 451	Barmer/Rajasthan
R11	Sg 384	2003	Local	Barmer/Rajasthan
R12	Sg 385	2003	Barmer local	Barmer/Rajasthan
R13	Sg 406	2003	Bikaner local	Bikaner/Rajasthan
H1	Sg 298	1999	W 504–1–1	IARI/New Delhi
H2	Sg 334	2001	HHB 67	Bhiwani/Haryana
H3	Sg 335	2001	HHB 67	Bhiwani/Haryana

Data recording

Data were recorded for latent period and disease incidence. Latent period was expressed as number of days from inoculation to sporulation on 50% infected seedlings

(Thakur et al. 1998). Disease incidence was recorded 14 days after inoculation as percent infected plants. Quantitative differences in the virulence levels of the isolates were determined by calculating virulence index (Thakur and Rao 1997) as:

$$\text{Virulence index} = \text{Percent disease incidence} \times \text{latent period}^{-1}$$

Spore collection and DNA extraction

Sporangia from the sporulating leaves infected by individual isolates were harvested in distilled sterilised water and filtered through doubled-layered muslin cloth. The spore suspension was centrifuged in 30 ml tube at 5000 rpm for 10 minutes, the supernatant was discarded and the pellet was used for the DNA extraction following the method described by Sastry et al. (1995).

AFLP analysis

The 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*R1 and *Mse*l 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*R1 and *Mse*l adapters at 20°C for 2 h. After terminating the reaction, the ligation mixture was diluted 10-fold with TE buffer and the fragments were preamplified in a thermal cycler (MJ Research, 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. Selective primers provided in the kit were used and the amplification was carried out according to the manufacturer's protocol. The *Eco*R1 primer was labelled with [$\gamma-^{32}$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.

Statistical analysis

The data on downy mildew incidence, latent period and virulence index were subjected to analysis of variance using GENSTAT statistical package to determine significant differences among isolates, host genotypes and their interactions (Payne 2002). Based on the proximity matrix, calculated by using Euclidian measure, the cluster analysis was done using the Average Linkage method to determine the similarity among the isolates and to classify isolates into virulence groups based on virulence index.

AFLP profiles of 46 isolates were used to construct a binary matrix. Each band was scored as present (1) or absent (0) across the isolates. The data were then analysed using Numerical Taxonomy System Version 2.2 (NTSYSpc). The proximity matrix was computed using the Jaccard's similarity coefficient and a dendrogram was constructed by unweighted pair group method of arithmetic averages (UPGMA) using the SAHN (Sequential Agglomerative Hierarical Nested) cluster analysis module (Rohlf 2000). Mantel test in NTSYSpc for matrix comparison was carried out to see the goodness of fit among disease incidence, latent period, virulence index and AFLP data (Mantel 1967). Finally, the PIC (Polymorphism Information Content) values for the three primer combinations were calculated using the formula given by Botstein et al. (1980).

Analysis of Molecular Variance version 1.55 (AMOVA) was used to estimate variance components for the AFLP patterns and to partition the total variance into 'within groups' and 'among groups' (Excoffier et al. 1992). To see the significance of variance components, 1000 permutations were used. Unbiased measures of identity and genetic distance among *S. graminicola* populations from seven states were calculated by Nei's distance (Nei 1978).

Results

Pathogenic variability

All the test isolates induced clear downy mildew symptoms on the susceptible lines 7042S and ICMP 451. However, differential reactions were observed on seven lines against the test isolates. The percent disease incidence varied from 0 to 100 across lines and isolates (Table 2). Isolate Sg 009 was found to be the least virulent with overall mean incidence 21.2% across the nine lines, whereas Sg 385 was most virulent with overall mean 84.8%, followed by Sg 383 (84.6%) and Sg 441 (74.0%). Pearl millet line 700651 exhibited the least downy mildew incidence (20.2%) across the 46 test isolates and the highest incidence (98.4%) was recorded on the susceptible check 7042S.

Significant variation was observed for the latent period, ranging from 4 to 13 days. The mean latent period across the differentials was maximum for isolate Sg 437 (9.8 days) and minimum for Sg 385 (4.4 days). Mean latent period across the isolates was maximum on P 310-17 and 700651 (8.5 days) and minimum on the susceptible check 7042S (5.2 days). The analysis of variance exhibited significant ($P < 0.001$) effect of isolates, host genotypes and their interaction on downy mildew incidence as well as latent period (Table 5).

Pathotype grouping

Based on percent downy mildew incidence, reaction of the differential hosts to the individual isolate was categorised as resistant ($\leq$10% incidence) or susceptible ($>$10% incidence). Differential reaction of the host genotypes to the test isolates was observed and on the basis of the reaction type, isolates were grouped into different pathotypes. The 46 isolates used in the study were grouped into 21 pathogenic groups/pathotypes (Table 3). Pathotype P 11 was most virulent infecting all the nine host differentials. A maximum of 12 isolates were included in this pathotype. Of the 12 isolates, six were from Gujarat, five from Rajasthan and one from Andhra Pradesh. P 5 was the second largest pathogenic group with six isolates; however, this pathotype was the least virulent and could infect only three differential lines. Out of 21 pathotypes, 15 were represented by single isolates and distinguished as different pathotypes. Pathotype P 10, P 12, P 13 and P 14 infected eight differentials followed by P 8, P 9, P 15 and P 19 being virulent on seven lines. Of the nine host differentials, P 310-17 showed resistance against 14 pathotypes followed by IP 18293 having resistance against 12 pathotypes.

