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Genetic Variability of Sorghum Charcoal Rot Pathogen (*Macrophomina phaseolina*) Assessed by Random DNA Markers

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Genetic diversity among selected isolates of *Macrophomina phaseolina*, a causal agent of charcoal rot (stalk rot) disease in sorghum was studied using PCR-RAPD markers. A set of ten isolates, from ten different *rabi* sorghum genotypes representing two traditional sorghum growing situations *viz*., Dharwad- a transitional high rainfall region and Bijapur- a semi-arid low rainfall region in South India. From a set of 40 random primers tested, amplicon profiles of 15 were reproducible. A total of 149 amplicon levels, with an average of 9.9 bands per primer, were available for analysis, of which 148 were polymorphic (99.3%). It was possible to discriminate all the isolates with any of the 15 primers employed. UPGMA clustering of data indicated that the isolates shared varied levels of genetic similarity within a range of 0.14 to 0.72 similarity coefficient index and it was suggestive that grouping of isolates was not related to sampling location in anyway. A high level of genetic heterogeneity of 0.28 was recorded among the isolates.

Keywords : sorghum, charcoal rot, *Macrophomina phaseolina*, genetic diversity, PCR-RAPD marker

The fungus *Macrophomina phaseolina*, is known to cause stalk rot or charcoal rot disease in more than 500 plant species worldwide, including important crops like sorghum, bean, cotton, soybean and corn under favorable conditions (Mihail and Taylor, 1992; Ali and Dennis, 1992). It is a common, soil borne, non-aggressive and pulverous pathogen that generally attacks the plants which are less vigorous due to moisture stress. In sorghum, *M. phaseolina* is a destructive pathogen, causing charcoal rot in post rainy season from September to January (called rabi season) in India (Anahosur and Patil, 1993; Subramanium, 1994 and Reddy, 1997) and in other part of the world (Mughogho and Pande, 1983). Charcoal rot in sorghum is characterized by severe lodging during grain filling stage, affecting the process of normal grain development and eventually reduced grain yield. Under favorable conditions complete lodging and a grain yield loss of about 64 per cent has been reported (Mughogho and Pande, 1983). Significant reduction in fodder quality of infected stalks is also a major concern (Manici et al., 1995; Mayek-perez, et al., 2001). Light brown discoloration of the sub-epidermal tissues in taproot and lower part of stem are the typical symptoms. In advanced stages, leaves turn yellow and wilt, but still remain attached to the plant. Under field conditions no control measure is fully effective, if conditions are favorable (soil moisture stress, coupled with high temperature during grain filling) for disease manifestation. Host plant resistance has been considered as the only practicable way to manage this disease. However, sorghum breeding efforts have not yielded any cultivar with satisfactory level of field resistance to charcoal rot so far. Polygenic nature of charcoal rot resistance, its interaction with moisture stress and temperature have compounded the breeding efforts for resistance to charcoal rot in sorghum.

All improved hybrids and high yielding cultivars are susceptible to charcoal rot in India. Crop rotation, post harvest removal of infected plant material, manipulation of planting dates, adequate and timely fertilization, planting density and supplementary irrigation during grain filling stage of the crop are the measures suggested to manage the disease. However, it is difficult to implement one or more of them under dry land conditions, where most of sorghum is grown in India. Though, fungicide treatment of seed and soil has been practiced in some cases, it is neither economical nor environmentally safe (Abawi and Pastor-Corrales, 1990). Hence, utilization of the host plant resistance is the most realistic alternative. Genetic variability and pathogenicity are the two key factors that influence the detection of resistance in the host plant and its management. Polykaryotic feature of *M. phaseolina* mycelium appears to make it highly variable in pathogenicity and cultural characters (Ali and Dennis, 1992, Dhingra and Sinclair, 1973 and Than et al., 1991). Interestingly, despite its wide host range, *Macrophomina* is a monotypic genus. So far, microsclerotial size, cultural characteristics, chlorate sensitivity and pycnidia formation are most often considered characters in variability studies of *M. phaseolina* (Mihail and Taylor, 1992, Cloud and Rupe, 1991 and Suriachandraselvan and

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Seetharam, 2000).

