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RAPD Analysis of Indian Isolates of Rice Sheath Blight Fungus *Rhizoctonia solani*

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Rice sheath blight fungus *Rhizoctonia solani* has a wide host range and is highly variable in pathogenecity, sclerotial production and cultural characteristics. In India, breeding for sheath blight resistant cultivars has been a priority area of research. However, lack of adequate information about the genetic variability of the fungal populations occurring in India, non-availability of appropriate markers and the non-availability of resistant donors are some of the limiting factors to achieve this objective. To assess the genetic variability in sheath blight fungus, 18 isolates collected from different rice growing regions of India were analyzed by using random amplified polymorphic DNA (RAPD) markers. The similarity values of RAPD profiles ranged from 0.41 to 0.85 with an average of 0.66 among all the isolates. The percentage polymorphism detected per primer varied from 79.2 to 100%. All the primers could be used to fingerprint the individual isolates. The cluster analysis using unweighted paired group method with arithmetic averages could distinguish between *R. solani* isolates as well as the virulent and avirulent isolates on rice.

Key words : rice sheath blight, *Rhizoctonia solani*, RAPD.

Rice sheath blight (ShB) fungus, *Rhizoctonia solani* Kuhn has a wide host range and is highly variable in pathogenecity, sclerotial production and cultural characterstics (1). Incidence of rice ShB disease has been increasing in recent years, because of unavailability of resistant cultivars or any other suitable economic disease management measures. By hyphal anastomosis, 12 anastomosis groups (AGs) were described within *R. solani*, which have been divided into 25 subgroups. Based on morphological and pathogenic characters, AG1 was divided into three subgroups *viz.*,1A, 1B and 1C. The rice ShB fungus belongs to AG1 and subgroup 1A (2). Due to lack of suitable morphological and biochemical markers in *R. solani*, there is no information on the mechanisms by which genetic variability is generated. Lack of suitable markers has also hindered studies on the population dynamics of the pathogen in soil and on the epidemiology of the pathogen. In absence of suitable morphological and physiological characteristics, identification of isolates of *R. solani* has proved very difficult, impeding resistance breeding efforts and other disease management strategies. With the advent of various molecular marker technologies, the studies in genetic diversity in plant pathogens have become feasible.

RAPDs have been successfully used to study populations of various plant pathogenic fungi (3,4). Genetic variation in isolates of *R. solani* AG2, 3, 4 and 8 has been analyzed using RAPDs and it was showed that all of the anastomosis groups and subgroups could be distinguished, though there was considerable variation within the groups (5). Genetic variation within the AG8 and AG9 was also studied using RAPD (6,7). The main advantages of the RAPD are the speed and the simplicity of the technique, and the fact that no prior DNA sequence information is required to generate the primers (8). Genetic differences between AGs and subgroups are also demonstrated with isozymes, pectic zymograms (ZG), Restriction Fragment Length Polymorphism (RFLP) analysis of ribosomal RNA genes (rDNA), PCR based methods and ribosomal internal-transcribed spacer (ITS) sequences (9-12). Various molecular markers were used to study genetic variation in different AGs, out of which, isozyme, RAPD, electrophoretic karyotyping, single copy nuclear (scn) RFLP were reported to be more suitable for diversity studies at population level. The rDNA RFLP, isozymes, scnRFLP and RAPD were more informative at the species level in *R. solani* (13). For Indian isolates of *R. solani*, the characterization has been done only through pathogenecity tests. Earlier, variability in five Indian isolates of *R. solani* was studied using isozymes (14). We report here the analysis of random amplified DNA polymorphism in 18 isolates of rice ShB fungus collected from various rice growing regions of India.

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Materials and Methods

The isolates of *R. solani* used in the study and their geographic locations are listed in Table 1. Stock cultures were obtained by single sclerotial culture and were maintained on the potato dextrose agar. All the isolates were grouped under anastomosis group 1 (AG1), but for one isolate (ALM) (15).