Virulence index

A high level of variation for the virulence index (0–25) was observed among the isolates (Table 4). Mean virulence index across the differential was minimum (3.54) for Sg 010 (least virulent) and maximum (19.65) for Sg 385 (highly virulent). Minimum and maximum virulence index values of 3.54 (Sg 010) and 14.75 (K-2004), 4.24 (Sg 348) and

Table 2. Downy mildew incidence of 46 isolates of *Sclerospora graminicola* on nine host differential lines.

	Downy mildew incidence (%) on host differential lines*									
Isolate	P 7-4	P 310-17	700651	7042R	852B	IP18292	IP18293	ICMP 451	7042S	Overall Mean
Sg 008	31	5	14	61	0	0	0	22	96	25.4
Sg 009	0	0	1	64	0	0	0	27	99	21.2
Sg 010	10	1	9	29	16	0	0	34	100	22.1
Sg 011	20	4	15	84	82	0	0	22	100	36.3
Sg 012	3	1	55	61	9	0	0	12	96	26.3
Sg 013	15	2	4	33	0	0	0	99	100	28.1
Sg 018	8	0	4	47	0	0	4	94	97	28.2
Sg 019	3	0	0	38	0	0	0	91	95	25.2
Sg 153	21	2	12	60	0	0	4	83	100	31.3
Sg 409	52	96	20	93	1	8	85	91	99	60.5
K-1977	9	2	5	30	3	0	0	94	99	26.8
K-1987	18	16	22	52	14	5	7	88	95	35.2
K-1997	21	10	6	22	4	3	4	97	100	29.6
K-1999	12	0	0	3	2	2	4	85	97	22.7
K-2001	12	5	5	39	3	2	6	87	96	28.3
K-2004	42	40	33	79	100	97	28	100	100	68.8
Sg 021	4	2	2	23	0	0	2	89	97	24.3
Sg 332	17	0	35	43	0	0	0	98	100	32.6
Sg 048	0	0	0	60	74	74	37	40	90	41.7
Sg 200	10	3	11	33	6	49	6	78	96	32.4
Sg 348	7	1	6	21	1	3	2	89	100	25.6
Sg 432	90	77	42	85	13	100	59	100	100	74.0
Sg 435	60	19	56	63	6	100	12	95	100	56.8
Sg 437	58	60	37	93	6	100	8	74	100	59.7
Sg 438	44	58	60	69	100	100	40	96	100	74.1
Sg 439	53	73	56	71	100	100	53	95	100	77.9
Sg 440	42	55	28	100	100	100	40	87	100	72.4
Sg 441	81	58	77	96	100	100	44	100	100	84.0
Sg 442	27	4	15	49	35	72	11	100	100	45.9
Sg 445	86	63	53	75	100	80	46	100	100	78.1
Sg 138	97	100	18	75	100	33	70	94	100	76.3
Sg 139	28	6	8	47	54	64	9	57	96	41.0
Sg 144	18	21	18	60	100	2	2	19	100	37.8
Sg 145	45	22	10	87	92	87	31	62	96	59.1
Sg 148	17	22	8	44	35	28	11	12	87	29.3
Sg 151	18	5	16	47	1	0	5	99	100	32.3
Sg 212	12	3	1	53	9	3	12	100	100	32.6
Sg 381	63	100	20	61	100	0	47	100	100	65.7
Sg 382	90	100	13	100	99	24	50	100	100	75.1
Sg 383	70	100	12	100	100	83	96	100	100	84.7
Sg 384	76	83	26	69	94	51	64	98	95	72.9
Sg 385	55	100	27	97	100	89	95	100	100	84.8
Sg 406	12	5	40	88	9	14	6	56	100	36.7
Sg 298	21	9	9	80	52	14	15	61	99	40.0
Sg 334	12	8	10	22	0	8	6	98	100	29.3
Sg 335	14	2	11	41	3	3	8	76	100	28.7
Mean	32.70	29.20	20.21	59.72	39.63	34.74	22.37	78.24	98.37	

*Mean of three replications. SE (m) for isolates = ±0.75, host differentials = ±0.35, and their interactions = ±2.37.

Table 3. Pathotyping of 46 isolates of *Sclerospora graminicola* based on disease reaction on nine host differential lines.

