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Morphological, pathogenic and genetic variability amongst sorghum isolates of *Colletotrichum graminicola* from India

J. LATHA[1], KUSUM MATHUR[2], P.K. MUKHERJEE[1]*, A. CHAKRABARTI[1], V.P. RAO[3] and R.P. THAKUR[3]
[1]*Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Trombay, Mumbai 400 085*
[2]*Department of Plant Pathology, College of Agriculture, Udaipur 313 001*
[3]*Genetic Resources and Enhancement Program, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru 502 324*

ABSTRACT: A total of 18 isolates of *Collectotrichum graminicola* infecting sorghum in Andhra Pradesh and Maharashtra, India, were characterised and compared for morphological, pathogenic and genetic diversity. The representative single-lesion isolates and their derivatives varied significantly for morphological traits - colony colour, growth pattern and sporulation on oat meal agar medium. These isolates also exhibited significant variation for disease reaction on a set of sorghum differential lines in greenhouse tests. The isolates from the local sorghum cultivars were although different from those of CSH 9, they did infect CSH 9 indicating a virulence shift in the *C. graminicola* population from the local sorghum to hybrid CSH 9. Genotypic variation was studied using the random amplified polymorphic DNA (RAPD) technique. A set of six random primers could differentiate the isolates. The cluster analysis indicated a very high genetic variability among isolates of C. *graminicola* and amogg variants from a single lesion isolates in RAPD profile.

Key words: *Colletotrichum graminicola,* sorghum, pathogenic variability, virulence, RAPD

Colletotrichum graminicola (Ces) Wils., which causes anthracnose of sorghum *(Sorghum bicolor* (L.) Moench), is a highly variable pathogen (Warren, 1986; Ali and Warren, 1987; Casela and Ferreira, 1995; Thomas, 1995). Based on differential reaction on sorghum lines, 44 races/pathotypes have been reported from different countries- Brazil (Ferreira and Casela, 1986; Nakamura, 1982), northern Nigeria (Ozolua *et al.,* 1986), US and Puerto-Rico (Ali and Warren, 1987; Cardwell ef a/., 1989), India (Pande *et al.,* 1991) and western Africa (Thomas, 1995). tt is well recognized that pathogenic variability poses difficulty in development and deployment of effective "host resistance, which is a dependable and economic means of disease management. in India, several sorghum hybrids with multiple disease resistance are cultivated commercially, and CSH-9 is one of the most popular commercial hybrids widely grown in several states. A number of leaf samples infected with anthracnose were collected from CSH-9 and local sorghum cultivars during surveys of farmers' fields in Andhra Pradesh and Maharashtra. This study was conducted to determine the comparative morphological, pathogenic and genetic diversity among the *C. graminicola* populations to understand the evolution of new virulence in this pathogen.

MATERIALS AND METHODS

The pathogen isolates

During field surveys in the rainy seasons 1990-1995 a large number of anthracnose-infected leaf samples were collected from several fields of hybrid CSH-9,. and local sorghum cultivars (LSC) in Maharashtra and from few LSC and breeding lines in Andhra Pradesh. The leaf samples were blotter dried and stored at 4°C until used. The

*Author for correspondence
E-mail; prasunm@apsara.barcernet.in

samples were visually examined for typical anthracnose lesion types and three representative samples with typical anthracnose lesions from CSH-9, one from LSC from Maharashtra, and four samples from Andhra Pradesh (from LSC or breeding lines) were selected for the study. From each collection 20 single-lesion leaf bits (2 mm x 2 mm) were cut, surface sterilized with 0.1% $HgCl_2$ solution for 2 min, rinsed twice in sterilized distilled water and aseptically transferred on to 2% oat meal agar (OMA). The cultures were incubated at 25°C under continuous fluorescent light for 7 days. A single fastest growing isolate was selected from each of the 20 cultures of each collection for further evaluation. When grown on OMA plates at 25°C for 7 days, several morphological variants from the five single-lesion isolates (Cg 10, Cg 42, Cg 52, Cg 88 and Cg 89) were identified and these were maintained on OMA. Thus a total of 18 isolates, including single lesion isolates and their morphological variants were analysed. The isolates were designated, as: Cg 10B and Cg 10C (Shadnagar, Mahbubnagar, Andhra Pradesh, from a local cultivar), Cg 22 (Vadalgi, Sholapur, Maharashtra, from CSH-9), Cg 42A and Cg 42B (Patancheru, Medak, A.P. from breeding line), Cg 52A and Cg 52B1 (Kottakota, Mahbubnagar, A.P. from local cultivar), Cg 88, Cy 88B, Cg 88C, Cg 88D, Cg 88E (Boti Bori, Nagpur, MS from CSH-9), Cg 89, Cg 89B1, Cg89B2 (Limba, Nagpur, MS from CSH-9), Cg 93 (Kolambi, Yawatmal, MS from local cultivar) and Cg 131A (Patancheru, Medak, A.P. from H112).

