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Molecular variability of *Macrophomina phaseolina* isolates using SSR marker

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ABSTRACT

An experiment was conducted at RARI, Durgapura, SKNAU, Jobner during *kharif* 2020 to analyze the genetic diversity of the 12 *Macrophomina phaseolina* isolates by using simple sequence repeat (SSR) markers. The SSR primers were tested for amplification of genomic DNA of *Macrophomia phaseolina* isolates. Total five primers of MB series were screened against twelve *Macrophomia phaseolina* isolates. All primes amplified scorable banding pattern. Level of polymorphism for SSR analysis ranged between 60–85.7%. The similarity matrix indicated that 12 isolates exhibited 42.82–100% similarity coefficient. The SSR analysis shows high molecular variability among the isolates grouped into five major clusters A, B, C, D and E. In the similarity matrix Mp-DPR was found to have higher value of similarity coefficient (1), whereas Mp-JSM was found to have lower value if similarity coefficient (0.42).

Keywords: Clusterbean, Genetic diversity, *Macrophomina phaseolina,* Molecular variability, SSR markers

Clusterbean [*Cyamopsis tetragonoloba* (L.) Taub.] eminently known as guar is a deep-rooted annual legume crop of family *Fabaceae* known for its drought and high temperature tolerance (Kumar and Rodge 2012). The production of clusterbean crop has been stagnant mainly because of its cultivation under rainfed areas, marginal and sub-marginal lands, low soil fertility and biotic stresses. Among, these diseases dry root rot caused by *Macrophomina phaseolina* has become a major biotic threat in several regions of the country and causes considerable economic yield losses because clusterbean is generally raised under moisture stress conditions and high temperature which is conducive for the development of dry root rot disease. The evidence suggests that it's primarily a root inhibiting fungus and produces tuber or cushion shaped, 1–8 mm (diameter) black sclerotia. These sclerotia serve as a primary means of survival. The high amount of variability also exists in this necrotrophic pathogen. Determination of fungal genetic variability based on molecular markers is reliable and independent technique to assess variability in the population of the pathogen (Kumar and Singh 2000). Hence, the present investigation was aimed to analyse the molecular variability among the isolates of *M. phaseolina* causing dry root rot of clusterbean by using SSR markers. The numbers of simple techniques were known that could be used to rapidly characterize *M. phaseolina* population in a particular area. However, evaluating genetic diversity information for *Macrophomina* management in field requires powerful discriminating, selective and reliable criteria for genotyping the isolates (Sharma *et al*. 2004). Furthermore, determination of fungal genetic variability based on molecular markers is reliable and independent technique to assess variability in the population of the pathogen.

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MATERIALS AND METHODS

Isolation and purification: Twelve *M. phaseolina* isolates viz. Mp-AWR (Alwar isolate), Mp-BKN (Bikaner), Mp-CUR(Churu), Mp-DPA(Durgapura-Jaipur), Mp-HMH(Hanumangarh), Mp-JJN(Jhunjhunu), Mp-JU(Jodhpur), Mp-JSM(Jaisalmer), Mp-NGO(Nagore), Mp-SIKR(Sikar), Mp-SNGR(SriGanganagar) and Mp-UDZ(Udaipur) were collected from different clusterbean growing areas of rajasthan and used in the present study. Dry root rot infested root samples of clusterbean showing typical symptoms were gently washed tap water for removing the soil particles adhering to the root surface. The washed root parts were cut into smaller under pieces and surface sterilized with 1% sodium hypochlorite solution for 1 min followed by three washings with sterilized distilled water. The surface sterilized pieces were transferred aseptically on potato dextrose agar slants and incubated at 28±2°C temperature in a BOD. incubator for 7 days. To obtain pure culture of *M. phaseolina*, single hyphal tip isolation technique was adopted (Rangaswami and Mahadevan 2004). Single hypha was demarcated under low power objective of microscope (10x) and cut with the help of dummy objective.

It was transferred aseptically on PDA slants with the help of an inoculating needle. The isolated fungus was identified as *Macrophomina phaseolina* on the basis of morphological and colony characters, viz. white to grey colony turning black with age, septate hyphae and blackish colour spherical, irregular and oblong type sclerotia.

DNA isolation: For DNA isolation, Erlenmeyer flasks (250 ml) containing 100 ml of potato dextrose broth were inoculated with 5 cm discs of actively growing culture of *M. phaseolina.* The cultures were placed on a rotary shaker (100 rpm) and incubated at 27°C for 2–3 days. Mycelia were harvested by filtration through cheesecloth, blotted dry with sterile paper towels and used immediately for DNA extraction. Total genomic DNA was isolated from the fungus following the CTAB method (Murray and Thompson 1980). The DNA obtained after extraction was confirmed by running it on 0.8% agarose gel (containing ethidium bromide @0.5 mg/ml) in a horizontal gel electrophoresis system.