	Reaction on host differential lines									
Pathotype	P 7-4	P 310-17	700651	7042 R	852 B	IP 18292	IP 18293	ICMP 451	7042 S	Isolate No.
1	S	**R**	S	S	**R**	**R**	**R**	S	S	A1,A9,M2,H3,R6
2	S	**R**	**R**	S	**R**	**R**	**R**	S	S	A6,H2,A13,A15,
3	S	**R**	**R**	S	**R**	**R**	S	S	S	R7
4	S	**R**	**R**	**R**	**R**	**R**	**R**	S	S	A14
5	**R**	**R**	**R**	S	**R**	**R**	**R**	S	S	A2, A7, A8, A11, M1, G2
6	**R**	**R**	**R**	S	S	**R**	**R**	S	S	A3
7	S	**R**	S	S	S	**R**	**R**	S	S	A4
8	S	S	S	S	S	**R**	**R**	S	S	A12,R3
9	S	S	S	S	**R**	**R**	S	S	S	A10
10	S	S	S	S	**R**	S	S	S	S	G4
11	S	S	S	S	S	S	S	S	S	A16,R11,R12,R1, R9,R10,G9,G8, G7,G6,G11,G3
12	S	S	S	S	S	**R**	S	S	S	R8
13	S	**R**	S	S	S	S	S	S	S	G10
14	S	S	**R**	S	S	S	S	S	S	R4,R5
15	S	**R**	**R**	S	S	S	S	S	S	H1
16	**R**	**R**	**R**	S	S	S	S	S	S	K1
17	S	**R**	**R**	S	S	S	**R**	S	S	R2
18	**R**	**R**	S	S	**R**	S	**R**	S	S	G1
19	S	S	S	S	**R**	S	**R**	S	S	G5
20	S	**R**	S	S	**R**	S	**R**	S	S	R13
21	**R**	**R**	S	S	**R**	**R**	**R**	S	S	A5

R = DM incidence $\leq$10%; S = DM incidence $>$10%.

16.46 (Sg 445), and 5.24 (Sg 148) and 19.65 (Sg 385) were observed among the isolates from Andhra Pradesh, Gujarat and Rajasthan, respectively. On the basis of virulence index, K-2004, Sg 445 and Sg 385 were designated as the most virulent isolates of Andhra Pradesh, Gujarat and Rajasthan, respectively. Mean virulence index across the isolates was minimum for 700651 (2.73) followed by IP 18293 (4.04) and maximum for susceptible check 7042S (19.60) followed by the other susceptible line ICMP 451 (14.86). The virulence index was highly influenced by isolate, differentials and their interactions (Table 5).

A dendrogram generated by the average linkage cluster analysis of virulence index of the test isolates clustered the 46 isolates into eight groups (Figure 1). However, isolate Sg 409 from Andhra Pradesh could not be assigned to any group. Group G II and G I were the major groups clustering 13 and 7 isolates, respectively. Several sub-groups were also observed within these two groups. Group G V had all the five isolates from Gujarat, whereas group G VII represented Rajasthan with six isolates.

AFLP analysis

A high level of polymorphism was observed among the 46 *S. graminicola* isolates collected from the major pearl millet growing states of India. A total of 297 bands were scored following selective amplification with three primer combinations. The number of bands

Table 4. Virulence index of 46 isolates of *Sclerospora graminicola* on nine host differential lines.