Table 1. Geographic location and virulence pattern of the Indian rice sheath blight fungal isolates

Isolate Code	Location, State	Disease Score[1]	Category
ADT	Aduthurai, Tamil Nadu	8.0	Virulent
ALM	Almora, Uttar Pradesh	0.0	Avirulent
CNM	Chinsura, West Bengal	8.0	Virulent
FBZ	Faizabad, Uttar Pradesh	8.0	Virulent
IAR	IARI, New Delhi	6.3	Moderately virulent
KPL	Kapurthala, Punjab	6.4	Moderately virulent
MCP	Moncompu, Kerala	7.0	Moderately virulent
MPM	Machilipatnam, Andhra Pradesh	7.3	Moderately virulent
MTU	Maruteru, Andhra Pradesh	9.0	Virulent
PAT	Patna, Bihar	7.3	Moderately virulent
PBR	Port Blair, Andaman	7.3	Moderately virulent
PNT	Pattambi Uttar Pradesh	7.3	Moderately virulent
PTB	Pattambi, Kerala	7.0	Moderately virulent
RNR	Rajendranagar, Andhra Pradesh	7.3	Moderately virulent
RS5	Chennai, Tamilnadu	6.6	Moderately virulent
RSP	RS Pura, Jammu and Kashmir	1.0	Avirulent
TNL	Tenali, Andhra Pradesh	8.3	Virulent
TTB	Titabar, Assam	7.3	Moderately virulent

[1]Disease Score (Score) = 0-9 scale of Standard Evaluation System (17)
(0-3.9: avirulent, 4-7.9: moderately virulent, 8-9: virulent).

Virulence test — Initially four rice cultivars (IR50, Kavya, Swarnadhan and Vikramarya) were screened to study the pathogenecity pattern of all the 18 isolates. Then, all the isolates were screened in a glass-house on IR50 (the most susceptible) in three replications, following the method of Bhaktavatsalam *et al* (16). One week after inoculation, the plants were scored using the standard evaluation system (score 0-9) (17). Based on the scores, isolates were classified as highly virulent (8.0-9.0), moderately virulent (4-7.9) and avirulent (0-3.9).

Isolation of genomic DNA — Actively growing mycelial plugs were inoculated in 25 ml of potato dextrose broth and incubated for 7 days at 27°C. DNA was extracted from 1 g of mycelial mat according to the modified protocol reported by Bennett and Nair (18). The crude DNA was purified by RNase treatment, and ethanol precipitated and dissolved in TE buffer, pH 8.0 (10mM Tris, 1mM EDTA).

RAPD analysis — Ten random decamer primers *viz.*, OPA06, OPA10, OPB01, OPC01, OPE20, OPF06, OPH18, OPQ01, OPR01 and OPZ20 obtained from the Operon Technologies Inc., USA, were used for PCR based amplification of the template DNA of the isolates. The amplification reactions were performed in a volume of 25μl containing 2.5 μl of 10x Taq DNA polymerase buffer, 2.5 U Taq polymerase (Bangalore Genei, India), 3.0 μl of 1.25mM dNTPs each, 15 ng of a single random primer and 20 ng of genomic DNA. The thermo-cycling profile consisted 1 cycle of initial denaturation at 94°C for 1 min followed by 34 cycles of 94°C for 1 min (denaturation), 36°C for 1 min (primer annealing), 72°C for 2 min (extension), followed by a final extension at 72°C for 6 min on the thermocycler model PTC 100-60 (MJ Res. Inc., USA). To visualize the profile of thus amplified products, a 10 μl aliquot from each of the reaction products was electrophoresed in 1.1% agarose gel (w/v) in 1x TBE, stained in ethidium bromide (1μg ml^{-1}), and photographed using a UV transilluminator with the Polaroid camera attachment. One kilo-base DNA ladder (Stratagene, USA) was used as the molecular size marker. The patterns generated were tested for their reproducibility.

Statistical analyses — A binary matrix of presence or absence (1/0) of polymorphs of a specific molecular weight in all the isolates with each primer was compiled.

Based on the matrix, simple matching coefficients (similarity values) between all possible paired combinations of the isolates were calculated as suggested by Sokal and Michener (19). The average similarity values were calculated for all pair-wise comparisons within and between the virulent, moderately virulent and avirulent groups. The similarity values were subjected to the sequential agglomerative hierarchal nested (SAHN) clustering using the unweighted pair-group method, arithmetic average (UPGMA) employing NTSYS-PC version 2.0 (20) to generate dendrogram displaying genetic relatedness among the isolates. Bootstrap values for the branches of the phenogram were generated using the Winboot program (21). The goodness of fit for the phenogram was determined by computing a cophenetic value matrix using the Cophenetic (ultrametric) (COPH) module and comparing this matrix with SAHN tree matrix using the matrix comparison (MXCOMP) module.

