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Characterization of downy mildew isolates of *Sclerospora graminicola* by using differential cultivars and molecular markers

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Abstract

Fourteen differential cultivar sets were used to analyze 27 downy mildew isolates of pearl millet, *Sclerospora graminicola* from different agroclimatic regions of India. Among 27 isolates, six distinct pathotypes were determined, the isolates were found to infect a narrow range of differential cultivars used and the disease incidence ranged between 0-98 per cent. Comparisons of RAPD and ISSR marker analyses were carried out on six *S. graminicola* pathotypes. A total of 20 random and 19 ISSR primers generated 152 and 297 bands and revealed 73.6 and 82.15% polymorphism which were able to distinguish the six pathotypes. In RAPD, maximum genetic distance index obtained was 0.1312 between pathotype-2 and pathotype-3 and minimum distance of 0.0231 was between pathotype-1 and pathotype-4. In ISSR 0.1302 was maximum between pathotype-4 and pathotype-6 and minimum distance was 0.0124 from pathotype-1 and pathotype-5. UPGMA dendrograms obtained from cluster analysis data gave similarity coefficient ranging from 0.27 to 0.88 and 0.40 to 0.83 in RAPD and ISSR, respectively. Clustering of six pathotypes within groups was not similar when RAPD and ISSR derived dendrograms were compared. RAPD and ISSR 3D scaling of ordination supported the UPGMA results and clarified relationships among six *S. graminicola* pathotypes.

Key Words**:** Pearl millet [*Pennisetum glaucum* (L.) R. Br.], *Sclerospora graminicola*, differential cultivars, DNA fingerprinting and molecular markers.

Sclerospora graminicola downy mildew izolatlarının farklı kültüvarlar ve moleküler markörler kullanılarak karakterizasyonu

Özet

Hindistan'ın farklı agroklimatik bölgelerinden seçilen 27 pearl millet, *Sclerospora graminicola* downy mildew izolatlarını analiz etmek için 14 farklı kultivar seti kullanılmıştır. 27 izolat arasında 6 farklı patotip belirlenmiştir. Bu izolatların kullanılan farklı kültüvarların bir kısmını enfekte ettiği ve hastalık insidansının yüzde 0 ila yüzde 98 arasında değiştiği bulunmuştur. Altı *S. graminicola* patotip ile RAPD ve ISSR markör karşılaştırma analizleri yapılmıştır. Toplam 20 rastgele ve 19 ISSR primerleri 152 ve 297 bantlarını oluşturmuştur, % 73.6 ve % 82.15 polimorfizmlerini açığa çıkarmıştır, 6 patotipi ayırt edebilmiştir. RAPD'de elde edilen maksimum genetik mesafe indeksi patotip-2 ve patotip-3 arasında 0.1312 ve minumum mesafe patotip-1 ve patotip-4 arasında 0.0231'dir. ISSR'da patotip-4 ve patotip-6 arasında 0.1302 maksimum değer, 0.0124 patotip-1 ve patotip-5 arasında minimum mesafe değeri olarak tespit edilmiştir. Grup analiz verilerinden elde edilen UPGMA dendogramlar RAPD için benzerlik katsayısını 0.27 ile 0.88 arasında, ISSR için benzerlik katsayısını 0.40 ila 0.83 arasında vermiştir. RAPD ve ISSR'dan elde edilen

dendogramlar karşılaştırıldığında 6 patotipin gruplar içinde gruplanması benzer değildir. RAPD ve ISSR 3D ölçeklendirmeleri UPGMA sonuçlarını desteklemiştir ve altı *S. graminicola* patotipi arasında ilişkiyi açıklamıştır.

Anahtar Sözcükler: Pearl millet [*Pennisetum glaucum* (L.) R. Br.], *Sclerospora graminicola*, farklı kültüvarlar, DNA parmakizi ve moleküler markörler.

Introduction

Downy mildew disease of pearl millet caused by the biotrophic, oomycete fungus *Sclerospora graminicola* [(Sacc). Schroet] is the major threat to pearl millet [(*Pennisetum glaucum* L.) R. Br.] productions resulting up to 80% yield loss as the grains are replaced by leaf-like structures in the earhead (Howarth and Yadav, 2002). The fungus is largely heterothallic (Michelmore et al., 1982; Idris and Ball, 1984), but homothallism may also exist (Michelmore et al., 1982). These characteristics of the fungus make it highly variable like its host, pearl millet, which is a highly outcrossing crop species. Pearl millet is the staple food for 90 million people of the semiarid tropics. In India, it is being grown in an area of 9.8 million hectares with an annual production of 7.01 million tones (Khairwal et al., 2004). In India, the monetary loss due to a single epidemic of downy mildew is estimated to be £7.8 million (Hash et al., 2003). Since 1981, several investigations have reported the presence of different pathotypes or races of *S. graminicola* in India, and to date, approximately 3 different pathotypes/races have been identified by (Shetty and Ahamad, 1981; Amruthesh, 2000). These studies have been sporadic, and no systematic characterization of S. *graminicola* pathotypes/races on a national scale has been carried out. Moreover, since different sets of differential cultivars were employed in each study, results are not directly comparable between the different reports. To overcome this problem, a group of 14 differential cultivars proposed from Indian Council of Agricultural Research (ICAR) and International Crop Research Institute for Semi-Arid Tropics (ICRISAT) under International Pearl Millet Downy Mildew Virulence Nursery (IPMDMVN) joint co-operation Programme system was initiated in 1976, so that the investigators working with *S. graminicola* allows comparison and compilation of data from different groups. Although differential cultivars are useful in determining pathotypes or races of plant pathogens, classification of pathogen population is often subjective, depending on the experience of the investigator. Mixed or segregating germ plasm and different growth and infection conditions can also exacerbate the problem. A possible solution is to combine data from differential cultivars with DNA based molecular marker analysis. Our interest is to study the variability of *S. graminicola* populations in India by combining analyses involving differential cultivars with molecular marker analysis. Isolates of the pathogen were obtained from geographically distinct regions of India in which commercial pearl millet cultivars are grown under very different conditions. The objective of the study were to i) Determine the variability of the pathogen populations in terms of pathotypes or races as defined by inoculation of differential cultivars; ii) Compare the variability of the *S. graminicola* isolates in pathotypes or races based on differential cultivars, and iii) Compare the efficiency of Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence repeats (ISSR) markers for classifying *S. graminicola* pathotypes.

