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Studies on Pathogen Variability in Blackgram root rot by *Macrophomina phaseolina*

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Abstract
Blackgram is subjected to different biotic constraints, root rot being the serious one. The root rot pathogen *Macrophomina phaseolina* was isolated from the diseased stems and roots of blackgram collected from seven different places of Tamil Nadu. Among the seven isolates screened, K_2m_7 collected from Kallipatti in Namakkal district was identified as a virulent culture. Variability among the seven *M.phaseolina* isolates analyzed by RAPD-PCR technique, grouped them into two clusters. Cluser A had six isolates and cluster B had one isolate.

Keywords: Blackgram, *Macrophomina phaseolina*, Isolates, RAPD

Introduction
Blackgram seeds are highly nutritious with protein (25-26%), carbohydrates (60%), fat (1.5%), minerals, amino acids and vitamins. Blackgram has been distributed mainly in tropical and sub-tropical countries, where it is grown mainly in summer season. It is grown in India, Pakistan, Sri-Lanka, Burma and some countries of South East Asia. In India, blackgram is very popularly grown in Andhra Pradesh, Bihar, Madhya Pradesh, Maharashtra, Uttar Pradesh, West Bengal, Punjab, Haryana, Tamil Nadu and Karnataka in an area of 3.29 million ha with a total production of 1.60 million tonnes and the average productivity is 485 kg/ha (Anonymous, 2008) [2]. In Tamil Nadu, blackgram is grown in area of 2.15 lakh ha with a production of 0.7 lakh tonnes during 2005-2006 (Ravindran and Anita, 2008) [10]. During 2010-2011, the productivity of blackgram is 528 kg/ha (Anonymous, 2011) [3]

Macrophomina phaseolina is soil and seed- borne pathogenic fungus produces cushion shaped black sclerotia (Wheeler, 1975) [14]. Its prevalence can be enhanced by different physiological and ecological factors such as low moisture content, high temperature and heat (Dhingra and Sinclair 1978) [5]. The disease recently observed in severe proportions in the blackgram growing areas of Tamil Nadu leading to severe loss in the yield. The disease may cause up to 100 per cent yield losses (Bashir and Malik, 1988).The loss in grain weight due to the disease in *Rabi* cultivars varies from 18.53 to 63.22 per cent (Anonymous, 1999) [1].

RAPD involves the use of single short random oligonucleotide sequence (called random primers) defined cyclic amplification of DNA, which expose the polymorphism, distributed throughout the genome. The amplified fragments are called random amplified polymorphic DNA (Williams *et al.*, 1990) [15]. Shekhar *et al.* (2006) [12] analyzed seven isolates of *M. phaseolina* incident of maize charcoal rot through RAPD marker for genetic diversity. The UPGMA cluster analysis for 706 loci score permitted for identification of three main clusters. Similarity matrix and Jaccards, similarity coefficient between the isolates indicated that the maximum genetic variation was among isolates of Arabhavi and Coimbatore with 70.8 per cent followed by Ludhiana and Coimbatore with 69.5 per cent. The most closely related isolates were Hyderabad and Delhi with an affinity percentage of 65.5 followed by Udaipur and Bangalore isolates with 62.9 percent similarity.

Monga *et al.* (2007) [7] analyzed 25 isolate of *R. bataticola* causal organism of cotton root rot using RAPD markers to study the relationship between molecular variability and pathogenicity. All the 15 primers used generated scorable polymorphic bands for the isolates showed the genetic polymorphism among the isolates based on the RAPD analysis. However, no strict correlation was observed between isolates grouped based on pathogenicity, morphological feature and RAPD finger printing.

Materials and Methods

Genetic variability among different isolates of *M. phaseolina*

DNA extraction

The isolates of *M. phaseolina* were cultured in potato dextrose broth for five days. 100-200 mg of mycelia mat was taken and macerated with CTAB buffer (Jaccard, 1998) [6]. After maceration 700 µl of solution was transferred into 1.5 ml centrifuge tube and incubated at 65 ^{0}C for 10 minutes. After incubation equal volume of chloroform; isoamyl alcohol (24:1) were added. Then the mixture was kept in a centrifuge at 10000 rpm for 10 minutes. The upper aqueous solution was taken and transferred into 1.5 ml centrifuge, then equal volume of chloroform; isoamyl alcohol (24:1) were added. The mixture was again centrifuged at 10000 rpm for 10 minutes. After centrifugation 300 µl of aqueous was taken to which 5 M sodium chloride, ice cold ethanol (5:2) were added. This mixture was kept at -27 ^{0}C for 1 hr or overnight. This was followed by centrifugation at 13000 rpm at 4^{o}C for 10 minutes. After centrifugation, equal volume of ethanol was added and kept for pellet drying. Then 50 µl sterile water was added to the pellet and stored at -27 ^{o}C, which is used as a DNA source.