Results and Discussion

Virulence test — The 18 isolates of *Rhizoctonia solani* were classified as virulent, moderately virulent and avirulent types, based on their reaction produced on a susceptible rice cultivar IR50 (Table 1). The isolates ADT, CNM, FBZ, MTU and TNL were assessed as virulent, the isolates ALM and RSP as avirulent, and the remaining 11 as the moderately virulent isolates.

RAPD polymorphism — Of the ten primers tried, the primer OPA06 failed to effect any amplification in all the 18 isolates tested. Other nine primers generated PCR products ranging from 0.34 to 2.0 kb. Of the 247 RAPD bands scored, only 10 bands (4%) were common among all the isolates. The percentage polymorphism detected per primer varied from 79.2 to 100% (Table 2). Some of the bands produced by the virulent isolates were not produced by the avirulent isolates. Similarly, some bands were produced by the avirulent isolates only. The primers OPC01, OPE20, OPH18, OPQ01 produced 100% polymorphic bands indicating their suitability for fingerprinting of the Indian rice ShB isolates. In some cases, though some unique bands were recorded, these unique bands may not be taken for identification of individual isolates, as their uniqueness may be challenged if more isolates are screened.

RAPD polymorphism and virulence — About 6.8% of the total bands were shared by all the virulent isolates. The present study could identify a few RAPD markers to differentiate between the ShB isolates that are virulent and avirulent on rice. The primers OPA10, OPB01, OPF06, OPR01 and OPZ20 produced at least one polymorph each that was common for all the isolates, while the primers OPA10, OPB01, OPE20, OPH18, OPQ01, OPR01, OPZ20 produced the bands specific for the virulent isolates only (Fig. 1). Thus RAPD analysis provided a quick method of not only differentiating among the isolates but also between the virulent and avirulent isolates. To validate the aforementioned association, a rice sheath blight isolate (RS319), unknown whether virulent or not, was subjected to RAPD analysis with the

Table 2. Summary of the RAPD data of rice sheath blight fungal isolates obtained with nine primers

Primer	Total No. of bands	% of bands polymorphic to all isolates	% of bands common to all the isolates	Bands common only for the virulent and moderately virulent isolates	
				Percentage	Size in bp
OPA10	35	94.3	5.7	5.7	990 340
OPB01	36	97.2	2.8	13.8	2030 1800 1300 1278 1200
OPC01	27	100	0.0	0.0	-
OPE20	21	100	0.0	9.5	1500 398
OPF06	24	79.2	20.8	0.0	-
OPH18	34	100	0.0	2.9	1283
OPQ01	20	100	0.0	10	1714 1501
OPR01	23	95.7	4.3	13	1664 1400 1225
OPZ20	27	96.3	3.7	7.4	1639 858