Materials and methods

Field survey

Pearl millet crops were surveyed in the farmers' fields of the state of Karnataka, Hyderabad, Maha-

rashtra, Andra Pradesh, Gujarat, Delhi, Haryana, Rajasthan and Madhya Pradesh, India for recording downy mildew disease. A roving survey was planned in each crop season to examine the crop from the tillering to flowering stages. Pearl millet fields nearest to the road were observed, and in each field, five random microplots (2 rows x 5 m) with at least 50 plants/microplot were examined for downy mildew symptoms.

Isolation of S. graminicola population from field survey and maintenance

Downy mildew infected leaf samples from genotypes that showed downy mildew incidence over 2-3 years in farmers' fields were collected to obtain oospores inoculum. After generating the disease by oospore inoculum, the single leaf was used to multiply the inoculum on the respective host to maintain the uniformity in the asexual spores of S. *graminicola*. Twenty seven *S. graminicola* isolates prevailing across different geographic regions of India were selected for the present study. Isolates were maintained on respective host cultivars in greenhouses at Department of Applied Botany and Biotechnology, Mysore and ICRISAT, Patencheru (Table 1).

Characterization of S. graminicola isolates

Host differential cultivars

To distinguish the pathotypes of 27 different *S. graminicola* isolates, the set of differential cultivars to identify intraspecific variations in pathogenicity of isolates of *S. graminicola* proposed from ICAR-ICRISAT joint co-operation Programme through International Pearl Millet Downy Mildew Virulence Nursery (IPMDMVN) was used (ICAR, 1996). The set consists of 14 cultivars viz., HB3,

Table 1. Pearl millet downy mildew isolates across the pearl millet growing areas in India

Sl. No.	Identity of *S. graminicola* Isolates	Host cultivars	Place of collection
1	Sg 021	7042 S	Ahmednagar, Gujarat
2	Sg 048	852B	Mysore, Karnataka
3	Sg 139	Nokha local	Jodhpur, Rajasthan
4	Sg 139	IP 18292-BP	Jodhpur, Rajasthan
5	Sg 150	834B	Jalna, Maharastra
6	Sg151	Nokha local	Durgapura, Rajasthan
7	Sg 153	7042 S	Patancheru, Andhra Pradesh
8	Sg 200	ICMP-451	Jamnagar, Gujarat
9	Sg 212	ICMP-451	Durgapura, Rajasthan
10	Sg 298	W 504-1-1	Pusa, New Delhi
11	Sg 332	MLBH-267	Aurangabad, Maharastra
12	Sg 334- local	843B	Bhiwani, Haryana
13	Sg 335-HHB67	843B	Bhiwani. Haryana
14	Sg 348	ICMP-451	Anand, Gujarat
15	Pat-K1977	7042 S	Patancheru, Andhra Pradesh
16	Pat-K1987	7042 S	Patancheru, Andhra Pradesh
17	DM-11	HB3	Gulbarga, Karnataka
18	DM-39	Kalucombu	Mysore, Karnataka
19	DM-27	MBH110	Aurangabad, Maharastra
20	DM-46	7042S	ICRISAT, Andhra Pradesh
21	DM-79	MLBH104	Jalna, Maharastra
22	DM-2005	HHB-67	Hissar, Haryana
23	DM-86	Ekanath 201	Dhule, Maharastra
24	DM-93	Proagro-501	Jalgaon, Maharashtra
25	DM-90	GK1004	Ellora, Maharastra
26	DM-71	ICMH-451	Gwalior, Madhya Pradesh
27	DM-19	BJ104	Nandikotkur, Andhra Pradesh

Kalukombu, MBH110, 7042S, MLBH104, HHB67, ICMP451, 843B, 852B, 700651, P310-17, 1P18292, 1P18293 and H77/833-2 known resistant and susceptible pearl millet lines of diverse geographical origin as potential differential lines. The number or pathotype designation given to an isolate is determined by the cultivars of the differential set that are infected by that isolate.

Inoculation of differential cultivars

Sporangia from each isolate were harvested from infected leaves during early morning and incubated for 15 min in dark and the released zoospores were suspended at a concentration of 4 x 10^4 zoospores/ml and inoculated to two-day old seedlings sown in earthen pot (12-15 cm diameter) for three continuous days. 10 seedlings of each cultivar were inoculated in each experiment, and the whole experiment was repeated at least twice. Inoculated plants were grown under 95% humidity for 15 days and then evaluated for downy mildew disease symptoms, symptoms were evaluated on a four-level scale described as 0-5% = highly resistant, 5.1-10% = resistant, 10.1-25% = susceptible and 25.1-100% = highly susceptible.

Molecular characterization of six S. graminicola pathotypes

DNA isolation

Six pathotypes, which were identified based on host differential cultivars, were used for molecular characterization. Sporangia of six different pathotypes were harvested in a sterilized MIRA cloth dispensed in ice cold sterile deionized water and fungal DNA was extracted (Sastry et al., 1995).