PCR amplification and electrophoresis: Five SSR (Simple sequence repeats) primers of MB-series which indicated a higher degree of polymorphism, were selected for diversity analysis study. Details of primers (5'–3') used for DNA amplification are given in Table 1. Amplification reactions were carried out in volumes of 20 µl containing 1 µl (50 ng) template DNA, 2 µl Iez. Reaction buffer (10 X), 0.5 mM dNTPs, 1 µl forward primer, 1 µl reverse primer, 2 µl of (25 mM) MgCl2, 0.3 µl µl of Taq polymerase (5 U/µl) and 12.2 µl sterile distilled water. Amplification was performed in a thermal cycler (Eppendorf, Germany) programmed for 35 cycles at a temperature regime of 94°C for 30 sec, 57°C annealing temperature for 45 sec, 72°C for 1 min after an initial denaturation at 94°C for 2 min. Following the cycling, the mixture was incubated at 72°C for 10 min and then kept at 4°C hold till electrophoresis.

Table 1 List of SSR primers

Primer	Monomorphic bands	Poly morphic	Unique Non-Un.	Total bands	Polymorphic %
MB-2	1	1	3	5	80.0
MB-5	1	3	3	7	85.7
MB-9	2	2	1	5	60.0
MB-10	1	3	-	4	75.0
MB-11	2	3	-	5	60.0

Separation on polyacrylamide gel electrophoresis and silver staining: SSR-PCR amplified product was separated on 1.0% polyacrylamide gel (PAGE) assembly. 1.0% of PAGE (100 ml) was made by using urea, acrylamide, bisacrylamide and 10X TBE. To the gel, 0.085–10% Ammonium per sulphate solution and 0.075% of TEMED were added before use. The gel solution was poured between the assembled glass plates, the comb was placed onto the gel and allowed to polymerize for 30 min at room temperature. The wells were rinsed with 1X TBE buffer and loaded 10 µl PCR products with 5 µl of 6X loading dye followed by loading of (1 µl) 100bp DNA ladder. The gel was run on 50 V till dye came closer to bottom. After electrophoresis, the gel was carefully placed in a plastic tray, rinsed with distilled water and gel staining procedure was followed. The gel was fixed in Fixer solution (30 ml Methanol, 1.5 ml Glacial acetic acid, and 270 ml distilled water) by shaking gently for 5 min. Fixer was removed and the gel was shaken for 3–5 min in 0.1% silver nitrate staining solution. Removing the staining solution, the developer (900 µl formaldehyde, 9 gm NaOH, 300 ml water) was transferred to the staining tray and shaken for 15–20 min gently until for yellow to dark black precipitate became noticeable. The developer was removed and the gel was rinsed again with distilled water. The gel was visualized under a white background and photographed.

Data analysis: Data were scored as the presence (1) or absence (0) of the individual band for each isolate (Table 2). This binary data was used to compute the similarity coefficient using the Jaccard similarity coefficient with the help of Numerical Taxonomy System Version 2.2 (NTSYSpc) (Rahlf 1994). The similarity matrix was used to construct the dendrogram by Un weighted pair group

Table 2 Similarity coefficient for SSR analysis

Mp-DPR	Mp-BKN	Mp-JJN	Mp-CUR	Mp-SIKR	Mp-JU	Mp-JSM	Mp-SGNR	Mp-NGO	Mp-HMH	Mp-AWR	Mp-UDR
1.00000											
0.94444	1.00000										
0.83333	0.88235	1.00000									
0.90000	0.85000	0.75000	1.00000								
1.00000	0.94444	0.83333	0.90000	1.0000							
1.00000	0.94444	0.83333	0.90000	1.00000	1.00000						
0.63157	0.66666	0.55555	0.57142	0.63157	0.63157	1.00000					
0.73684	0.77777	0.87500	0.75000	0.73684	0.73684	0.55555	1.00000				
0.75000	0.70000	0.77777	0.76190	0.75000	0.75000	0.42857	0.77777	1.00000			
0.76190	0.71428	0.78947	0.69565	0.76190	0.76190	0.45454	0.70000	0.80000	1.00000		
0.77272	0.72727	0.63636	0.78260	0.77272	0.77272	0.61904	0.71428	0.65217	0.66666	1.00000	
0.77272	0.72727	0.63636	0.78260	0.77272	0.77272	0.54545	0.71428	0.65217	0.60000	0.82608	1.0000

method of arithmetic average (UPGMA) using the SAHN (Sequential Agglomerative Hierarchal Nested) cluster analysis module (Rohlf 2000).