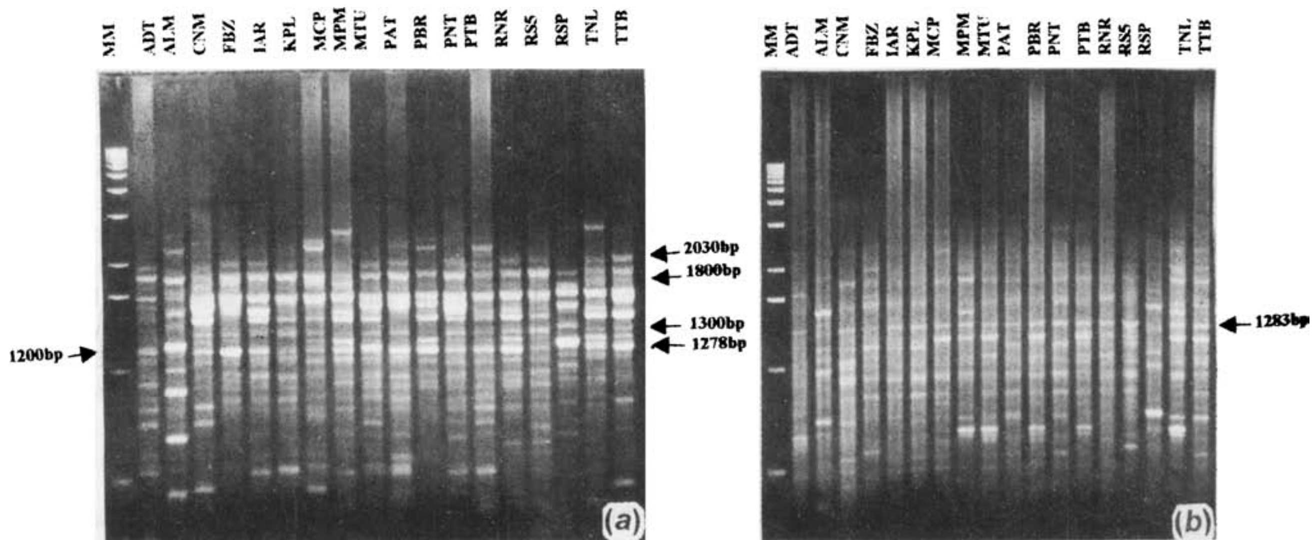

Fig. 1. RAPD patterns of 18 Indian sheath blight fungal isolates; **(a)** with OPH18 primer, and **(b)** with OPB01 primer. Bands specific to virulent isolates are marked. MM : 1 kb ladder.

OPA10 primer and the isolate was identified as a virulent one. Subsequent standard glass-house screening technique confirmed the virulent nature of the isolate. However, there was no difference in RAPD patterns between the virulent and moderately virulent isolates. Similar observations were reported by Yang *et al* (6) on *R. solani* isolates of AG8. The presence of bands specific for virulence group can be exploited further for molecular identification of rice sheath blight fungal isolates. Balali *et al* (22) have reported DNA fingerprinting probe specific to isolates of *R. solani* AG3. Group specific probes can be of great value in identification of isolates and would be very useful in studies on population dynamics and epidemiology of the pathogen.

Genetic similarity among R. solani isolates — The similarity coefficients among RAPD profiles of *R. solani* isolates were higher than 40%. The maximum similarity value (0.85) was obtained between IAR and KPL, and the minimum (0.41) was obtained between RSP and PBR and RSP and TTB isolates. The average similarity value was the highest (74%) among the isolates of moderately virulent group with 11 isolates, followed by the virulent group with 5 isolates (70%) and the least in the avirulent group with only two isolates (62%). This trend observed may be due to the influence of the number of isolates falling under each group. On the other hand, the between group average similarity value was the highest between the virulent and moderately virulent groups (73%) and the least between the avirulent and virulent as well as the avirulent and moderately virulent groups (46%), indicating genetic distinctness of the avirulent isolates from the virulent and moderately virulent isolates. The high similarity values obtained among the virulent isolates may be attributed to the clonal nature of the propagation prevalent in the pathogen. According to Milgroom (23) asexual reproduction results in offspring that are genetically identical to each other and their parent (or nearly so, but for mutation), the result of which is a clonal population structure. On the other hand, the meiotic phase associated with sexual reproduction results in independent assortment of chromosomes and recombination within chromosomes, causing greater genetic diversity. In the light of larger variation between AGs and smaller variations between the isolates within AGs, probably both sexual and asexual reproduction may be occurring simultaneously in *R. solani*. However, to verify this, a larger set of isolates in a given population will have to be tested. Analysis of pathogen populations of *R. solani* in rice based cropping systems using RFLP of mitochondrial DNA and PCR fingerprinting with primers targeted to variable number tandem repeats (VNTR) varied around one single pattern suggesting they are relatively closely related (24). Yang *et al* (6,7) have reported that isolates of AG8 within ZG group were characterized by identical RAPD patterns suggesting that isolates were clonal or closely related. Another case of possible clonality of multiple isolates of AG3 isolates was presented by Balali *et al* (10), wherein, for the 136 isolates examined, only 38 DNA fingerprint patterns could be established and one DNA fingerprint was associated with approximately 30% of the isolates. Together, these studies suggest clonal population structure for AG3 and AG8.