PCR amplification and electrophoresis

Twenty RAPD primers of 10-base oligonucleotide and 20 microsatellite repeat primers (ISSR) was used for PCR amplification. Amplification of DNA was performed in 10mM of Tris-HCl pH 9.0, 50mM of KCl, 1.5mM of $MgCl_2$, 0.2 mM of dNTPs, 0.2µM of primer, 0.6 units of Taq DNA Polymerase enzyme (Bangalore Genei, Bangalore, India) and 50ng of DNA per 20 µl reaction using a

Table 2. Fingerprint patterns generated using the 20 RAPD 10-base oligonucleotide primers in six pathotypes of *S. graminicola*

Sl No	RAPD-Primers	Total no of bands amplified	No of polymorphous bands	PIC value (%)
1	OPA-05	06	05	83.3
2	OPA-07	07	05	71.4
3	OPA-11	05	05	100.0
4	OPA-14	08	06	75.0
5	OPA-15	08	06	75.0
6	OPB -02	06	04	50.0
7	OPB -03	09	06	44.4
8	OPB -06	07	05	71.4
9	OPB -09	12	12	100.0
10	OPB -12	07	07	100.0
11	OPC-07	09	06	66.6
12	OPC-10	07	06	85.7
13	OPC-12	07	05	71.4
14	OPD-09	09	06	66.6
15	OPS-02	06	06	100.0
16	OPS-09	08	06	75.0
17	OPL-18	08	06	75.0
18	OPM-04	07	06	85.7
19	OPN-20	10	09	90.0
20	OPX-09	06	05	83.3
	Total	152	112	73.6

Thermocycler (UNO II-BIOMETRA) for 40 cycles. PCR conditions included initial denaturation at 94^0 C for 4 min, each cycle comprised 1 min denaturation at 94^0 C, 1 min annealing at primer specific temperature (Table-3), 2 min extension at 72^0 C and a final extension for 10 min at 72^0 C.

Amplified products were mixed with bromophenol blue/xylene cyanol loading dye and were analyzed in an electrophoresis unit (Maxicell, EC-360 M) on 1.5-1.8% agarose gels using 1X Tris-Borate-EDTA buffer (pH –8.3) at 60-65 Volts. The gels were stained with ethidium bromide and then visualized under UV light.

Scoring of amplified bands/fragments

Each fragment that was amplified using RAPD and ISSR primers was treated as a unit character and scored in terms of binary code (1/0=+/-). Only reproducible bands were considered for scoring. Total number of fragments scored from 6 pathotypes by 20 primers were analyzed and tabulated.

Data analysis

To better understand the patterns of variation among isolates, clustering with Unweighted Pair Group with Arithmetic Average (UPGMA) was performed. The data was boot strapped to get 200 replicate data sets which were used to calculate Nei's genetic distance (Nei, 1978) with PHYLIP 3.6a3 statistical package (Felsenstein, 2002). A similarity matrix was constructed based on Dice coefficient (Dice, 1945) using the SIMQUAL program in the NTSYSpc, version 2.01 statistical package. Cluster analysis of the similarity matrix was performed with UPGMA using SAHN program in NTSYSpc, version 2.01. A graphical representation of the estimated genetic similarities between pathogen isolates was obtained by 3D plot on the basis of the similarity matrix as input using EIGEN program from NTSYS pc 2.01. The three-dimensional scaling was generated in order to highlight the resolving power of the ordination separately.

Results

Field survey

A total of 27 isolates which showed typical downy mildew incidence viz., cottony growth on the lower surface of the leaf, earhead malformation/leafy growth of the earhead, stunted growth with sporu-

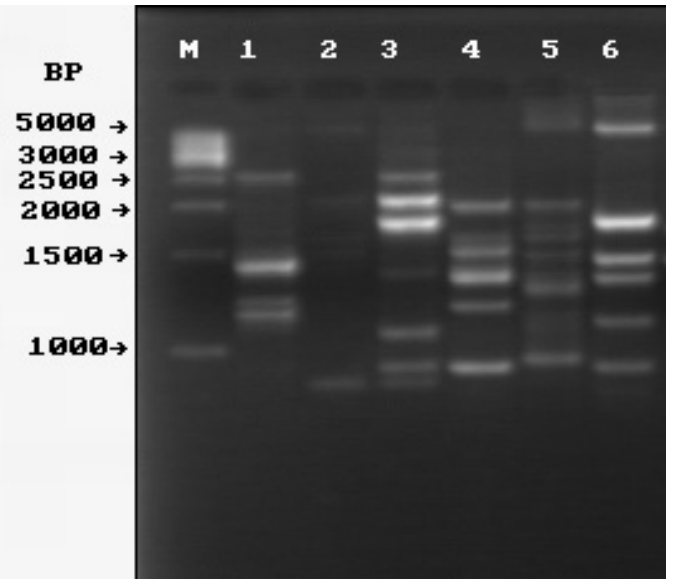

Figure 1. 1.8% agarose gel electrophoresis showing DNA fingerprint of 6 pathotypes of *S. graminicola* amplified with 10-base oligonucleotide OPB-09 produced maximum 12 bands with 100% polymorphism.

lation were collected from the respective field and tabulated (Table 1).

Infection of individual differential cultivars

Pathotype numbers give an indication of the variability of the pathogen in terms of pathogenicity. However, the occurrence of distinct pathotypes does not directly imply a wide range of resistance and avirulence interactions. To obtain more detailed information on these interactions, the number of isolates infecting each individual cultivar was determined and the results indicated that only 6 pathotypes were characterized based on infection of differential cultivars (Table 4). Analysis of the downy mildew disease incidence caused by six pathotypes on the host differentials revealed significant differences between the pathogenic populations and their reaction to the host cultivars. Diseased plants showed one or more typical downy mildew symptoms. The disease incidence ranged between 0-98 percent. Pathotype-1 and 4 were highly virulent on HB3 and 7042S, respectively and recorded 98% and 97% of disease incidence but the pathotype-4 does not infect or infects only a few plants of the cultivar 852B. Pathotype-1 was highly virulent on 5 host cultivars viz., HB3, 7042S, 843b, 852B and 700651 with disease incidence of 98, 78, 38, 66 and 27 % respectively, pathotype-2 was highly resistant to all the differential cultivars tested except for cultivar Kalukombu-a local land race cultivar which recorded 33 % disease incidence and this pathogenic population had no or very low virulence on the other host cultivars as it recorded 0 % incidence in cultivars HB3, MBH110, HHB67, 843B 852B, 700651, P310-17, IP18292, IP18293 and H77/833-2, while in ICMP-451 it was 7% and 4% in 7042S and MLBH-104 cultivars. Pathotype-3 was highly virulent on only one cultivar MBH110 and caused 85% disease incidence. Pathotype-4 was highly virulent on 4 host cultivars 7042S, HB3, 843B and H77/833-2 which recorded disease incidence of 97, 58 42 and 39 % respectively. The pathotype- 5 was highly virulent on 3 host cultivars and showed maximum disease incidence of 82 % on MLBH104, followed by 70 and 66 % disease incidence on ICMP-451 and 843B host differentials. Field surveys of pearl millet crops in Haryana during 2003–2005 indicated high incidence of downy mildew on several new hybrids and disease incidence varied considerably within and across sever-