	Virulence index*									
Isolate	P 7-4	P 310-17	700651	7042R	852B	IP18292	IP18293	ICMP 451	7042S	Overall Mean
Sg 008	3.44	0.55	1.41	12.22	0	0	0	3.72	19.15	4.50
Sg 009	0	0	0.47	10.58	0	0	0	3.80	19.79	3.85
Sg 010	1.10	0.25	0.80	5.70	1.67	0	0	5.66	16.67	3.54
Sg 011	2.55	0.40	2.14	16.77	13.73	0	0	3.18	20.00	6.53
Sg 012	0.02	0.32	6.1	12.16	1.47	0	0	2.05	19.24	4.60
Sg 013	1.85	0.47	0.28	5.57	0	0	0	19.77	20.00	5.33
Sg 018	0.89	0	0.37	7.80	0	0	0.38	18.82	19.33	6.98
Sg 019	0.20	0	0	7.64	0	0	0	18.25	19.01	5.29
Sg 153	2.95	0.81	1.42	11.99	0	0	0.38	16.53	20.00	6.00
Sg 409	10.39	19.26	2.54	23.23	1.44	0.69	21.37	22.70	24.74	14.04
K-1977	1.10	0.57	0.46	5.97	0.26	0	0	15.64	19.79	4.87
K-1987	1.98	1.75	2.26	10.34	1.59	0.58	0.75	17.55	18.94	6.19
K-1997	3.08	1.26	0.67	4.33	0.38	1.05	0.51	19.32	20.00	5.62
K-1999	1.56	0	0	0.36	0.20	0.89	0.45	10.65	19.32	3.71
K-2001	1.42	0.73	0.49	7.86	0.35	0.53	0.57	12.45	15.99	4.49
K-2004	7.08	6.71	5.53	19.63	25.00	19.41	4.42	20.00	25.00	14.75
Sg 021	0.33	0.49	0.31	4.59	0	0	0.26	17.83	19.38	4.80
Sg 332	1.76	0	3.86	7.64	8.62	6.10	4.06	19.54	20.00	7.95
Sg 048	0	0	0	10.05	0	0	0	5.70	17.99	3.79
Sg 200	1.45	0.29	1.15	5.82	0.70	4.46	0.52	13.83	16.04	4.92
Sg 348	0.73	0.09	0.51	3.34	0.48	1.18	0.30	14.85	16.67	4.24
Sg 432	9.29	11.02	4.23	14.10	1.49	13.69	7.73	16.67	15.87	10.45
Sg 435	6.46	2.17	6.53	10.76	0.88	14.29	1.43	11.84	15.28	7.74
Sg 437	6.75	5.19	3.50	10.30	4.25	8.21	0.90	6.64	11.96	6.41
Sg 438	5.35	6.46	6.45	11.47	15.28	11.67	4.45	12.62	14.29	9.78
Sg 439	6.98	9.54	6.56	11.87	15.08	13.69	6.73	15.92	14.29	11.18
Sg 440	5.28	6.91	3.81	16.67	13.65	13.10	5.00	14.54	15.08	10.45
Sg 441	10.60	6.72	8.65	19.24	12.04	16.67	5.75	20.00	21.67	13.48
Sg 442	3.17	0.66	1.93	6.74	4.10	8.82	1.66	21.67	20.83	7.73
Sg 445	16.21	11.97	10.25	17.50	25.00	13.28	8.91	21.67	23.33	16.46
Sg 138	19.33	20.00	3.06	15.06	20.00	5.46	13.94	18.75	20.00	15.07
Sg 139	4.72	0.64	0.93	7.80	6.79	8.46	1.00	9.53	15.97	6.20
Sg 144	3.63	3.87	2.89	11.98	20.00	0.83	0.08	3.13	20.00	7.38
Sg 145	7.42	2.00	1.66	21.77	18.42	14.55	5.20	12.42	24.00	11.94
Sg 148	2.96	2.42	2.52	7.55	7.00	4.71	1.54	2.01	16.43	5.24
Sg 151	2.05	0.52	2.39	9.34	0.38	0	0.46	19.79	20.00	6.10
Sg 212	1.93	0.37	0.72	13.14	2.24	1.28	2.21	25.00	25.00	7.99
Sg 381	15.69	20.00	2.86	15.18	25.00	0	11.73	25.00	25.00	15.61
Sg 382	18.09	20.00	2.44	20.00	19.82	3.41	9.90	18.89	20.00	14.73
Sg 383	11.62	25.00	1.66	25.00	25.00	13.90	22.44	25.00	25.00	19.40
Sg 384	15.12	16.58	5.21	13.84	18.89	8.52	12.78	19.54	18.93	14.38
Sg 385	13.86	20.00	5.41	24.17	25.00	14.77	23.63	25.00	25.00	19.65
Sg 406	2.05	1.21	8.09	22.03	1.23	2.32	0.77	11.23	25.00	8.21
Sg 298	3.07	0.83	1.05	13.29	6.46	1.31	1.84	10.16	16.50	6.06
Sg 334	1.65	0.90	0.99	3.41	0	0.69	0.64	19.62	20.00	5.32
Sg 335	2.21	0.37	1.23	8.12	0.41	0.47	1.24	15.20	25.00	6.03
Mean	5.21	4.98	2.73	11.82	7.48	4.98	4.04	14.86	19.60	

*Mean of three replications. SE (m) for isolates = $\pm$0.22, host differentials = $\pm$0.09, and their interactions = $\pm$0.65.

Table 5. Analysis of variance for downy mildew incidence, latent period and virulence index by the *Sclerospora graminicola* isolates on host differentials.

Source of variation	df	Downy mildew incidence		Latent period		Virulence index	
		MS	F-value	MS	F-value	MS	F-value
Isolate (I)	45	12519071	817.31*	48.67	83.56*	507.28	388.24*
Host genotype (H)	8	101414.10	5924.72*	273.94	371.76*	4342.286	3403.42*
I X H	360	1504.72	87.91*	6.45	8.75*	61.20	47.97*
Residual	736	17.12		0.74		1.28	

*Significant at ($P < 0.001$).