Attempts to classify the isolates of this fungus based on colony morphology using specified media and chlorate sensitivity tests have indicated that isolates with similar morphology need not be genetically identical; hence the use of molecular tools has been suggested to study genetic variation among isolates (Su et al., 2001). Restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), restriction analysis of ribosomal DNA and random amplified polymorphic DNA (RAPD) analysis have been useful to unveil genetic variability in fungal population (Henrion et al., 1992; Chen et al., 1992). However, for *Pythium,* restriction analysis of ITS region did not prove to be a suitable method for detecting variability (Chen et al., 1992). RAPD markers, which have wider genome coverage, detect larger number of loci, simple to accomplish, and do not require any prior knowledge of DNA sequence. RAPD markers have been considered suitable for measuring genetic relatedness, detecting variation within and between *M. phaseolina* populations (Alvaro et al., 2003, Fuhlbohm, 1997 and Jana et al., 2003). The decamer primers commonly used in this technique do produce polymorphic amplicons; repeatability of the technique is a concern (Virk et al., 1995). Using RAPD markers *M. phaseolina* on different crops have been studied (Jana et al., 2003). However, understanding the genetic variability among isolates of *M. phaseolina* occurring on sorghum in traditional post rainy situation is essential for area wide disease management. In this paper, we report on utility of the technique in uncovering differences among a set of selected isolates of *M. phaseolina*, collected on sorghum from transitionally high rain fall and semi-arid low rainfall situation in South India.

Material and Methods

Sampling locations. Infected sorghum stalks of ten different popular cultivars (D1 - DSV–4, D2 - GRS–1, D3 - SPV–1650, D4 - SPV–1359, D5 - Bidar local, D6 - Raichur local, D7 - Annigeri local from Dharwad and B1 - M–35-1, B2 - CSH–13R, B2 - E36–1 from Bijapur) were collected from charcoal rot affected fields of two locations - Regional Agricultural Research Station (RARS), Bijapur (semi-arid zone) and Main Agricultural Research Station (MARS), Dharwad (high rainfall area). Sample collection was made during February to May 2004.

Isolation of fungus. Each infected stalk was thoroughly washed and dried separately at room temperature before its actual use for isolation of the fungus. Selected region of the stalk was cut into 5-7 mm sections and 5 to 8 such pieces were separately surface sterilized with 0.1% $HgCl_2$ for 1 min followed by four rinses with sterile water and inoculated on potato dextrose agar (PDA) plates. Plates were incubated at 26±1°C in darkness for 6 days. Small individual colonies were collected along with small piece of medium and lightly ground in sterile water. A single microsclerotium was collected from each sample under microscope and transferred to PDA plates, separately. Suspension of microsclerotia was obtained from these cultures and dispersed on fresh water agar plates. Pure culture of each isolate was obtained after 48 h of incubation, by selecting growing hyphae from a single sclerotium, which, were collected on acidified PDA medium for microscopic conformation of hyphal and conidial characteristics. Confirmed pure cultures were maintained on PDA slants at 26± 1°C for 6 days and stored in darkness at 6°C for further use.

Genomic DNA isolation. In order to isolate genomic DNA from mycelium of each isolate, stock culture was grown in potato dextrose broth (PDB) at 26±1°C for 12 days. Mycelia were centrifuged at 3,000×g for 8 min and the mycelial pellet was rinsed with sterile water and gently squeezed to remove excess moisture. These samples were immediately used for genomic DNA isolation following a modified protocol, essentially derived from Hegedus and Kachatourians (1996) and Lee and Taylor (1990). About 3 g of fungal mat was homogenized using pestle and mortar in 4 ml of 2% sodium dodecyl sulfate (SDS) for 5 min. To this, 6 ml of lysis buffer (2.5 mM EDTA, 1% Triton X and 50 mM Tris-HCl, pH 8.0) was added and the suspension was extracted with equal volume of phenol:chloroform (1:1) and centrifuged at 10,000 rpm for 10 min. The supernatant was taken into a fresh tube and $1/10^{th}$ volume of 3 M sodium acetate and 2.5 volume of isopropanol were added, mixed by gentle inversion and kept at 2°C for 30 min. The contents were centrifuged at 10,000 rpm for 10 min at 4°C. The DNA pellet was washed with 70 % ethanol, briefly air-dried and resuspended in 300 µl of $T_{10}E_1$ (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). The RNA contamination from the genomic DNA was removed by adding 5 µl of RNase (10 mg/ml) to each tube, DNA was redisolved by taping the pellet and incubated at 37°C for 30 min. Genomic DNA was also further purified by a phenol-chloroform treatment and precipitation with ethanol and sodium acetate and finally dissolved in 100 µl of $T_{10}E_1$. The exact amount of DNA in samples was quantified by the spectrophotometric method (Sambrook and Russel, 2001).

