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*Full Length Research Paper*

# Characterization of molecular variability in *Rhizoctonia solani* isolates from different agro-ecological zones by random amplified polymorphic DNA (RAPD) markers

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***Rhizoctonia solani*** **is a plant pathogenic fungus which is the causal organism of sheath blight of rice. Yield losses due to the sheath blight, caused by** ***R. solani*** **Kuhn is reported to range from 5.2 to 50%, depending on environmental conditions, crop stages at which the disease appears, cultivation practices and cultivars in India. Rice sheath blight has become a major constraint to rice production during the last two decades. In India, breeding for sheath blight resistant cultivars has been a priority area of research. Sheath blight disease caused by** ***R. solani*** **Kuhn was first recorded as minor disease of rice in West Bengal (a major rice growing province of India), later the disease was referred to as a major one in West Bengal, probably second only to blast in its crop damage potential. The present work was done to assess the molecular variability in 22 isolates of sheath blight fungus, collected from four different ecological regions of West Bengal, ranging from coastal, alluvial, red-lateritic to terai belts using random amplified polymorphic DNA (RAPD) markers. A total of 267 reproducible and scorable polymorphic bands ranging approximately as low as 200 bp to as high as 1500 bp were generated with eight RAPD primers. The similarity values of RAPD profiles ranged from 0.41 to 0.94 with an average of 0.67 among all the isolates. Most of the isolates collected from similar agro-ecological location clustered together in the present study. Our results indicate high genetic variability in the pathogen population in different epidemiological regions of West Bengal. Our study also reveals host-specific banding profile for the two isolates RS21 and RS22, which were collected from cabbage and were confirmed to belong to anastomosis group 4 (AG4).**

**Key words:** *Rhizoctonia solani,* rice, molecular variability, randomly amplified polymorphic DNA.

## INTRODUCTION

*Rhizoctonia solani* Kuhn (Teleomorph: *Thanatephorus cucumeris*), is a widely distributed soil-borne plant parasitic-saprophytic fungus (Mirmajlessi et al., 2012). Sheath blight (ShB) disease caused by *R. solani* Kuhn, is becoming a major constraint to rice production, especially in the intensified cultivation system (Jayaprakashvel and Mathivanan, 2012). The emergence of *R. solani* as an economically important rice pathogen has been attributed to the intensification of the rice-cropping systems with the development of new short-statured, high-tillering, high yielding varieties, high plant densities and an increase in nitrogen fertilization. These factors promote disease spread by providing a favorable microclimate, due to a denser leaf canopy with an increased leaf-to leaf and leaf-to-sheath contact. Incidence of rice sheath blight disease has increased in recent years, because of the unavailability of resistant cultivars or any other suitable economic disease management measures. Sheath blight disease caused by *R. solani* Kuhn was first recorded as minor disease of rice in West Bengal (Roy, 1978). Later

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**Table 1.** Geographic locations of the Indian rice sheath blight isolates.

| Agroecological zone | Isolate |
|---|---|
| Alluvial | RS1, RS9, RS15, RS16, RS17, RS18, RS19, RS20, RS21*, RS22* (isolated from cabbage) |
| Red-Lateritic | RS2, RS3, RS5, RS6, RS7, RS8, RS9, RS10, RS11, RS12, RS13, RS14 |
| Coastal | RS4 |