Morphological variations

Mycelial plugs (5-mm diameter) were removed from the advancing margins of 7-day-old cultures of each isolate and transferred onto fresh OMA in petriplates (90 mm diameter). Three replicates of each isolate were maintained and incubated for 10 days at 25°C with 12 h photoperiod. The colony morphology wais assessed for growth characteristics, mycelial type, color, acervuli, number of setae, appressoria and sclerotia.

Pathogenic variability

Seven sorghum lines Were used as host differentials for assessing the pathogenic variability of the isolates. Seeds were surface-sterilized with 0.1% $HgCl_2$ for 3 min, washed thoroughly with distilled sterilized water, dried at room temperature (25°C), and were sown in 18 cm square plastic pots filled with autoclaved potting mixture (black soil: sand: farmyard manure, 3:2:2 v/v/v). Five plants per pot and each plant as a replicate was used for each sorghum line and the pathogen isolate. The isolates were grown at 25°C in 2% oat meal broth for 5 days and the conidia separated by filtering through a double-layered muslin cloth. Twenty-one day-old plants (5-6 leaf stage) of each line were spray-inoculated, using atomisers, with an aqueous spore suspension ($1x10^5$ Conidia /ml) containing Tween-20 (1 ml/l) with each of the 18 isolates. Polyethylene sheets were used to separate plants when inoculating with different isolates to eliminate inoculum drift The plants were air-dried and kept in a humidity chamber (>95% RH) for 24 h. The plants were then transferred to a greenhouse at 25 ± 2°C. The experiment was repeated once to confirm the results. The plants were scored for disease reaction types: R (resistant- no symptoms, hypersensitive lesions or chlorotic flecking), MR (moderately resistant- red spots or necrotic spots without acervuli) and S (susceptible- lesions with acervuli).

Genetic variability

Total genomic DNA from the fungal isolates was extracted according to Mukherjee (1999).The fungus was grown on 2% oatmeal broth at 25°C. with 100 rpm shaking, for 4 days. The mycelium *was* collected *on* sterile Whatman No. 1 *filter* papers, blotted dry, and ground to fine powder in liquid nitrogen, using a pestle and mortar. Rest of the procedure was the same as described earlier (Mukherjee 1999). DNA was finally suspended in sterile water. For RAPD analysis, six random primers from kit A of Operon Technologies, Inc. (OPA1-CAGGCCCTTC; OPA2-TGCCGAG.CTG; OPA5-AGGGGTCTTG; OPA9-CGGTAACGCC; OPA10-GTGATCGCAG; OPA15-TTCCGAACCC) were. used. These primers were selected after a primary screening of all the 20 primers from kit A. For amplification, 50 ng of DNA, 0;2 mM primer, 0.1 mM each dNTPs and 1.0 U Taq polymerase (Genei) were used. The denaturation temperature was 94°C for 1 min and extension temperature 72°C for 2 min. An annealing temp. of 37°C (for 1 min), which is used normally for RAPD, was used except for primers OPA1, OPA2, OPA9 and

OPA15, which have higher thermodynamic Tm, 40°C was used. This modification improved the results by eliminating the non-specific and non-reproducible bands'. The amplified products were size separated on 1.5% agarose gel, stained with ethidium bromide and photographed using a polaroid camera. The bands were scored as 1 (if present) or 0 (if absent), data analyzed using TreeconW software (Yves Van de Peer, Department of Biochemistry, University of Antwerp, Belgium) and a phylogenetic tree constructed.

RESULTS

Morphological variations

The isolates exhibited variable colony characteristics, such as mycelial growth, color, presence or absence of acervulus, number of setae, appresorium and sclerotium on OMA plates at 25°C (Table 1). Distinct differences were recorded for the formation of acervulus, appresorium and sclerotium within single lesion isolates and their derivatives. Mycelial growth varied from profuse raised to raised feity, and submerged wooly. Colony color varied from greyish white to lilac-grey, white and grey; and acervulus formation was either distinct or indistinct A large variation was recorded for the number of setae formed in each acervulus and it ranged, from 1-4 in Cg 88 and its derivatives to 20-30 in Cg 89B-2. Isolates Cg 10B and 10C were similar for mycelial growth and color, but varied for acervulus, number of setae and appresorium. Cg 22 had white felty growth and abundant sporulation, and Cg 93 had. greyish white felty growth. Isolate Cg 88 had lilac-white-grey colony with several appressoria and sclerotia. Appressoria and sclerotia were also present in Cg 22, Cg 89 and Cg 93. Of the four derivatives of Cg 88, Cg 88E had distinct acervuli, but no appressoria or sclerotia; Cg 88B had both appressoria and sclerotia; Cg 88C had lilac white colony and no appressoria/ sclerotia; and Cg 88D had grey colony and no appressoria. Three of the

Table 1. Morphological and cultural variations among Isolates of *Colletotrichum graminicola,* 10 days after incubation on oat meal agar at 25°C.