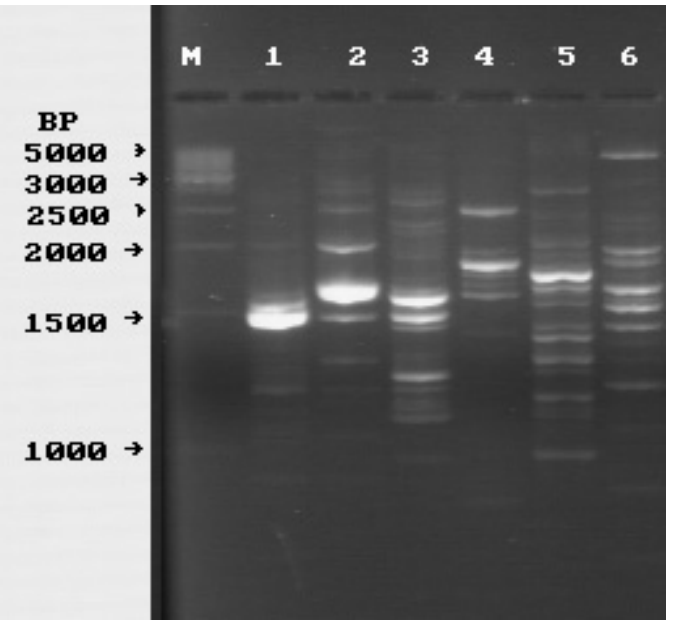

Figure 2. 1.8% agarose gel electrophoresis showing DNA fingerprint of 6 pathotypes of *S. graminicola* amplified with dinucleotide repeat 17899 B $(CA)_6$ GC produced maximum 19 bands with 100% polymorphism.

al cultivars. Virulence data indicate that emergence of a new isolate specific to HHB67, which has been most popular public sector hybrid and occupies over half of the pearl millet in Haryana and also grown in Rajasthan and Madhya Pradesh. Virulence pattern of *S. graminicola* population suggested that the isolate from HHB67 genotype could be different from existing genotypes. This new HHB67 isolate was grouped as pathotype-6 based on host differential reaction. The pathotype-6 showed maximum disease incidence of 65 % on HHB67 host differential and recorded susceptible disease reaction of 15 and 13 % on MLBH-104 and H77/833-2 cultivars.

Out of 27 asexual zoospores inoculated to host differential cultivars, two of the 14 differential cultivars were highly susceptible to at least twelve *S. graminicola* isolates. The percentage of isolates tested from all sites that were pathogenic on the individual pearl millet cultivars varied from 26 to 98%. Thirteen isolates were found to be highly virulent on differential cultivar HB3 ranging from 27 to 98% disease, twelve asexual isolates was highly virulent on cultivar 7042S, disease incidence ranging from 29 to 97% disease incidence. Six isolates showed virulent nature on MBH-110 and MLBH-104 differential cultivars, three isolates were highly virulent on HHB67, all the 27 asexual isolates showed resistant reaction on differential cultivar IP-18292 and IP-18293, except for isolate viz., Sg139a which recorded 11 % disease incidence on IP-18292.

Identification of pathotypes of S. graminicola

A summary of the pathotypes found in each state and the number of isolates corresponding to each pathotype are presented (Table 5). From the 27 isolates analyzed to 6 pathotypes, eight isolates viz., Sg-21, Sg-48, Sg-153, Sg-200, Sg-212, DM-11, DM-86 and DM-90 were classified as pathotype-1, five isolates viz., Sg-348, Pat-K-1977, DM-46, DM-71 and DM-19 were grouped in pathotype-4, isolates Sg-298, Pat-K-1987, DM-79 and DM-93 were grouped in pathotype-5, three isolates were classified in pathotype-3 and pathotype-6 respectively, whereas isolate DM-39 was grouped in pathotype-2 based on virulence assessment on 14 differential cultivars (Table 6).

Table 3. Fingerprint patterns generated using the 20 ISSR repeat primers in 6 pathotypes of *S. graminicola*

Sequence	ISSR Primer	Annealing Temp (^{O}C)	No. of bands generated	No. of polymorphous bands	PIC value (%)
$(CT)_8TC$	814	40	15	12	80.0
$(CT)_8AC$	844 A	40	14	11	78.5
$(CT)_8GC$	844 B	40	15	15	100.0
$(CA)_6AC$	17898 A	45	15	13	86.6
$(CA)_6GT$	17898B	45	14	12	85.7
$(CA)_6AG$	17899 A	45	18	15	83.3
$(CA)_6GC$	17899 B	45	19	19	100.0
$(GA)_6GG$	HB 08	48	16	12	75.0
$(GT)_6GG$	HB 09	48	14	11	78.5
$(GA)_6CC$	HB 10	48	17	14	82.3
$(GT)_6CC$	HB 11	48	16	13	81.2
$(CAC)_3GC$	HB 12	45	18	14	77.7
$(GAG)_3GC$	HB 13	45	14	11	78.5
$(CTC)_3GC$	HB 14	45	13	13	100.0
$(GTG)_3GC$	HB 15	48	18	13	72.2
$(GA)_9T$	ISSR-16	48	14	11	78.5
$(GA)_9C$	ISSR-17	48	16	12	75.0
$(TAG)_4$	ISSR-18	40-55	00	00	00
$(GACA)_4$	ISSR-19	48	14	10	71.4
$(GGAT)_4$	ISSR-20	50	17	13	76.4
			297	244	82.15