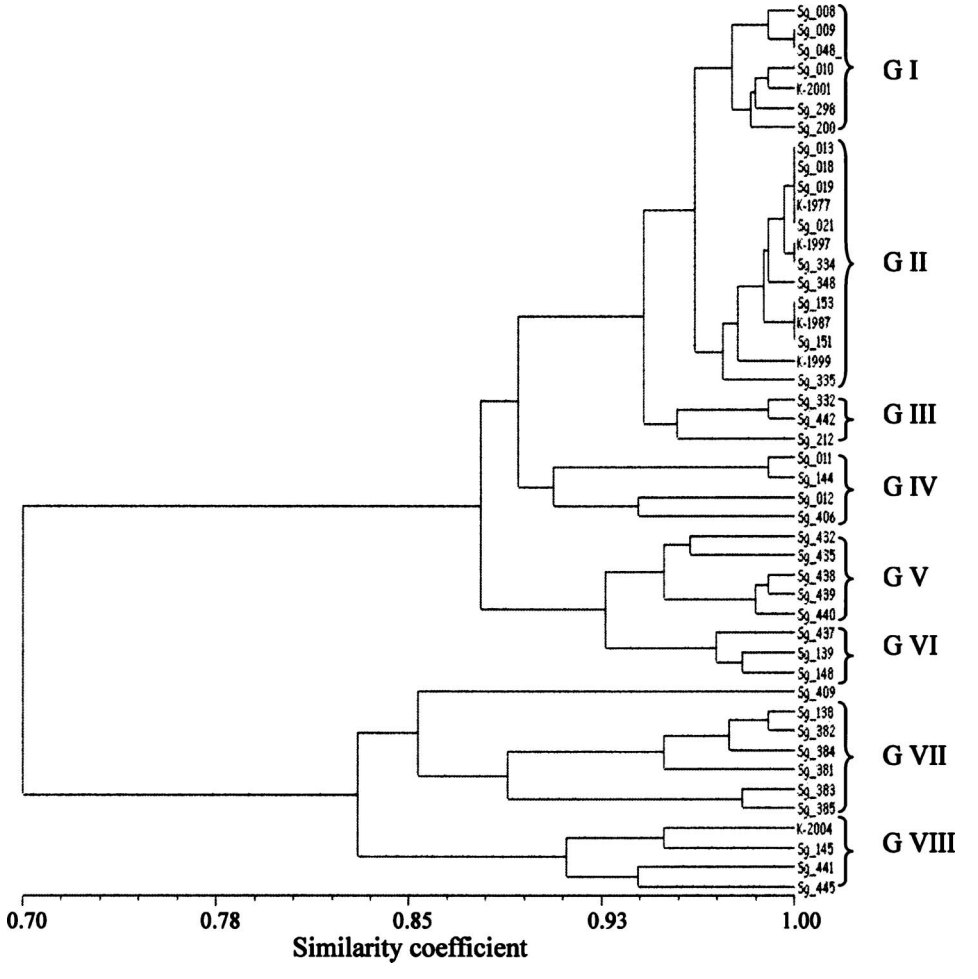

Figure 1. Dendrogram depicting genetic relatedness among 46 isolates of *Sclerospora graminicola* based on virulence index.

generated by the primer combinations E-TT/M-CAG, E-AT/M-CAG and E-TG/M-CAT were 101, 101 and 95, respectively. All the 297 amplicons were polymorphic. Very high polymorphic information content (PIC) values of 0.985, 0.982 and 0.980 were observed for

the primer combinations E-AT/M-CAG, E-TT/M-CAG and E-TG/M-CAT, respectively. However, no region/host specific amplicon was observed.

Phenetic analysis

Jaccard's similarity coefficients ranged from 0.03 (between isolates K-1997 from Andhra Pradesh and Sg 385 and Sg 406 from Rajasthan) to 0.50 (between isolates Sg 008 and Sg 011 from Andhra Pradesh). Similarity coefficients between isolates within Andhra Pradesh ranged from 0.05 (between K-1997 and Sg 011) to 0.5 (Sg 008 and Sg 011), within Gujarat from 0.13 (Sg 437 and Sg 445) and 0.36 (Sg 432 Sg 347) and within Rajasthan it ranged between 0.08 (Sg 145 and Sg 406) and 0.32 (Sg 384 and Sg 383).

The UPGMA based dendrogram generated using Jaccard similarity coefficients clustered the test into seven groups (Figure 2), however, with a low similarity value around 0.2. Two isolates from Andhra Pradesh and one each from Rajasthan and Delhi could not be clustered into any of these groups. Group A1 was represented by nine isolates, all from Andhra Pradesh. Similarly, group A VI clustered all the five isolates from Rajasthan. Group A III was the largest group with 11 isolates representing most of the states. A II, A IV, and A VII were small groups clustering only three isolates in each group.

Analysis of molecular variance

Analysis of molecular variance (AMOVA) revealed that 93.2% of the variance among the 46 AFLP patterns was due to the differences among the isolates within states and 6.8% was due to differences among the states (Table 6). However, despite the little contribution of variation among states towards the total variance, the value was statistically significant at $P < 0.01$. Genetic distance among the pathogen populations from different states was very low (Table 7). Maximum genetic distance was observed between isolate from Karnataka and populations from other states.

Discussion

Variation in the pathogen population for virulence on the host genotypes is required for the selection of host-specific virulence (Thakur et al. 1992). Differential reaction of the hosts towards pathogen isolates was observed both for disease incidence as well as latent period showing variation in the *S. graminicola* populations. Seventeen isolates observed incompatible reaction on one or more host differentials indicating qualitative resistance in pearl millet towards the matching avirulences. However, level of downy mildew incidence was quite variable in most of the isolates on the test differentials. Differentials with $\leq$10% downy mildew incidence were categorised as resistant and with $>$10% incidence as susceptible. Based upon the reaction type isolates were grouped in 21 pathotypes. Occurrence of 21 pathotypes in the populations of 46 isolates further supports the presence of different virulences in the pathogen and the presence of different R genes/QTLs in the differential hosts. Of the 21 pathotypes, P 11 was virulent on all the nine differentials indicating that virulence has evolved against the R genes/QTLs present in these genotypes. Of the 12 isolates representing the pathotype, six were from Gujarat, five from Rajasthan and only one from Andhra Pradesh, indicating a prevalence of more virulent populations of *S. graminicola* in Rajasthan and Gujarat. Rajasthan and Gujarat are the major pearl millet growing states and cultivation of large numbers of hybrids with diverse genetic make up may account for the increased virulence. Moreover, most of these isolates are the