PCR-RAPD analysis. DNA samples for all the ten isolates were subjected to PCR-RAPD analysis by amplifying the genomic DNA in a total volume of 25 µl containing 10-12 ng of DNA. The reaction buffer consisted of 2.5 µl of 10X buffer (500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl pH

8.0), 0.2 μM random primer (Operon Technologies Inc., Alameda, CA, USA), 0.2 mM of each dNTPs and 0.5 U of *Taq* polymerase (Bangalore Genei Pvt Ltd, Bangalore). The PCR amplification conditions were: initial denaturation at 94°C for 5 min followed by 39 cycle of denaturation at 94°C for 2 min, primer annealing at 36°C for 1 min, primer extension at 72°C for 2 min, and a final primer extension at 72°C for 8 min. The amplification product in 20 μl was separated in 1.2% agarose gel using 1X TBE (89 mM Tris-HCl, 89 mM boric acid and 2 mM EDTA pH 8.0), stained with ethidium bromide (0.50 μg/ml) and visualized under UV transilluminator. Lambda DNA (*Eco*RI+*Hin*dIII) double digest (Bangalore Genei Pvt. Ltd) was used as marker in separate lane. Amplification and gel separation were done at least twice with each random primer to obtain the reproducible amplicon pattern. A total of 40 random primers from A and C kits of Operon Technologies (USA) were tested and eventually 15 primers, that produced reproducible amplicons patterns were considered for final analysis.

Data analysis. The PCR-RAPD amplification pattern was converted into binary data matrix, the presence of band at an amplicon level was scored as 1 and its absence as 0. The binary data was analyzed using standard procedure in NTSYS-PC (version 2.1; Exeter Biological Software, Setauket, NY) software package (Rholf, 2000). The data was subjected to the SIMQUAL option to obtain association coefficients using Jaccard's coefficient (Jaccard, 1908) of similarity to generate a similarity matrix. Clustering analysis was performed with the unweighted pair-group method using arithmetic averages (UPGMA) in the SAHN (sequential, agglomerative, hierarchical and nested clustering method) module of NTSYS-PC.

Results

PCR-RAPD markers proved to be informative and useful in detecting the genetic diversity among the 10 isolates of *M. phaseolina*. Amplification of genomic DNA of all isolates produced large number of distinct amplicons; the size of amplicon was detected by comparing with a DNA ladder ranged approximately from 200 bp to 3500 bp (Fig. 1). Very rarely, some amplicons were larger than 3500 bp. Of the two primer kits (20 of KitA and 20 of KitC) employed, repeatable amplicon pattern was obtained for 15 primers. The primer amplicon levels and the number of polymorphic amplicons were: A1 – 13/13, A2 – 14/14, A3 – 18/18, A5 – 10/10, A7 – 10/10, A9 – 9/9, A10 – 8/8, A11 – 9/9, A13 – 11/10, C11 – 12/12, C12 – 3/3 , C13 – 5/5, C15 – 8/8, C16 – 9/9, C20 – 10/10 respectively. A total of 149 amplicon levels from 15 primers were available for the analysis, of