Fingerprinting of S. graminicola pathotypes by R APD and ISSR

The six pathotypes namely DM-11, DM-39, DM-27, DM-46, DM-79 and DM-2005 were analyzed with 20 RAPD 10-base oligonucleotide primers. Scoring of bands was carried out considering the dark and prominent bands. However, bands of lower intensity but with high reproducibility were also included in analysis (Table 2). Of the 20 RAPD primers tested for polymorphism with the 6 pathotypes, a total of 152 bands were obtained from PCR amplification with 20 primers using template DNA from 6 pathotypes. Subsequent scoring of the bands revealed 73.6% polymorphism between *S. graminicola* pathotypes. Primer OPB - 09 produced a maximum of 12 bands showing 100% polymorphism (Figure 1), followed by 10

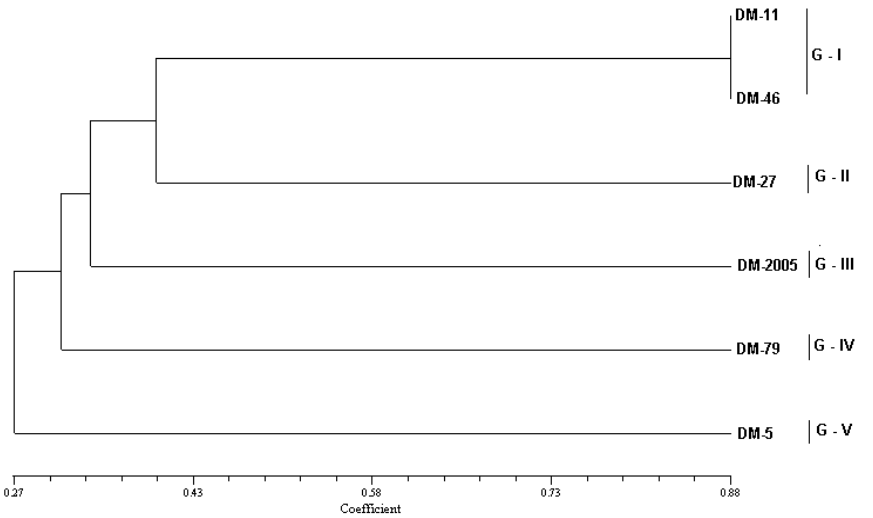

Figure 3. Dendrogram based on RAPD polymorphisms of six pathotypes of *S. graminicola* of Pearl Millet by Un-weighted pair group method of averages (UPGMA) cluster analysis.

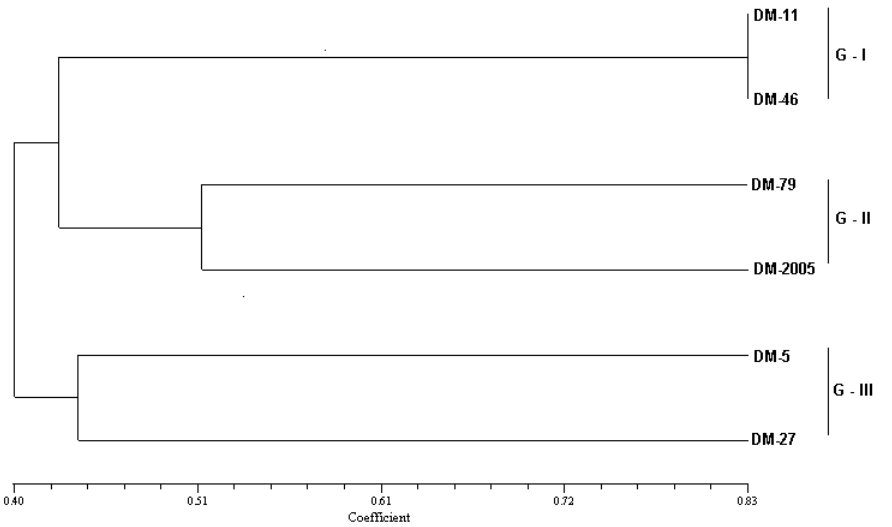

Figure 4. Dendrogram based on ISSR Polymorphisms of Six pathotypes of *S. graminicola* of Pearl Millet by Un-weighted pair group method of averages (UPGMA) cluster analysis.

bands by OPN-20.

The microsatellite primers used included seventeen 3' anchored, one tri nucleotide and two tetra nucleotide microsatellite repeat primers (ISSR). Scoring of bands was carried out considering the dark and prominent bands. However, bands of lower intensity but with high reproducibility were also included in analysis (Table 3). Out of the 20 ISSR primers screened, trinucleotide primer-18 $(TAG)_4$ failed to amplify the template DNA at different annealing temperatures during repeated trials. A total of 297 bands were obtained from PCR amplification with 19 primers using template DNA from six pathotypes. Subsequent scoring of the bands revealed 82.15% polymorphism between *S. graminicola* pathotypes. The results indicated that ISSR fingerprints of *S. graminicola* detected a high level of diversity among the isolates and that ISSR markers could be a powerful alternative for fingerprinting and diversity analysis. Dinucleotide repeat 17899 B $(CA)_6$ GC produced a maximum of 19 bands showing 100% polymorphism (Figure 2), followed by 18 bands by trinucleotide repeat 17899 A $(CA)_6$ AG and HB 15 $(GTG)_3$ GC. Further, a unique band of approximately 600 bp band was detected in isolates Sg 048, Sg 153, Sg 212, DM-11 and DM-90 from PCR amplification with Dinucleotide Repeat 17898 A $(CA)_6$ AC which was found to be associated to *S. graminicola* pathotype-1.