the disease was referred to as a major one in West Bengal, probably second only to blast in its crop damage potential (Biswas, 2000). Yield losses due to this disease is reported to range from 5.2 to 50%, depending on environmental conditions, crop stages at which the disease appears, cultivation practices and cultivars in India (Rajan, 1987; Sharma and Teng, 1996). In India, breeding for sheath blight resistant cultivars has been a priority area of research. However, lack of adequate information on the genetic variability of the fungal population occurring in India, non-availability of resistant donors and the non-availability of appropriate markers are some of the limiting factors for developing suitable strategies for control measure (Neeraja et al., 2002). Till date, no reportable data exists on the genetic diversity among the sheath blight isolates of *R. solani* in West Bengal. Diversity within rice sheath blight isolates has been studied by morphological characterization and pathogenicity testing and also by various molecular techniques. Perhaps, evaluation of the genetic diversity in pathogen isolates has been an initial step towards understanding the population structure. Molecular techniques have become reliable and are highly suitable tools for identifying pathogen species and for assessing genetic variation within collections and populations (Sundravadana et al., 2011). With the advent of various molecular marker technologies, the studies of genetic diversity in plant pathogens have become feasible. Random amplified polymorphic DNA (RAPD) markers have been successfully applied to numerous filamentous fungi in different fields of experimental mycology (Pollastro et al., 2000). RAPD offers a promising, versatile and informative molecular tool to detect genetic variation within population of plant pathogens (Chiochetti et al., 1999). The main advantages of RAPD are the speed and the simplicity of the technique, and also the fact that closely related strains of a pathogen can be distinguished without prior knowledge of the nature of polymorphic regions by the use of RAPD. Variability in molecular characters is used for determining resistant cultivars (Thirumalasamy et al., 2006) and for the evaluation of the germplasm resistant line (Shekhar et al., 2006). Understanding the genetic structure of pathogenic fungi is critical for developing appropriate strategies for disease management; however, little is known about the genetic structure of *Rhizoctonia* spp. in natural population (Vilgalys and Cubeta, 1994). Thus, the present study wasundertaken to assess the molecular variability using RAPD markers to distinguish the isolates collected from different agro-ecological zones of West Bengal.

## MATERIALS AND METHODS

### Fungal isolates and culture maintenance

Twenty two (n = 22) isolates of *R. solani* used in the study were collected from paddy fields of different agroecological regions of West Bengal. Stock cultures were obtained by single sclerotial culture and were maintained on the potato dextrose agar (PDA) and designated as RS1 to RS22 (RS– *R. solani*). *Rhizoctonia* isolates were confirmed both by their morphological as well as by ITS sequencing. The isolates of *R. solani* used in the study and their geographic locations are listed in Table 1.

### Isolation of genomic DNA

Actively growing mycelial plugs were inoculated in 50 ml of potato dextrose broth and incubated for 5 days at 28°C. High quality genomic DNA isolation was done for ten isolates of *R. solani* according to Guha et al. (2006). Isolated DNA was quantified on 1% Agarose gel. Final working DNA concentration for setting up PCR was about 20 to 30 ng/ul.

### RAPD analysis

In preliminary experiments, a total of 20 primers were screened for RAPD analysis, of which eight were selected and used for the present study based on the reproducibility of the PCR amplification. All PCRs with these eight RAPD primers were repeated three times to assess reproducibily. These primers amplified fragments of 200 to 1500 bp. These primers: OPW05 (5'-GGCGGATAAG-3'), OPK14 (5'-CCCGCTACAC-3'), OPK20 (5'-GTGTCGCGAG-3'), OPW11 (5'-CTGATGCGTG-3'), OPS19 (5'GAGTCAGCAG-3'), OPX13 (5'-ACGGGAGCAA-3'), OPX01 (5'-CTGGGCACGA-3'), OPD12 (5'CACCGTATCC-3') were used for PCR-based amplification of the template DNA of the isolates. The amplification reactions were performed in a volume of 25 µl containing 2.5 µl of 10x Taq DNA polymerase buffer, 2.5 U Taq polymerase, 4 µl 2.5 mM dNTPs mix, 20 ng of a single random primer and 30 ng of genomic DNA. The thermocycling profile consisted of 1 cycle of initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 30 s (denaturation), 36°C for 1 min (primer annealing), 72°C for 1 min 30 s (extension), followed by a final extension at 72°C for 7 min. Amplified PCR products were subjected to 1.5T% agarose gel electrophoresis with 1.0x TBE as running buffer. The banding patterns were visualized under UV trans-illuminator with ethidium bromide staining. The DNA banding profiles were documented in the gel documentation system and compared with 1 kb DNA ladder. The patterns generated were tested for their reproducibility. Each amplified band was considered as RAPD marker and recorded for all isolates. Data was entered using a matrix in which all the

observed bands or characters were listed. The RAPD pattern of each isolate was evaluated; assigning character state '1' to all the bands that could be reproducible and detected in the gel and '0' for the absence of the band. The data matrix thus generated was used to calculate Jaccard similarity coefficient for each pair wise comparison. The similarity coefficients were subjected to Sequential Agglomerative Hierarchical Nested (SAHN) clustering using the unweighted pair-group method on arithmetic average (UPGMA) cluster analysis employing NTSYS-PC version 2.0 to generate dendrogram displaying genetic relatedness among the isolates.