Isolate designation	Mycelial growth	Color	Acervulus	No. of setae/ acervulus	Appressoria	Sclerotia
Cg 10B	Profuse, raised	Greyish white	Distinct	2-3	+	-
Cg 10C	Profuse, raised	Greyish white	Indistinct	5-6	-	-
Cg 22	Raised, felty	White	Distinct	2-3	+	+
Cg 42A	Raised, felty	White	Distinct	1-2	+	-
Cg 42B	Raised, felty	White	Distinct	2-3	+	-
Cg 52A	Raised, wooly	Lilac-grey	Distinct	2-10	-	-
Cg 52B1	Raised wooly	Lilac-grey	Distinct	2-4	+	-
Cg 88	Raised, felty	Lilac- white grey	Distinct	1-4	+	+
Cg 88B	Raised, felty	Grey	Indistinct	1-2	+	+
Cg 88C	Submerged, felty	Lilac-white	Indistinct	1-2	-	-
Cg 88D	Raised, felty	Grey	Indistinct	1-2	-	-
Cg 88E	Submerged, felty	White-grey	Distinct	3-4	-	-
Cg 89	Raised, felty	Greyish white	Distinct	1-30	+	+
Cg 89A	Raised, felty	Greyish white	Indistinct	1-2	+	-
Cg 89B-1	Submerged, wooly	Grey	Distinct (Aggregates)	10-15	-	+
Cg 89B-2	Submerged, felty	White	Distinct (Pin-heads like)	20-30	-	-
Cg 93	Raised, felty	Greyish white	Distinct	3-4	+	+
Cg 131A	Submerged, felty	Greyish	Distinct	5-8	+	+

+ = Present; - = Absent

Cg 89 derivatives were also distinct morphological variants - Cg 89A had several appressoria but no sclerotia; Cg 89B-1 had several acervuli aggregates measuring up to 0.5 mm and sclerotia, while Cg 89B-2 had several small pinhead-like acervuli but no appressoria or sclerotia.

Pathogenic variation

. The isolates induced variable reaction types on the sorghum lines (Table 2). Only two (Cg 88E and Cg 89A) of the 18 isolates induced S reaction on the highly resistant sorghum line A2267-2, while five isolates induced S reaction on IRAT-204. However, there were no isolates that induced uniform disease reaction on all seven sorghum lines. Of these, Cg 88 was the most virulent as it induced S reaction on six lines and MR reaction on one line (A2267-2). Cg 22.induced S reaction on four and MR on three lines. Isolate Cg 89 was the least virulent and caused only R/ MR reaction on all the seven lines. Among the variants of Cg 88, Cg 88C and Cg 88E infected all the seven lines, but these two varied in reaction on specific lines. Isolate Cg 88C was similar to its parental isolate Cg 88 in inducing S reaction on six lines and MR on one. Isolate Cg 88E was different in virulence from the above two and it induced S reaction on six lines (including A2267-2) and MR on CSH 9. Isolate Cg 88D induced S reaction on six line's and R on A2267-2, while Cg 88B induced S reaction on six lines and R on A2267-2. Similarly, the Cg 89 variants also varied amongst them as well as from the parental isolate. Isolate Cg 89A was the most virulent and infected all the. seven lines and induced S reaction on six and MR on IRAT 204. Isolate Cg 89B-1 infected six lines, but induced S reaction on two lines (IS 3758 and IS 3089), MR on three and R on one. Isolate Cg 89B-2 infected five lines with MR reaction and was avirulent on A 2267-2 and IS 3758.