Genetic distance was calculated for RAPD and ISSR fingerprints obtained to establish the relatedness between the isolates. In RAPD, the maximum distance index obtained was 0.1312 between pathotype-2 and pathotype-3, which originated from Mysore, Karnataka and Aurangabad, Maharashtra respectively. The minimum distance index value of 0.0231 was obtained between pathotype-1 and pathotype-4 of Gulbarga, Karnataka and ICRISAT, Andhra Pradesh respectively, while in ISSR 0.1302 was maximum between pathotype-4 and pathotype-6 from ICRISAT, Andhra Pradesh and Hissar, Haryana and minimum distance of 0.0124 from pathotype-1 and pathotype-5 of Gulbarga, Karnataka and Dhule, Maharashtra. The distance matrix based on RAPD and ISSR data sets was used to construct a dendrogram. The UPGMA Dendrogram obtained from the cluster analysis of RAPD and ISSR data gave similar clustering pattern, with similarity coefficient ranging from 0.27 to 0.88 and 0.40 to 0.83 in RAPD and ISSR respectively.

The dendrogram based on RAPD showed some variation in the clustering of the *S. graminicola*

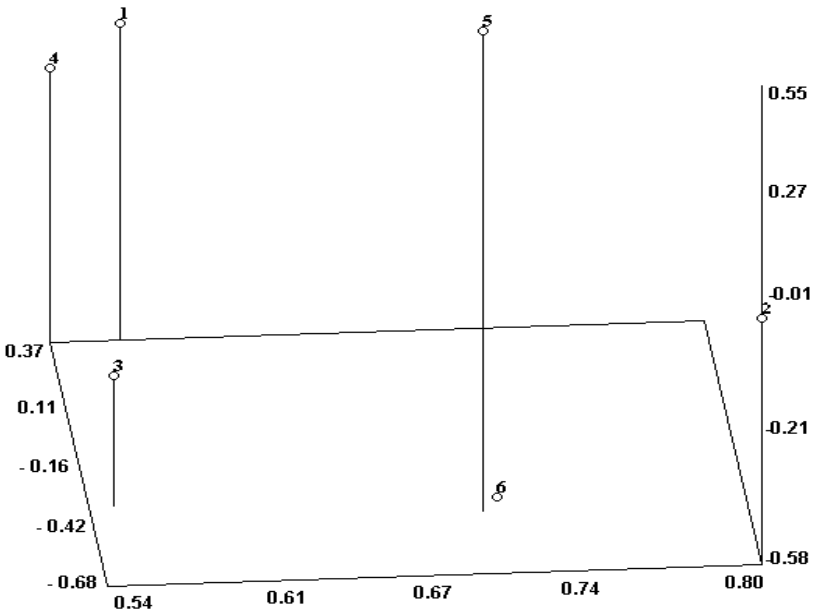

Figure 5. Three-dimensional scaling generated by NTSYS of six pathotypes of *S. graminicola* based on the combination of data obtained with 20 RAPD primers.

pathotypes. Clustered with six pathotypes into five groups (Figure 3), DM-11 (pathotype-1) and DM-46 (pathotype-4) are grouped together in Group I, while DM-27 (pathotype-3), DM-2005 (pathotype-6), DM-79 (pathotype-5) and DM-39 (pathotype-2) formed separate distinct groups (II-V). Clustering of ISSR pattern revealed major three groups DM-11 (pathotype-1) and DM-46 (pathotype-4) are grouped together in Group I, Group II consisted of DM-79 (pathotype-5) and DM-2005 (pathotype-6), while DM-39 (pathotype 2) and DM-27 (pathotype 3) placed in Group III (Figure 4).

Three-dimensional scaling

The three Dimensional ordination confirms the cluster analysis is one of the multivariate approaches of grouping based on the similarity coefficient values of the component traits of the entities. RAPD and ISSR 3D scaling ordination supported the UPGMA results and clarified relationships among six *S. graminicola* pathotypes (Figure 5 and 6).

Discussion

Traditionally, pathogenic variability has been assessed by morphological markers or through virulence surveys that use a set of host-differentials containing different resistant genes (Thakur et al., 1998). In the past decade, there has been a dramatic increase in the studies on genetic variations in populations of plant pathogens. A basic requirement for these studies is the availability of genetic markers that can be used to assay variations among individuals. The types of markers that have been used have progressed first from virulence and fungicide resistance, to mating types, isozymes and vegetative incompatibility (Michelmore et al.,

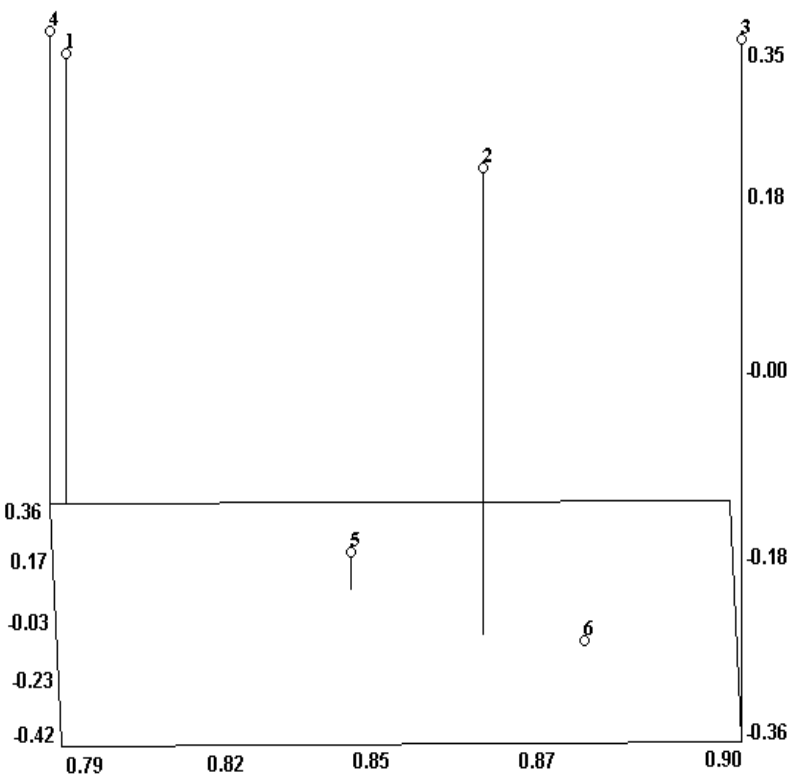

Figure 6. Three-Dimensional scaling generated by NTSYS of six pathotypes of *S. graminicola* based on the combination of data obtained with 20 ISSR primers.