## RESULTS

### DNA profile analysis

RAPD analysis was carried out on the 20 *R. solani* isolates together with two control strains of *R. solani* of different anastomosis groups (AG4). A total of 267 reproducible and scorable polymorphic bands ranging approximately as low as 200 bp to as high as 1500 bp were generated with eight RAPD primers and they could well display the genetic polymorphisms among the isolates. Parts of the amplification results are shown in Figure 1A and B. Out of the 267 RAPD bands scored, only 12 (5%) were common among all the isolates. Although in some isolates, unique bands were found, but they were not considered for identification of these isolates as their uniqueness may be challenged on increasing the number of isolates while screening. This was in accordance with the findings of Neeraja et al. (2002). The similarity values of RAPD profiles ranged from 0.41 to 0.94 with an average of 0.69 among all the isolates.

### Genetic similarity and cluster analysis

A dendrogram was generated by SAHN subroutine clustering using Jaccard coefficient as presented in Figure 2. The estimated similarity ranged from 41 to 94%, reflecting wide range of variability among the diverse collection of isolates at their molecular level. The maximum similarity value (0. 94) was obtained between isolates RS2 and RS3, both of which belong to red-lateritic region. Based on UPGMA analysis, all the isolates were classified into three major clusters; A, B and C. The first group consists of 18 isolates, which further constituted two subgroups A1 and A2. Subgroup A1 contained 14 isolates (isolate nos. RS1, RS2, RS3, RS4, RS5, RS6, RS7, RS8, RS9, RS10, RS11, RS12, RS13 and RS14) with an average overall similarity which is more than 70% genetic. All these isolates were collected from red-lateritic zone of West Bengal, except RS1, RS9 and RS4 which belong to alluvial and coastal zones, respectively. Subgroup A2 is formed from 4 isolates (isolates RS15, RS16, RS18 and RS20), all of which belonged to alluvial zone. They shared the highest genetic similarity of 82% between isolate RS15 and RS18. The second major cluster, B consisted of two isolates (isolate nos. RS17 and RS19), both of which belonged to alluvial zones but shared a genetic similarity of less than 60% among them and even less than 45% between some other isolates of subgroup A2 which also consisted of isolates belonging to similar agroecological region. The two control strains of *R. solani*, RS21 and RS22, belonging to different anastomosis group (AG4), did not share any of the groups and formed a separate major cluster C. These two isolates shared a genetic similarity of 94%. Overall, RS19 and RS16 were most diverse, showing a similarity of only 41%, followed by RS16 and RS7 which shared a genetic similarity of 43%. The present study could also identify distinct banding pattern with the isolates RS21 and RS 22, which belonged to different anastomosis groups and were obtained from cabbage.

## DISCUSSION

RAPD-PCR has been successfully used for studying inter and intra-specific variability among populations from different as well as the same geographical regions (Sundravadana et al., 2011). The results of our present study indicate significant level of variation among the 22 isolates of *R. solani*. The range of variation estimated is from 41 to 94%, among the 22 isolates. It is noteworthy that such wide variability has been found only among the few isolates used in our present study. On analyzing 24 isolates of *R. solani* from different locations and crops using 11 RAPD primers, Sharma et al. (2005), reported a similarity index of 0.16 to 0.53 and the markers revealed host-specific band profiles. Our present study also reveal host-specific banding profile for the two isolates RS21 and RS22. Most of the isolates collected from similar agroecological location clustered together in the present study. This lends support to the findings of Toda et al. (1999) and Sharma et al. (2005). Moreover, another significant finding from the present study is that although the isolates in cluster A2 and major cluster B were collected from similar geographical regions, it is noteworthy that the two isolates RS17 and RS19 of cluster B shared less than 45% similarity with the isolates of cluster A2 and thus formed a separate major cluster. These results are in consonance with that of Sharma et al. (2005), indicating high genetic variability in the pathogen population in different epidemiological regions of West Bengal. High genetic variability present in *R. solani* population has significant implications for effective control and resistance breeding strategies against sheath blight of rice disease in India. The separate cluster C formed by two isolates, evidently supports the credibility of the RAPD technique used in the present study for distinguishing different anastomosis group of *R. solani*. Another exceptional finding from the present study showed the isolate RS1, RS9 and RS4 fall within the same cluster A, although, these isolates were obtained from different agro-ecological zones. This might be due to