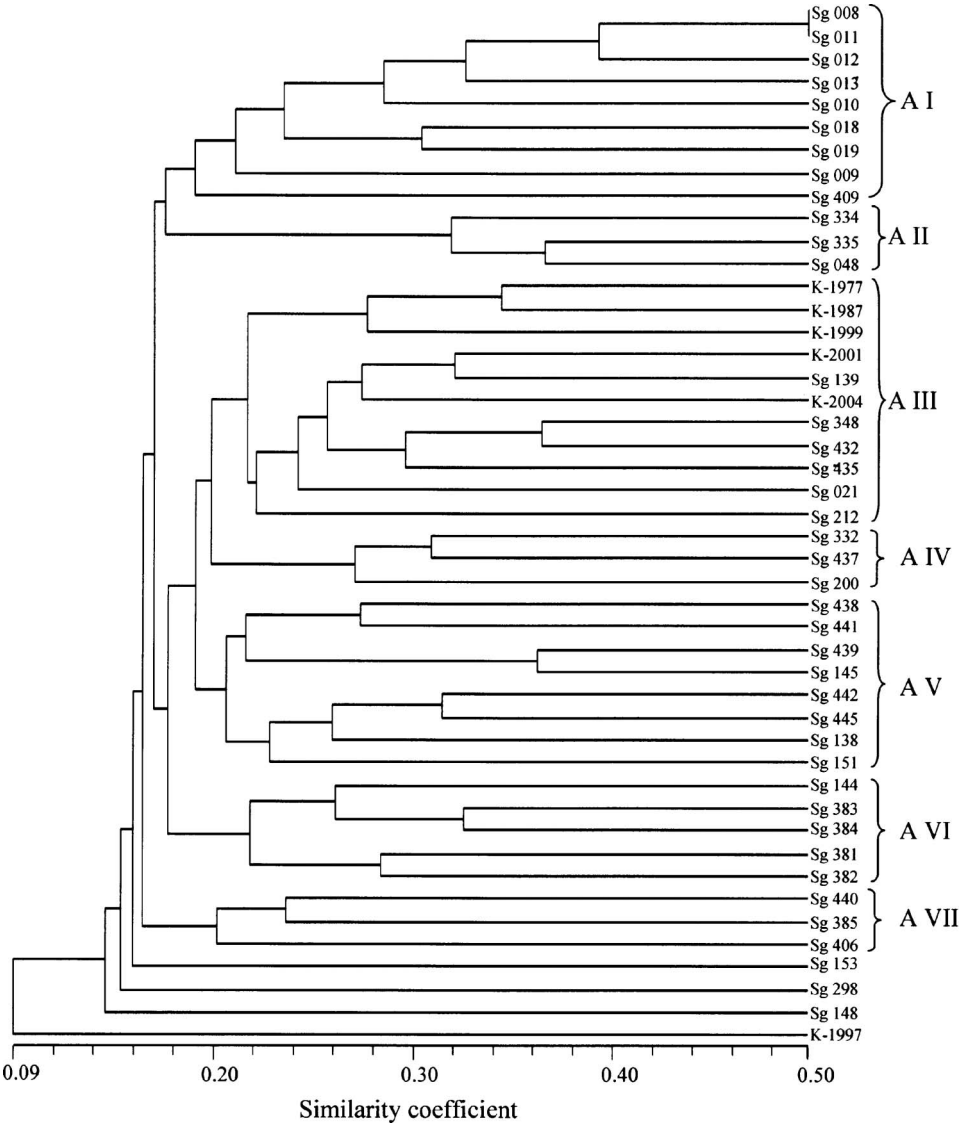

Figure 2. Dendrogram depicting genetic relatedness among 46 isolates of *Sclerospora graminicola* based on similarity coefficient calculated from AFLP fingerprints.

recent collections from their respective sites, further supporting evolution of more virulent strains over time. Similarly, isolates K-1977, K-1987, K-1997, K-1999, K-2001 and K-2004 collected over the years from the same host 7042S and from same location Patancheru, Andhra Pradesh, were grouped in different pathotypes, exhibiting temporal variation in *S. graminicola* populations.

In pearl millet downy mildew pathosystem, disease incidence levels indicate quantitative differences for virulence in the pathogen and resistance in the host. Quantitative variation in the *S. graminicola* isolates was studied by calculating the virulence index from two independent measures of pathogenicity, disease incidence and latent period (Thakur and Rao 1997). Grouping of the isolates based on virulence index, to some extent, supported the

Table 6. AMOVA for partitioning AFLP variation in *Sclerospora graminicola* isolates.

Source of variation	df	Variance component	% total	*P*
Among states	6	0.00836	6.8	0.009
Within states	39	0.1149	93.2	
Total	45			

Fixation index (Fst) = 0.068.