RESULTS AND DISCUSSION

Molecular markers are important tools for analysis of molecular diversity in any research programme. SSR markers have been used in many fungal species as a genetic marker for assessment of genetic diversity and proved highly efficient for assessment of genetic diversity and successful in characterizing individual genotype. Present investigation was undertaken to find out genetic relationship among 12 isolates of *M. phaseolina*, causal agent of dry root rot in clusterbean using SSR markers. Genetic variation was detected among 12 isolates of *M. phaseolina* using 5 SSR primers of MB Series (Table 1). Primer MB-2 produced 5 bands and average level of polymorphism was 80%. MB-2 showed 1 monomorphic band and 4 polymorphic bands (1 Unique and 3 Non-Unique). Primer MB-5 produced 7 bands and average level of polymorphism was 85.7%. MB-5 showed 1 monomorphic band and 6 polymorphic bands (3 Unique and 3 Non-Unique). Primer MB-9 produced 5 bands and average level of polymorphism was 60%. MB-9 showed 2 monomorphic bands and 3 polymorphic bands (2 Unique and 1 Non-Unique). Primer MB-10 produced 4 bands and average level of polymorphism was 75%. MB 10 showed 1 monomorphic bands and 3 polymorphic bands (3 Unique and 0 Non-Unique). Primer MB-11 produced 5 bands and average level of polymorphism was 60%. MB-11 showed 2 monomorphic bands and 3 polymorphic bands (1 Unique and 3 Non-Unique). Based on Jaccard's coefficients, a genetic similarity matrix was constructed to assess the genetic relatedness among the isolates of *M. phaseolina*. Genetic similarity coefficient of 12 isolates of *M. phaseolina* based on SSR analysis is given in Table 2. In the similarity matrix Mp-DPR was found to have a higher value of similarity coefficient (1) whereas, Mp-JSM was found to have a lower value (0.42). A dendrogram was generated by unweighted pair group method with UPGMA subprogramme of NTSYS-pc. The dendrogram for pooled data showed five clusters. In the dendrogram the isolates were grouped into five major clusters. First group i.e. cluster A, includes isolates belonging to Mp-DPR, Mp-SIKR, Mp-JU, Mp-BKN and Mp-CUR. Second group i.e. cluster B includes isolates belonging to Mp-JJN AND Mp-SGNR. Third group named cluster C includes isolates belonging to Mp-NGO and Mp-HMH. Fourth group or cluster D includes isolates belonging to Mp-AWR and Mp-UDR and fifth group i.e. cluster E includes Mp-JSM. It shows that Mp-DPR has higher value of similarity coefficient with Mp-SIKR, Mp-JU, Mp-BKN and Mp-CUR. Mp-JSM has lower value of similarity coefficient. The similarity matrix indicated that twelve isolates exhibited 42.828 to 100% similarity coefficient. Similarly, Walunj *et al.* (2018), also showed genetic diversity among the *Rhizoctonia bataticola* isolates by using Simple Sequence Repeat (SSR) markers and obtained similar results. Lohar *et al*. (2021) also analyzed the genetic diversity of *Fusarium solani* isolates by using six Simple Sequence Repeat (SSR) markers. They obtained two major clusters in the dendrogram, Cluster A and Cluster B and the similarity matrix indicated that six isolates of *Fusarium solani* exhibited 0.880–0.323% similarity coefficients for SSR primer and results of their findings support the present investigations.

It is difficult to distinguish these species using traditional morphological and cultural differences. To understand better, the existence of variation among the isolates of M. *phaseolina,* SSR markers have been used in the present investigation. In SSR analysis total five primers were screened against twelve isolates of *M. phaseolina*. All prime amplified scorable banding pattern. Level of polymorphism for SSR analysis was 60–85.7%. The similarity matrix indicated that twelve isolates exhibited 42.82–100% similarity coefficient. The SSR analysis shows high molecular variability among the isolates and grouped into five major clusters A, B, C, D and E. From the above observations, it is amply clear that all the isolates belonging to one geographical location have not come under a single group, reflecting the fact that the variation is independent of geographical nearness of agro climatic zone. These findings indicate a clear variation among the isolates and strongly supports the possibility of existence of pathotypes in *M. phaseolina* among diversified geographical situations.

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