The limited genetic variability and recovery of similar genotypes from various geographic regions in the present study are strongly indicative of clonal population structure for AG1 as well, and support the conclusions of earlier studies that AGs cluster together genetically and there is no correlation between geographical location and AG. The choice of the multilocus genetic marker like RAPD showing high levels of variation to assess genetic identity among isolates suspected being clonal in origin is well rewarded in the present study, supporting the inferences of Cubeta and Vilgalys (13) that methods such as RAPD profiling and DNA fingerprinting could be used in conjunction with hyphal anastomosis testing to assess hypothesis concerning clonal structure in the populations.

Cluster analysis — The SAHN/UPGMA analysis of the RAPDs also grouped the isolates into two major clusters bifurcating at 0.46 similarity (Fig. 2). The obtained cophenetic correlation value of r = 0.976 indicated that the UPGMA cluster analysis was statistically significant. Clear separation of the two clusters of the avirulent and virulent isolates based on RAPD polymorphism is in good agreement with and confirms the observations of the virulence test. A close association between the virulence and molecular polymorphism based on RAPDs in the present study suggests the utility of molecular typing for quick diagnosis of virulent isolates. Two avirulent isolates ALM and RSP contributed a major cluster and the remaining 16 virulent isolates formed the loose second major cluster. The latter cluster had the isolates branching from each other between 0.70 to 0.85 similarity, indicating relative genetic uniformity in the isolates at the RAPD loci analyzed. Bootstrap analysis showed 100% support

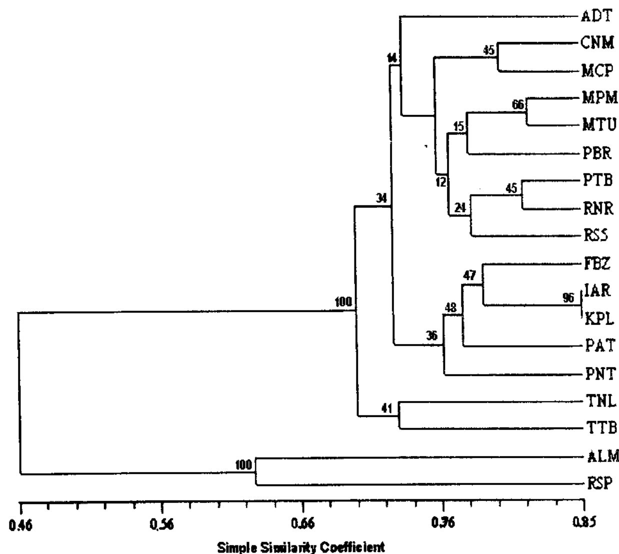

Fig. 2. Phenogram of 18 sheath blight fungal isolates using nine RAPD primers by UPGMA based on Simple matching coefficients.

to the branching of avirulent and virulent groups. Also, low bootstrap values within the virulent cluster again indicate lack of robustness in further sub-grouping among the isolates of the group.

At International Mycological Institute (IMI), UK, one of the two avirulent isolates used in the present study, ALM from Almora is identified as *Rhizoctonia oryzae-sativae*, one of the causal agents of *Rhizoctonia* sheath complex in rice, while all the other 17 isolates were identified as *R. solani* (15). The separation of this isolate from the rest of the *R. solani* isolates by cluster analysis, evidently supports the credibility of the RAPD technique used in the present study. Though the other isolate RSP was identified as *R. solani*, its avirulent nature and its out-grouping from the other virulent isolates along with ALM, suggests that it may be belonging to subgroup 1C (micro-sclerotial type), while the rest of isolates may belong to either of the other subgroups in AG1 *viz.,*1A (sasaki type), 1B (web blight type) . This supports the observations of Kuninaga and Yokasawa that the isolates from 1C type fuse well with 1A and 1B types and they are not pathogenic on rice (25). Kuninaga *et al* (26) have reported existence and role of bridging isolates in the evolution of *Rhizoctonia* by transferring genetic material from one isolate to other normally incompatible isolates that belong to different anastomosis groups. The isolate RSP may be considered as a possible bridging isolate between *R. oryzae-sativae* and *R. solani.*

In summary, our results demonstrate that RAPD markers have potential as a means of identifying the rice sheath blight fungal isolates and increasing our understanding the ecology and biology of this fungus by providing measurements of genetic relatedness and variation within the isolates. Though the primers and isolates used were a few in number, they could effectively differentiate the virulent isolates from avirulent isolates and could distinguish and characterize all the isolates studied at DNA level.

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