Genetic variations

Of the eighteen isolates from different localities/ single lesions, the isolates 88, 88B, 88C, 88D &

Table 2. Disease reactions induced by 18 isolates of *Colletotrichum gramlnicola* on seven sorghum lines by artificial inoculation in a greenhouse

Isolate	Sorghum lines						
	A2267-2	IRAT-204	IS 3758	IS 8354	IS3089	IS18442	CSH-9
Cg 10B .	R	R	S	MR	S	S	MR
Cg 10C	R	R	S	S	S	S	R
Cg 22	MR	MR	MR	S	S	S	S
Cg 42A	R	R	S	MR	MR	S	MR
Cg 42B	R	R	S	MR	MR	S	MR
Cg 52A	R	MR	S	S	S	S	S
Cg 52B1	R	R	S	S	S	S	R
Cg 88	MR	S	S	S	S	S	S
Cg 88B	R	S	S	S	S	S	S
Cg 88C	MR	S	S	S	S	S	S
Cg 88D	R	S	S	S	S	S	S
Cg 88E	S	S	S	S	S	S	MR
Cg 89	R	MR	R	MR	MR	MR	MR
Cg 89A	S	MR	S	S	S	S	S
Cg 89B-1	MR	MR	S	R	S	MR	MR
Cg 89B-2	R	MR	R	MR	MR	MR	MR
Cg 93	R	MR	S	S	MR	S	S
Cg 131A	R	R	S	MR	S	S	R

Disease reaction as R = resistant - no symptoms, 'or chlorotic flecking; MR = moderately resistant - necrotic spots without acervuli; and S = susceptible - lesions with acervuli

88E (all from a single lesion) were exactly the same with respect to the RAPD profile with the six primers tested. The same was true for 89B1 and 89B2; though 89B1/B2 differed considerably from 89 and 89A (all derived from a single lesion). A!! the isolates (except the two groups mentioned above) were different from each other, even though some originated from the same lesion (Fig. 1,2). By and large, two major clusters were formed: 89B1/B2 as one & the rest as one. There was no effect of place of isolation, rather two isolates from two different provinces (e.g.: 42A from Andhra Pradesh and 52A from Maharashtra) were closer to each other than two isolates originating from even the same lesion (e.g.: 42A and 42B).

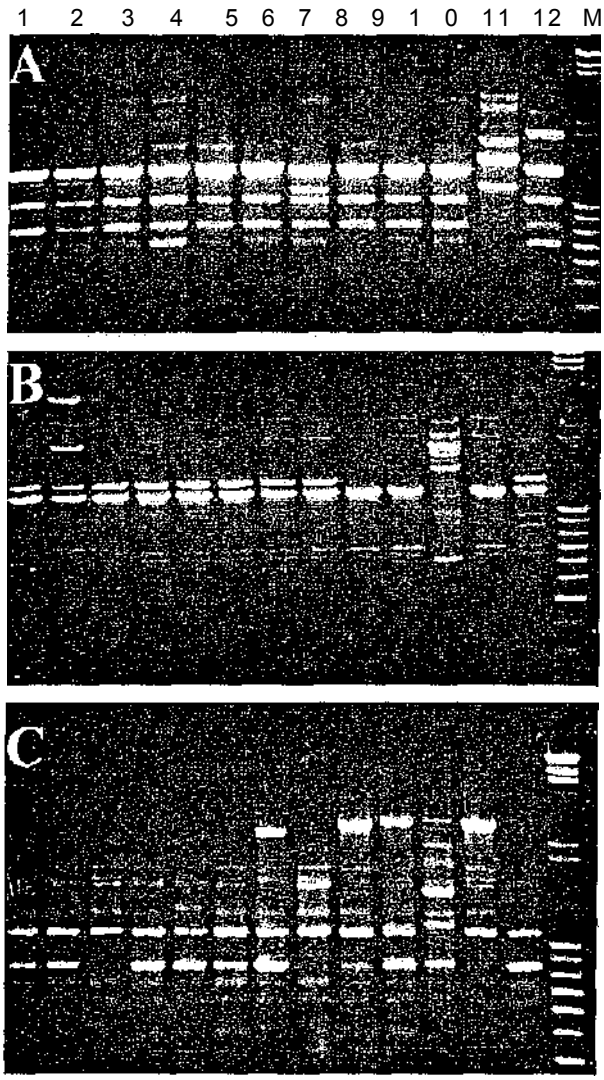

Fig.1. RAPD profile of *Colletotrichwn graminicoia* isolates using random primers OPA1 (A), OPA2 (B), and OPA10 (C). 1=Cg 10B; 2= Cg 10C; 3= Cg 22; 4= Cg 42A; 5= Cg 42B; 6= Cg 52A; 7= Cg 52S1; 8= Cg 88; 9= Cg 89; 10= Cg 89A; 11= Cg 89B1; 12= Cg 93; 13= Cg 131A. M= Molecular weight marker