1988). The potential for development of new races/pathotypes in the pathogen population makes it mandatory to monitor the pathogen population regularly to detect changes in virulence. Field survey data are valuable to estimate the relative prevalence and distribution of pathotype, to monitor the origin and spread of new virulent pathotype, and to detect shift towards virulence to resistance used in commercial genotypes. The information thus developed can be used in breeding programs before the new race/pathotype becomes widespread. Six distinct pathotypes were determined in the 27 isolates examined in this study. In Karnataka pathotype-1 and pathotype-2 was found, in Maharashtra, pathotype-3 and 5 were found, pathotype-4 was found in Andra Pradesh and pathotype-6 was found in Haryana. Pathotype-1, 2, 3, 4 and 5 was distributed in southern region of India, whereas pathotype-6 was from northern geographic region of India. The differences in the pathogen populations between the two regions probably reflect the differences in germplasm used the agricultural practices employed in each region (Thakur et al., 1999). The pearl millet cultivars grown in Karnataka and Andra Pradesh, mixture of land races are grown in combination with maize. In Maharashtra and Haryana, popular hybrids are grown to meet the needs of the market. It is also interesting to note that variability of the pathogen in the southern regions, where in a group of 14 isolates, 5 distinct pathotypes were found, whereas in Haryana where only one or two main HHB cultivars has been grown for many years, one type of pathotype-6 was identified. In the southern regions, the mixture of land races in close proximity to the cultivated forms may lead to an increase in the pathogen diversity in this region. Virulence to host differential lines has been the traditional means of assessing genetic variation in plant pathogens and knowledge on the changing genetic structure of pathogen population will facilitate management of diseases (Thakur and Mathur, 2002). The 6 distinct pathotypes in this study infect only six of the IPDMVN differential cultivars, pathotype-1 from Karnataka infects 7 differential cultivars at varied level of downy mildew disease incidence, remaining 5 pathotypes infected at least two to five of the fourteen differential cultivars examined, except one isolate DM-39 from Karnataka classified as pathotype-2 infects only one differential cultivar Kalukombu. Based on the host cultivar differentials variations in the pathogenic populations of *S. graminicola* were first reported by Shetty and Ahamad (1981) who have shown that pathogenic population-infecting 23A based hybrids such as the HB3

Table 4. Reactions of host differentials to Six different pathotypes of *S. graminicola*. Values are means of four independent replications based on total and diseased plants. Highly resistant (≤10%) and highly susceptible (≥25%).

HOST DIFFERENTIAL CULTIVARS	Percentage downy mildew disease incidence pathotypes					
	1	**2**	**3**	**4**	**5**	6
HB3	**98**	00	09	58	00	**02**
KaluKombu	00	**33**	02	00	00	**00**
MBH110	08	00	**85**	07	09	**00**
7042S	78	04	09	**97**	06	**03**
MLBH104	03	04	00	02	**82**	**13**
HHB67	00	00	02	04	03	65
ICMP451	15	07	00	06	70	**00**
843B	38	00	09	42	66	**02**
852B	66	00	08	21	10	**00**
700651	27	00	07	26	03	**05**
P310-17	16	00	07	12	09	**07**
1P18292	06	00	00	02	02	**00**
IP18293	04	00	00	04	02	**00**
H77/833-2	03	00	04	39	00	**15**

and also inbred lines like the 852B has been referred to as pathotype-1. The pathogenic population of *S. graminicola* infecting the local cultivar Kalukombu and not infecting most of the elite hybrids has been considered as pathotype-2. Shetty and Ahamad (1981) have also successfully differentiated these two pathotypes of *S. graminicola* on the basis of differences in several morphological features, differences in the number of nuclei per sporangium, seed-borne nature and soluble protein apart from Pathogenecity tests. It was demonstrated that in pearl millet, oospores are produced in young seedlings when compatible isolates are inoculated simultaneously (Michelmore et al., 1982). This indicated that the primary determinant of oospore production is the coexistence of compatible mating types in the same zones of the host tissue and *S. graminicola* is heterothallic. Heterothallic downy mildews require the presence of two mating types to form oospores. The significance of heterothallism in the epidemiology is however uncertain as homothallic isolates also occur. Homothallism also existed in downy mildew pathogen in which a nucleus within the same thallus can fuse with another nucleus of same mycelium (i.e., homokaryosis). Second mating type is not required for sexual reproduction. Self-fertile; a dikaryon or diploid develops from single meiospores.

A compilation of data from other reports in which the IPDMVN differential sets was used 9 of 15 isolates did not infect either 700561, IP-18293 and IP-18292. Two of the five isolates that did not infect, did infect HB3 and 7042S. The genetic basis of resistance in IP-18293 and IP-18292 is unknown but it is expected to be different from those of P 7-4 and P310-17, since isolates specifically infecting IP lines have been observed (Thakur Amruthesh, 2000; *et al.,* 2003). The preferential infection of the 14 differential cultivars probably reflects the adaptation of the pathogen within India to overcome resistance genes to which it is most commonly exposed. This is probably also true of ICMR-01004 and ICMR-01007 cultivars which is derived from H77/833-2 and ICMP-451 lines and contains QTLs resistant genes (Qi et al., 2004). A recent study on selection of host cultivar specific virulence in Oomycete populations has provided evidence for the occurrence of a gene for gene relationship in many host pathosystems (Kamoun, 2003).

Molecular markers are especially useful in biotrophic pathogens, where little is known on the genetic make up of a phytopathogenic Oomycete. Direct analysis of polymorphism in DNA has recently emerged as an important tool that can provide useful information on variation both within and between species. Molecular variability studied in the pathotypes of *S. graminicola* is new to literature and these studies clearly established the classification of races/pathotypes described.