Table 7. Unbiased measures of identity and genetic distance (Nei 1978) among *Sclerospora graminicola* populations from seven states. Nei's genetic identity is shown above diagonal, genetic distance below diagonal.

	AP	Maharashtra	Karnataka	Gujarat	Rajasthan	Delhi	Haryana
AP		0.973	0.802	0.988	0.990	0.901	0.943
Maharashtra	0.028		0.799	0.981	0.976	0.879	0.933
Karnataka	0.221	0.224		0.809	0.807	0.753	0.855
Gujarat	0.012	0.019	0.212		0.993	0.900	0.941
Rajasthan	0.010	0.025	0.214	0.007		0.907	0.944
Delhi	0.104	0.129	0.284	0.105	0.098		0.870
Haryana	0.059	0.069	0.157	0.061	0.058	0.140	

region-specific grouping of isolates. Group G VII clustered isolates with high virulence index and all the six isolates in this group were from Rajasthan. Similarly, G V contained all the five isolates from Gujarat. Both virulence index as well as disease incidence data reveals that Rajasthan populations of *S. graminicola* are highly virulent. Group G I included the least virulent isolates from Andhra Pradesh, Karnataka, Delhi and Gujarat. However, most of them were collected in the 1990s and might have been eliminated by now from the natural population of *S. graminicola* with the evolution of more virulent and better-fit pathotypes. Virulence index could also establish differences in resistance level among the host differentials. Differential line 700651 recorded minimum mean virulence index (2.73) across the 46 isolates indicating that resistance in this genotype is comparatively stable and might be governed by several QTLs for downy mildew resistance. A number of QTLs for host plant resistance have been reported in pearl millet against different pathotypes of *S. graminicola* (Hash and Witcombe 2001; Jones et al. 2002).

DNA fingerprinting using AFLP exhibited a high degree of variation in the *S. graminicola* populations. Variation at the DNA level has been reported in *S. graminicola* through RAPD (Sastry et al. 2001), RFLP (Sastry et al. 1995; Sastry et al. 1997) and AFLP (Singru et al. 2003; Sivaramakrishnan et al. 2003; Pushpavathi et al. 2006b). This high genotypic variability is because of frequent recombination events taking place in the natural populations of *S. graminicola*. The occurrence of both the mating types, Mat A and Mat B, as well as homothallism have been reported from the Indian isolates of *S. graminicola* (Pushpavathi et al. 2006a). Low similarity values among the isolates observed in the present study further demonstrate the high level of variation available in the pathogen. Of the seven groups generated through the cluster analysis of AFLP data, group A I included all the isolates from Andhra Pradesh, whereas group A VI was represented by Rajasthan isolates. Region-specific grouping corresponding to host gene pools has been reported in *Colletotrichum lindemuthianum* based on molecular and virulence markers (Sicard et al. 1997).

AMOVA reported differences among geographical regions/states, which were significant but represented only 6.8% of the total variation. This variation between states might be because of clustering of some isolates from Andhra Pradesh as well as from Rajasthan in the region-specific groups. Populations in Andhra Pradesh seem distantly related to those of Rajastahn and Gujarat. Similarly, Manici and Bonora (2007) observed that most of the variation in Italian isolates of *Rhizoctonia* spp. from strawberry occurred within each of the four geographic regions from where the isolates were collected. Very poor correlation was observed following matrix comparison of AFLP data with pathogenicity data. Therefore, no association between grouping of the isolates based on molecular data and pathogenicity data could be established. Sharma et al. (2002) made similar observations with *Magnaporthe grisea* isolates and concluded that molecular polymorphism is largely independent of virulence polymorphism. These differences are expected because few genes for virulence and/or pathogenicity are responsible for pathogenic variation, whereas DNA markers like AFLP detect variability among the entire genomes (Andebrhan and Furtek 1994). However, four isolates from Rajasthan (Sg 381, Sg 382, Sg 383 and Sg 384) shared the same virulence as well as DNA fingerprinting group.

Our results confirm the occurrence of new virulences of *S. graminicola* in major pearl millet growing areas in India. However, differential set used in this study cannot further discern these virulences. Therefore, lines with diverse resistance genes/QTLs need to be identified as supplementary differentials. AFLP as well as pathotyping successfully detected the spatial and temporal variation in the *S. graminicola* population from different genotypes. However, association of DNA fingerprinting groups with the pathotyping could not be established. Therefore, allele-specific markers such as SSRs or markers designed for pathogenicity genes may be used to establish the relationship between pathotyping and molecular analysis.

References

Andebrhan T, Furtek DB. 1994. Random amplified polymorphic DNA (RAPD) analysis of *Crinipellis perniclosa* isolate from different hosts. Plant Pathol. 43:1020–1027.

Ball SL, Pike DJ. 1984. Intercontinental variation in *Sclerospora graminicola*. Ann Appl Biol. 104:41–51.

Botstein D, White RL, Skolnik M, Dawis RW. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphism. Am J Hum Genet. 32:314–331.

Excoffier L, Smouse P, Quattro L. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial restriction data. Genetics. 131:479–491.

Hash CT, Witcombe JR. 2001. Pearl millet molecular marker research. Int Sorg Mill Newsletter. 42:8–15.