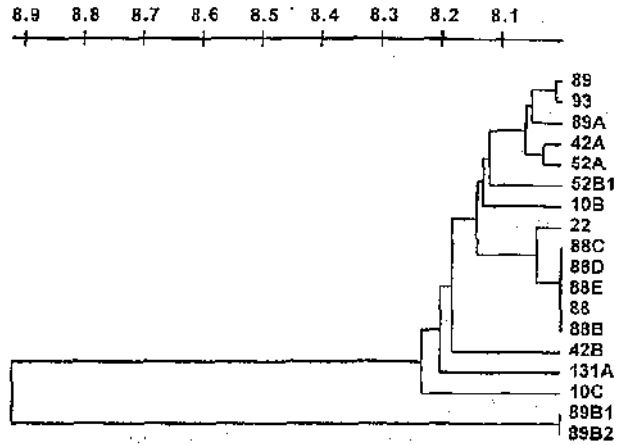

Fig.2. Phylogenetic tree of *Colletotrichum graminicola* isolates

DISCUSSION

Differentiation of pathogenic races is well demonstrated in the host-pathogen systems where the genes for resistance are known and in several of these, Mendelian inheritance of virulence is established (Shaner *et al.,* 1992). In the sorghum-anthracnose system the identity and number of the genes for resistance are not yet well known and so the pathogenic race differentiation is presently possible through virulence analysis on the host differentials (Frederiksen and Rosenow, 1971; Thakur, 1995). In this study, thesorghum lines used clearly differentiated the isolates based on disease reactions.

Isolates of *C. graminicola* from the commercial hybrid CSH-9, and the LSC from different locations in Maharashtra state were distinct pathotypes, and two of these Cg 88 and Cg 89 (both from CSH-9) behaved as heterogeneous populations providing morphological and pathogenic variants. It was also observed that some isolates, such as Cg 89B-2, were avirulent or less virulent than the original isolate which were in accordance with those of Some *et al.,* (1996) for heterogeneity and variations in virulence among populations of *Plasmodiophora brassicae on Brassica* spp. Several pathotypes are known to-existin the-populations-of-the different pathogens (Irwin and Cameron, 1978; Ou, 1980; Tu, 1986) and influence their survival and

pathogenic fitness (Liu *et ai,* 1996; Park *et a*/., 1995; Tapsoba and Wilson, 1996).

The variants with higher virulence in a pathogen population are of interest because these are more virulent on sorghum lines resistant to the predominant pathotypes. These results also indicate the risks of using single-lesion isolates that could be heterogeneous, for evaluation of host resistance, as these would provide inconsistent results. Such variable pathotypes within populations may affect the rate of disease development and induce infection in more 'host lines, and thus may have implications for cultivar resistance stability.

The isolates from CSH-9 exhibited S reaction to A2267-2, which was found resistant to C. *graminicola* isolates from several Indian locations both under field and greenhouse tests (ICRISAT, 1996; Pande *et a*/., 1991). This information and the availability of the pathogenically variable isolates virulent on different resistant cultivars/lines are useful for determining the resistance loci in the host (Crute, 1992), for which there is so far no information in the sorghum -C. *graminicola* system. Existence of morphologically variable pathotypes has also been reported in other species of *Colletotrichum,* such as *C. gloeosporioides* (Irwin and Cameron, 1978) and *C. trifolii* (O'Neili, 1996). However, in the present study, isolates from a single host cultivar (CSH-9) from different locations within one state were found to be distinct pathotypes and these were different from the one from the LSC. The results indicate a virulence shift in *C. graminicola* populations from LSC to CSH-9. In view of the evolution of new virulence in C. *graminicola* populations specific to CSH-9 it would be useful to monitor the resistance stability of the existing commercial hybrid cultivars through field survey and virulence analysis. This is important for strategic utilization and deployment of effective resistance to sorghum cultivars.

RAPD analysis of C. *graminicola* isolates indicated that sorghum isolates of *C. graminicola* from India are hyper-variable with distinct genetic variations (Fig. 2). Genetic variability in C. *graminicola* isolates from various plants and places have been studied earlier using RAPD (Guthrie *et ai,* 1992; Browning *et al,* 1999; Backman *et al.,* 1999) and RFLP (Rosewich *et ai,* 1998) markers. Based on the genetic analysis of *C. graminicola* population in a'sorghum disease nursery, Rosewich *et al.* (1998) inferred that asexual reproduction, rather than genetic drift and gene flow was the major contributor to the genetic structure of C. *graminicola* population. The present finding suggests that the isolates from even the same lesion are genetically distinct, which further strengthens the hyper-variable nature of this. pathogen. It should be noted, however, that the groupings based on RAPD data could not be correlated to the ones based on morphology/ pathogenicity in this study.

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