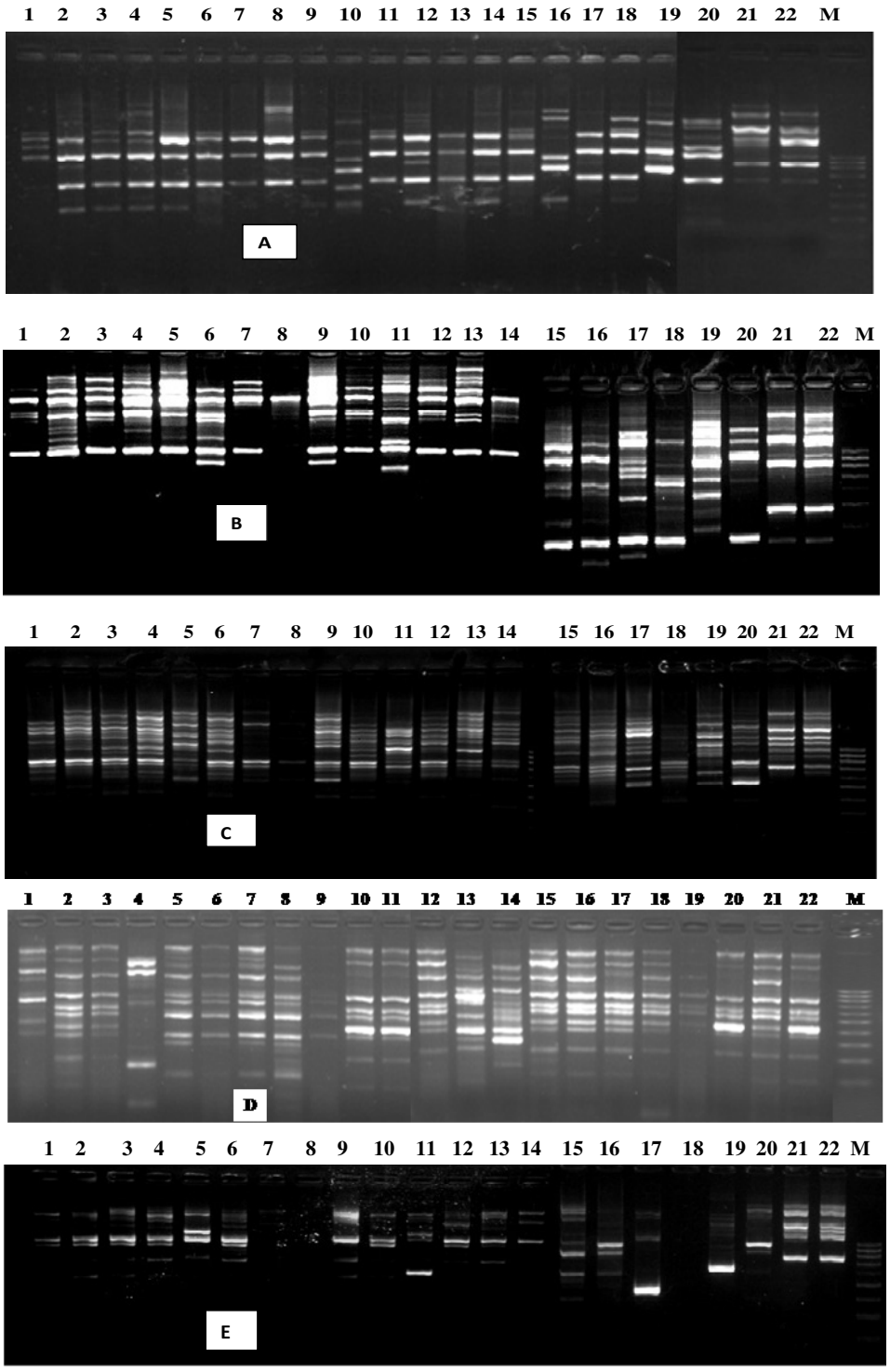


**Figure 1.** RAPD patterns of 22 sheath blight isolates; A, with OPW05 primer; B, with OPK14 primers; C, with OPK20 primer; D, with OPD12 primer; E, with OPX01 primer.