RAPD-PCR analysis of *M.phaseolina*

Several types of DNA markers have been used widely for linkage mapping *viz.*, RFLPs, RAPDs (Williams *et al.*, 1990) [15], SSRs or microsatellites, AFLPs and SNPs in combination with DNA chip technology. For the assessment of genetic diversity, seven isolates of *M.phaseolina* of Tamil Nadu, a random primer OPB 17 was used to carry out RAPD analysis. Amplification was performed in a total volume of 25 µl reaction containing 10 X PCR buffer, 1.5 mM $MgCl_2$, 1 µmM of primer, 1 mM of dots and 1.5 unit of Taq DNA Polymerase (Genei). PCR amplications were carried out in a Eppendoff Thermal Cycler with the PCR conditions of initial denaturation at 95^{o}C for 5 min followed by 35 cycles at 94^{o}C for 30S, 37^{o}C for one min, 72^{0}C for one min and final extension at 72^{o}C for seven min. The PCR products were separated electrophoretically in 1.8% agarose gel using 1X TAE buffer (Sambrook *et al.* 1989) [11]. The gels were stained with Ethidium bromide and photographed using Alpha Imager EC (Alpha Innotech corporation). The sizes of the amplified products were estimated using medium range DNA rules (Genei).

Analysis of RAPD-PCR results

The RAPD-PCR bands obtained for each isolates were scored based on their presence (1) or absence (0). The experiments were repeated twice for each isolates to confirm the repeatability and only reliable and repeatable bands were considered. Genetic similarity between pairs was calculated according to Jaccard's similarity coefficient (Jaccard, 1998) [6] followed by cluster analysis by the UPGMA in the SAHN programme of NTSYS-pc version 2.02 (Rahlf, 1994) [8]. The relationship of the seven *M.phaseolina* isolates were inferred from the dendrogram constructed based on UPGMA cluster analysis.

Result and discussion

The banding pattern generated for seven *M. phaseolina* by using the random primer OPB 17 is depicted in Fig 8. The results of this RAPD analysis showed that the primer generated 4-14 bands and the size of these amplicons was ranged between 500-3000 bp. The dendrogram obtained from the fingerprints of the above said two primers has group as all the seven *M. phaseolina* isolates into two main clusters *viz.* A & B with genetic similarity of 15-64 %. Among the seven *Macrophomina phaseolina* isolates, six *M. phaseolina* isolates were grouped in cluster A with genetic similarity of 17.5% and the remaining one *M. phaseolina* isolates were grouped in cluster B. Further, cluster A was subdivided into sub cluster A1 and A2 with a genetic similarity of 17.5%. The sub cluster A1 was further divided into A1a and A1b with genetic similarity of 28%. The sub cluster A1a was further divided into A1a1 and A1a2 with genetic similarity of 41.5%. And sub-cluster A1b was further divided into A1b1 and A1b2 with a genetic similarity of 51.5%. In sub-sub-subcluster A1a1, the *M. phaseolina* isolates of local cultivar obtained from, Kallipatti and Thopapalayam showed 64 % similarity. To conclude, the phylogenetic analysis indicated that there is a wide variation among the *M. phaseolina* isolates subjected in this study and also its polyphyletic nature. However this phylogenetic analysis has not grouped the *Macrophomina phaseolina* isolates based on their geographical origin or the host from which they were isolated which is very important from the management point of view (Plate1).

Tarakanta *et al.* (2003) [13] showed that single RAPD primer A13 could be used to identify and discriminate several isolates of *M. phaseolina* and *Fusarium* sp. obtained from 20 hosts including soybean, cotton, chickpea and safflower. Rajkumar (2004) [9] observed that ten isolates of *M. phaseolina* representing Dharwad and Bijapur region proved pathogenic to sorghum, molecular profiling using RAPD markers indicated that the genetic differences among isolates and were species specific finger print to *M. phaseolina* was identified. Isolates were grouped using molecular and virulence data.

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