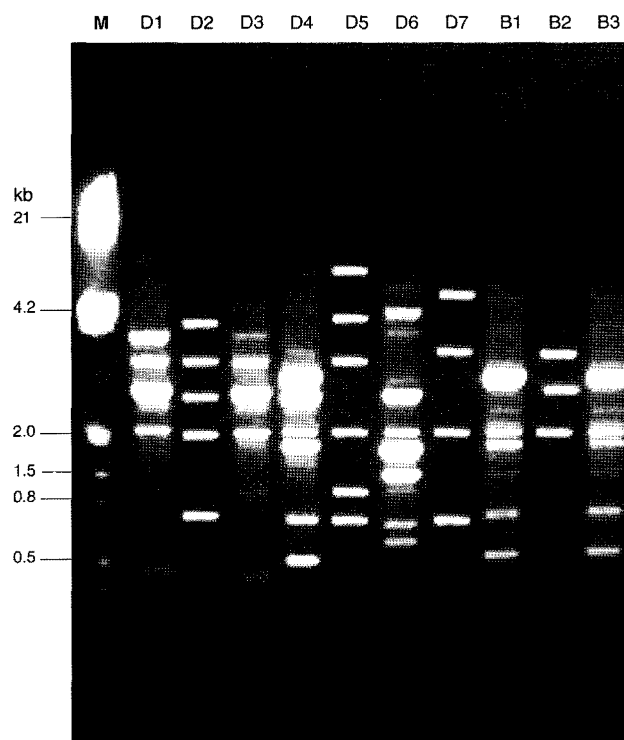

Fig. 1. PCR-RAPD profiles of ten isolates of *Macrophomina phaseolina* obtained with random primer A13. The gel was stained with ethidium bromide D1 to D7 and B1 to B3 are *M. phaseolina* isolates from Dharwad and Bijapur locations respectively. M: molecular weight marker, Lambda DNA double digested with *Eco*RI and *Hin*dIII.

which 148 amplicons were polymorphic (99.7%). The number of amplicons ranged from 3-18, with an average of 9.9 per primer with high polymorphism. A typical PCR-RAPD profile from primer A13 shown in Fig. 1.

All isolates of *M. phaseolina*, irrespective of their sampling location and source genotype showed a common amplicon of about 1800 bp by the primer A13 (Fig. 1). A13 could be potentially used to discriminate *M. phaseolina* isolates from other fungal species. However, any of the 15 primers employed in this study can be used in order to discriminate *M. phaseolina* isolates. Similarity coefficient generated using band sharing data among isolates is presented in the Table 1. A lowest genetic similarity coefficient of 0.06 was observed between isolates D5 and B1; D7 and B1, while the highest genetic similarity coefficient of 0.71 was between D1 and D3 which was closely (0.70) followed by D2 and D5. Jaccard's similarity coefficient matrix was analyzed with UPGMA by using NTSYS and a dendrogram was constructed (Fig. 2). All of the ten isolates were grouped into 2 major clusters, X and Y. Cluster X was further sub-grouped into two sub-clusters -X.1 and X.2; X.1 consisted of D1, D3, D6 - all from Dharwad location, while X.2 also consisted of 3 isolates - B1 and B3 in X.2.2 from

Table 1. Similarity matrix for ten isolates of *Macrophomina phaseolina* calculated based on band sharing data of PCR-RAPD analysis

isolate	D_1	D_2	D_3	D_4	D_5	D_6	D_7	B_1	B_2	B_3
D_1	1									
D_2	0.28	1								
D_3	0.71	0.17	1							
D_4	0.23	0.13	0.23	1						
D_5	0.20	0.70	0.14	0.09	1					
D_6	0.33	0.12	0.31	0.28	0.09	1				
D_7	0.20	0.70	0.19	0.12	0.66	0.09	1			
B_1	0.26	0.11	0.35	0.56	0.06	0.21	0.06	1		
B_2	0.20	0.22	0.14	0.09	0.21	0.15	0.31	0.13	1	
B_3	0.14	0.15	0.22	0.47	0.09	0.20	0.09	0.69	0.14	1