Idris MO, Ball SL. 1984. Inter- and intracontinental sexual compatibility in *Sclerospora graminicola*. Plant Pathol. 33:219–223.

Jeger MJ, Gilijamse E, Bock CH, Frinking HD. 1998. The epidemiology variability and control of the downy mildews of pearl millet and sorghum, with particular reference to Africa. Plant Pathol. 47:544–569.

Jones ES, Breese WA, Liu CJ, Singh SD, Shaw DS, Witcombe JR. 2002. Mapping quantitative trait loci for resistance to downy mildew in pearl millet: field and glasshouse screens detect the same QTL. Crop Sci. 42:1316–1323.

Kolmer JA, Long DL, Hughes ME. 2006. Physiologic specialisation of *Puccinia triticina* on wheat in the United States in 2004. Plant Dis. 90:1219–1224.

Manici LM, Bonora P. 2007. Molecular genetic variability of Italian binucleate *Rhizoctonia* spp. isolates from strawberry. Eur J Plant Pathol. 118:31–42.

Mantel NA. 1967. The detection of disease clustering and a generalised regression approach. Cancer Res. 27:209–220.

Michelmore RW, Pawar MN, Williams RJ. 1982. Heterothallism in *Sclerospora graminicola*. Phytopathology. 72:1368–1372.

Nei M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics. 89:583–590.

Payne RW. 2002. The Guide to GenStat (R) Release 6.1. Part 2: Statistics. Oxford: VSN International Ltd.

Pushpavathi B, Thakur RP, Chandrashekara Rao K. 2006a. Fertility and mating type frequency in Indian isolates of *Sclerospora graminicola*, the downy mildew pathogen of pearl millet. Plant Dis. 90:211–214.

Pushpavathi B, Thakur RP, Chandrashekara Rao K, Rao VP. 2006b. Characterisation of *Sclerospora graminicola* isolates from pearl millet for virulence and genetic diversity. Plant Pathol J. 22:28–35.

Rohlf FJ. 2000. NTSYS-pc: Numerical Taxonomy and Multivariate Analysis System Version 2.2. Setauket. New York: Exeter Software.

Sastry JG, Ramakrishna W, Sivaramakrishnan S, Thakur RP, Gupta VS, Ranjekar PK. 1995. DNA fingerprinting detects genetic variability in the pearl millet downy mildew pathogen (*Sclerospora graminicola*). Theor Appl Genet. 91:856–861.

Sastry JG, Sivaramakrishnan S, Rao VP, Thakur RP, Singru RS, Gupta VS, Ranjekar PK. 2001. Genetic basis of host specificity in *Sclerospora graminicola*, the pearl millet downy mildew pathogen. Indian Phytopathol. 54:323–328.

Sastry JG, Sivaramakrishnan S, Thakur RP, Gupta VS, Ranjekar PK. 1997. Genome characterisation of *Sclerospora graminicola*, the causal fungus of downy mildew of pearl millet. World J Microbiol Biotechnol. 13:693–697.

Sharma TR, Chauhan RS, Singh BM, Paul R, Sagar V, Rathour R. 2002. RAPD and pathotype analyses of *Magnaporthe grisea* populations from the north-western Himalaya region of India. J Phytopathol. 150:649–656.

Sicard D, Michalakis Y, Dron M, Neema C. 1997. Genetic diversity and pathogenic variation of *Colletotrichum lindemuthianum* in the three centers of diversity of its host, *Phaseolus vulgaris*. Phytopathology. 87:807–813.

Singru R, Sivaramakrishnan S, Thakur RP, Gupta VS, Ranjekar PK. 2003. Detection of genetic variability in pearl millet downy mildew (*Sclerospora graminicola*) by AFLP. Biochem Genet. 4:361–374.

Sivaramakrishnan S, Thakur RP, Kannan S, Rao VP. 2003. Pathogenic and genetic diversity among Indian isolates of *Sclerospora graminicola* from pearl millet. Indian Phytopathol. 56:392–397.

Thakur RP, Pushpavati B, Rao VP. 1998. Virulence characterisation of single-zoospore isolates of *Sclerospora graminicola* from pearl millet. Plant Dis. 82:747–751.

Thakur RP, Rao VP. 1997. Variation in virulence and aggressiveness among pathotypes of *Sclerospora graminicola* on pearl millet. Indian Phytopathol. 50:41–47.

Thakur RP, Rao VP, Amrutesh KN, Shetty HS, Datar VV. 2003. Field surveys of pearl millet downy mildew – effects of hybrids, fungicide and cropping sequence. J Mycol Plant Pathol. 33:387–394.

Thakur RP, Shetty KG. 1993. Variation in pathogenicity among single-oospore isolates of Sclerospora *graminicola*, the causal organism of downy mildew in pearl millet. Plant Pathol. 42:715–721.

Thakur RP, Shetty KG, King SB. 1992. Selection for host-specific virulence in asexual populations of *Sclerospora graminicola*. Plant Pathol. 41:626–632.

Williams RJ. 1984. Downy mildew of tropical cereals. Adv Plant Pathol. 3:1–103.