Molecular markers such as RAPD are widespread in their distribution throughout the genome having higher degree of polymorphism (Cramer et al., 2003). However, ISSR markers span short chromosomal sequence between SSR's and hence are easily assayable than the RAPD markers (Parsons et al., 1997). In addition, ISSR markers also mark gene rich regions as in the case of virulence of a pathogen especially with respect to pathogenic oomycetes. Most of the ISSR markers identified are randomly distributed in their respective genomes and high level of polymorphism observed in their study using ISSR primers is due to variation in the number of tandem repeat motif at a specific locus (Sharma et al., 2005).

Table 5. Pathotype grouping of *S. graminicola* isolates studied based on host differentials

Isolate number	Place of Origin	Host differential cultivar	Designated Pathotype
DM-11	Gulbarga, Karnataka (DMRL)	HB3	1
DM-39	Mysore, Karnataka	Kalukombu	2
DM-27	Aurangabad, Maharashtra	MBH110	3
DM-46	ICRISAT, A.P.	7042S	4
DM-79	Dhule, Maharashtra	MLBH104	5
DM-2005	Hissar, Haryana	HHB-67	6

The results presented here also demonstrate the efficiency of molecular markers for studying *S. graminicola* populations. Where 20 RAPD primers gave a total of 145 band and 20 ISSR primers produced 297 bands, a double fold differences. Correlation analyses carried out between the two data sets showed very low similarities obtained from RAPD data and those from ISSR data. This may result from the fact that ISSR markers are more widely dispersed throughout the genome. Although 6 pathotypes were determined for the 27 isolates, a much higher diversity was observed at the genotype level, The evolution of isolates from different agro climatic zones demonstrated significant variation levels of polymorphism among 27 isolates of *S. graminicola* in RAPD (73.6%) and ISSR (82.15%) markers. The number of total polymorphic and discriminant fragments is higher for ISSRs than RAPD. The unique band of approximately 600 bp band detected from ISSR primer 17898 A $(CA)_6$ AC was found to be associated to *S. graminicola* pathotype-1. It can be speculated that the isolates with distinct DNA profiles are likely to contain the highest number of novel alleles. Therefore the identity of the unique amplicon can be a useful marker for detection/linked with pathotype specific markers (Perez et al., 2000; Radisek et al., 2004; Travensolo et al., 2005; Usami et al., 2007). Clustering of isolates within groups was not similar when RAPD

Table 6. Host differentials Reactions of 27 downy mildew isolates of *S. graminicola* to six pathotypes. values are means of four independent replications based on total and diseased plants. Highly resistant (≤0%) and highly Susceptible (≥ 25%)

***S. graminicola* isolates**	**Percentage downy mildew disease incidence of 27 isolates of *S. graminicola* to six pathotypes**						**Pathotypes assigned**
	HB3	Kalukombu	MBH110	7042S	MLBH104	HHB-67	
Sg 021	45	**00**	**01**	**37**	**01**	**00**	Path-I
Sg 048	91	**00**	**08**	**68**	**03**	**00**	Path-I
Sg 139	04	**00**	**31**	**34**	**00**	**16**	ND
Sg 139a	09	**00**	**16**	**09**	**00**	**05**	ND
Sg 150	**06**	**00**	**54**	**08**	**00**	**02**	Path-III
Sg151	**00**	**00**	**29**	**34**	**00**	**11**	ND
Sg 153	75	**00**	**01**	**57**	**02**	**00**	Path-I
Sg 200	31	00	01	**29**	**03**	**01**	Path-I
Sg 212	60	00	04	**44**	**03**	**01**	Path-I
Sg 298	03	00	00	**02**	**29**	**09**	Path-V
Sg 332	07	00	32	**07**	**00**	**01**	Path-III
Sg 334- local	10	00	00	**00**	**40**	**47**	Path-VI
Sg 335-HHB67	07	00	00	**02**	**27**	**59**	Path-VI
Sg 348	**30**	**00**	**04**	**44**	**00**	**01**	Path-IV
Pat-K1977	**27**	**00**	**04**	**11**	**03**	**00**	Path-IV
Pat-K1987	**00**	**00**	**09**	**06**	51	01	Path-V
DM-11	98	00	08	78	03	00	Path-I
DM-39	00	33	00	04	04	00	Path-II
DM-27	09	02	85	09	00	02	Path-III
DM-46	58	00	07	97	02	04	Path-IV
DM-79	00	00	09	06	82	03	Path-V
DM-2005	02	00	00	03	13	65	Path-VI
DM-86	77	00	04	53	01	03	Path-I
DM-93	00	00	10	05	44	09	Path-V
DM-90	55	00	03	49	00	00	Path-I
DM-71	27	00	00	42	02	00	Path-IV
DM-19	45	01	09	66	00	00	Path-IV

* ND – Not determined

and ISSR derived dendrograms were compared. These differences may be attributed to marker sampling error or the level of polymorphism detected. In general, when a comparison is carried out between various molecular marker systems it has been evident that ISSRs represent an efficient and suitable marker system for discriminating among closely related pathogenic isolates like *Phytophthora* and *Pythium* (Cooke and Lees, 2004). Our study also demonstrates the discriminatory power of ISSR to clearly distinguish and separate oomycetes pathogen *S. graminicola* populations.

By extending the analysis to include *S. graminicola* isolates from other regions of the country, we hope to further test the relationship between haplotype and pathotype groups. A method to predict the pathotype group of new isolates on the basis of the present study would greatly facilitate the analysis of *S. graminicola* populations within India and could be extended to include populations from other regions. We conclude that the combined strategy of inoculating differential cultivars and using molecular markers is extremely efficient for the study of populations of *S. graminicola*. The results obtained suggest that isolates of *S. graminicola* can be separated into genetic basis of molecular markers and groups of haplotypes can be correlated to pathotype groups. These strategies will restrict the development of virulent populations for a longer time and provide durable control of the disease in the pearl millet growing areas.

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