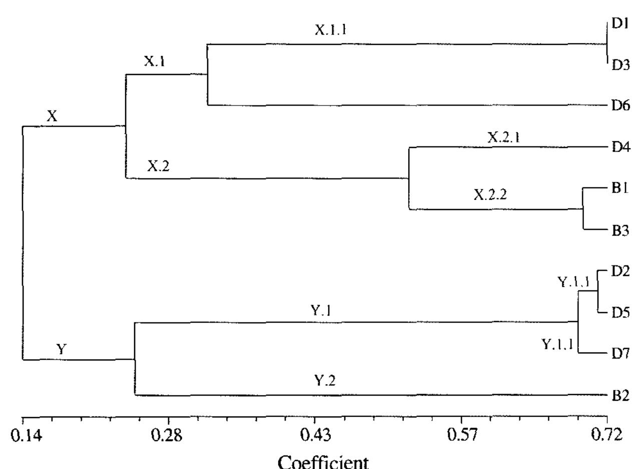

Fig. 2. Dendrogram constructed based on Jaccard coefficients with UPGMA-SAHN clustering method among 10 isolates of *M. phaseolina* using 149 PCR-RAPD loci. The scale below the dendrogram represents genetic similarity coefficients calculated according to Jaccard (1908). The branches are labeled by isolate.

Bijapur location and D4 in X.2.1 from Dharwad location. Similarly, cluster Y further was sub - divided into Y.1 and Y.2; Y.1 consisted of 3 isolates - D2, D5 (in Y.1.1) and D7 (in Y.1.2), all from Dharwad location; while Y.2 consisted of B2 isolate from Bijapur location.

Discussion

PCR-RAPD analysis is one of the simplest tools available for the analysis of genetic differences at DNA level without demanding any prior information about the genomic sequences. Being extremely sensitive amplicon patterns are not considered completely reproducible. However, preliminary data on genetic diversity which could quickly ascertained, is a useful first step. The preliminary information provided in the present study indicates that RAPD data can be used to distinctly identify individual isolates, provided the isolates themselves are characterized based on other parameters. Any of the 15 markers used could discriminate *M. phaseolina* isolates. The level of polymorphism was high (148/149; 99.7%). Overall, *M. phaseolina* isolates were found to have a very low genetic similarity coefficient index ranging from 0.14 to 0.72. Interestigly, the genetic heterogeneity was 0.28. Though *M. phaseolina* is not known to have sexual stage in its life cycle, a high genetic heterogeneity is maintained among the populations. Perhaps, the fusion between vegetative cells or para sexual recombination must have contributed for the greater variability (Carlile, 1986). It is possible that the primer employed might have picked polymorphism from the most variable regions of the genome. We believe that this finding will be useful in further characterization of the isolates of this fungus and assist in sorghum breeding program for charcoal rot resistance.

Jana and associates (2003) also reported very high level of genetic variability in *M. phaseolina* isolates, representing different host plants. An in-depth analysis using more robust markers like SSRs or AFLP must reveal more facts in this regard. Furthermore, single monomorphic band generated by A13 primer across 10 isolates in the present study was also reported earlier (Jana et al., 2003), which can be developed as SCAR (sequence characterized amplified region) marker to detect and differentiate *Macrophomina* from *Fusarium* species and also to enumerate the fungus in soil sample through quantitative PCR techniques.

The grouping pattern of isolates did not suggest location specific variability and specialization, though specific pairs of isolates did show some similarity. Little difference in genetic similarity observed between D5 and B1; D7 and B1 supports the idea that the isolates selected over the regions posses wide genetic variation. However, the clustering pattern of isolates shown by dendrogram represents apparent genetic similarity based on the genomic region represented by the amplicon. The dendrogram separated the isolates into two major clusters with intermix of isolates from both sampling locations. All isolates belonging to a geographical location

have not come under a single cluster, suggesting that the variability is independent of geographical proximity. Sub - clustering pattern within each major cluster did not follow any definite trend vis-vis sampling location. RAPDs based oligo-nucleotide fingerprinting has been successfully used to assess the genetic and pathogenic diversity in several fungal species (Carter et al., 2003, Jamil et al., 2000, Sharma et al., 1999 and Sharma et al., 2003). Before the RAPD data is employed for objective classification based on fungal parameters (aggressiveness, pathogencity, geographical and crop colonization), data from large number of primers is needed so that the most part of the genome is represented. Besides the use of more robust SSR and AFLP markers, RAPD markers could also be converted to the dependable SCARs. The study shows that the amount of variability in *Macrophomina phaseolina* is high, basically a saprophyte and opportunistic pathogen, high genetic variation severs it well to survive in nature.

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