the presence of shared clones among different populations. Our results are in conformity with the findings of Linde Rosewich et al. (1999) who found that in Texas, clones were identified in populations separated by up to 280 km using RFLP fingerprint probe. It becomes clear that managing Rhizoctonia diseases therefore remains a major challenge despite the recent useful knowledge developed on the biology and epidemiology of the

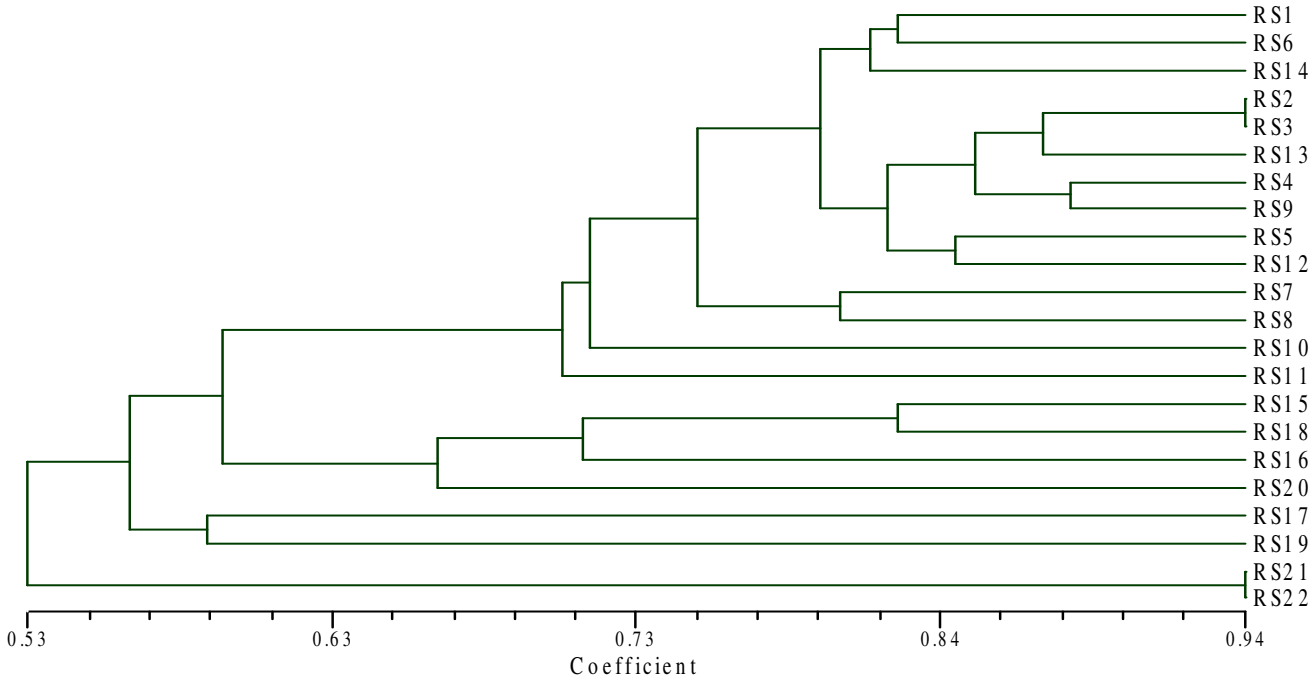


**Figure 2.** RAPD based dendrogram of *R. solani* isolates constructed using unpair group arithmetic average (UPGMA) and similarity matrices computed according to Jaccard's coefficient.

pathogen (Tsror, 2010). Also, it could be a cause of great concern for the plant pathologists because a day might come when a single fungicide would not be effective in controlling all the isolates even if they belong to same host plant and similar agro-ecological conditions.

In summary, our results demonstrate that RAPD markers have potential as a means of identifying the rice sheath blight fungal isolates and also increased our understanding of the ecology and biology of this fungus by providing measurements of genetic relatedness and variation within the isolates (Neeraja et al., 2002). Though the primers and isolates used in this study were relatively few in number, they could effectively establish the molecular variability among the sheath blight isolates of West Bengal, suggesting that RAPD can still be considered as a reliable, efficient and effective marker technology for determining genetic relationships in *Rhizoctonia* spp.

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