Research Paper

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Molecular Analysis of Genetic Variability in Rhizoctonia solani AG-7 Isolates Affecting Cotton Crop (Gossypium hirustum) in Semi Arid Tropics

Biotechnology

KEYWORDS : Universally primed-PCR, fingerprinting, Rhizoctonia solani, anastomosis group

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ABSTRACT

Rhizoctonia disease, caused by Rhizoctonia solani is one of the most important fungal diseases in cotton fields in Northern India. A total of 25 isolates of Rhizoctonia solani AG-7 obtained from diseased cotton seedlings showing typical symptoms of damping-off were characterized using universally primed PCR (UP-PCR) fingerprinting. Of 35 primers tested, two primers produced polymorphic amplification patterns with individual-specific bands. Amplification products obtained from the two UPs AA2M2 and L45 ranged between 0.3kb to 1.9kb. A combined dendrogram of 25 Rhizoctonia solani AG-7 isolates was prepared by using similarity coefficients of 30 DNA markers generated by UP primer AA2M2 and L45. At 0.27 similarity index the isolates divided into two groups. There was high genetic variability among the isolates collected from different locations. The results suggest that UP-PCR having long primer and higher annealing temperature, would be very useful, more sensitive and reliable marker in characterizing genetic diversity in R. solani.

INTRODUCTION

Rhizoctonia solani Kühn causes seedling damping-off, sore shin/ root rot of cotton seedlings (Gossypium hirsutum L.) (1). R. solani is composed of genetically isolated groups. Rhizoctonia spp. is major threats in cotton production worldwide, causing damping-off on young seedlings of cotton crop. Despite the significance of the cotton crop in several regions of the world and high economic importance of Rhizoctonia spp. attacks on this crop, only a few reports have characterized Rhizoctonia spp. isolated from this host. According to these reports R. solani AG-4 is the predominant anastomosis group (AG) infecting cotton crop. (2, 3). In addition, isolates of R. solani AG-7 (4, 5, 6) and AG-13 (1) have been recovered from diseased cotton seedlings. Characterization of Rhizoctonia populations within a cropping region can help in the development of environmentally friendly and sustainable crop management systems. Although the occurrence of Rhizoctonia spp. is well documented in India, there are no studies related to the occurrence of AGs and subgroups of R. solani. Therefore, this study was initiated to characterize the isolates of R. solani. AG-7 isolated from cotton seedlings.

The UP-PCR fingerprinting technique is a useful tool for the characterization and grouping of fungal strains in order to explain their genetic relatedness (7). UP-PCR markers have been used in studies on the genetic variation in fungal pathogens, including Fusarium spp. (8, 9), in monitoring strains of interest as well as for identification of Trichoderma strains (7) and to investigate the relationships among timothy (Phleum retense L.) genotypes (10). These circumstances provide an interesting opportunity to survey the diversity of R. solani in the cotton fields. The aim of this study was to analyze the genetic and biological variability of R. solani infecting cotton crop in the field of semi arid tropic region of northern India.

MATERIALS AND METHODS

Fungal strains and DNA extraction

Infected cotton root samples showing typical symptoms of root rot were collected from 50 fields across the cotton growing regions of northern India in the year of 2008, 2009 and 2010. Fungal strain of Rhizoctonia solani AG 7 were isolated from infected cotton roots and deposited in the Microbial Type Culture Collection (MTCC) IMTECH, Chandigarh, India, the MTCC number given to the culture was 9993. The nucleotide sequences of Rhizoctonia solani strain MV-1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8 ribosomal RNA gene, complete sequence and internal transcribed spacer 2 partial sequence was deposited at NCBI genbank under accession number JX576189.1. The fungal cultures were grown on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) in the dark and maintained in a growth chamber adjusted to 25-28°C for 5 days. Mycelium was separated from the agar substrate using a sterile spatula, and ground in liquid N2 with a sterile mortar and pestle. Aliquots of approximately 50mg of ground mycelium were used for DNA extraction using the method of Vandemark et al. (2000) (11) with minor modifications. DNA was suspended in 80 µl of sterile distilled water. DNA concentration was estimated by means of absorbance at 260 nm and 280 nm wavelength by using Nano-Drop spectrophotometer (ND-1000).

UP-PCR amplification

The UP-PCR primers were screened from the literature and procured from OPERON Technology, USA (Table 2). UP-PCR was performed as described by Lubeck et al., (1999) (7). Alternatively, UP-PCR was performed in an automated thermal cycler (PTC-100TM, MJ Research Inc., USA). The reaction mixture (25-µl) contained 50 ng of fungal genomic DNA, 0.4 mM dNTPs, 3.5 mM MgCl2, 0.4µM of primers, 1.25 units of Taq DNA polymerase (Banglore genei, Banglore) and 1×PCR buffer (10 mM Tris- HCl, pH 8.3, 50 mM KCl, and 0.1%Triton X -100) (Banglore genei, Banglore). UP-PCR conditions were as follows: first denaturation step at 94°C for 4 min followed by 30 cycles of denaturation at 92°C for 50 s, primer annealing at 52 to 56°C for 30 s, and primer extension at 72°C for 60 s. A final extension was performed at 72°C for 5 min. Amplification products were electrophoresed on 1.7 % agarose gel (SRL) in 0.5× TBE buffer solution. Gel was photographed using Gel Documentation System (Gene Genius Syngene, UK). Annealing conditions and concentration of Mgcl2 were optimized by performing a series of trial PCR amplifications for different primers. In UP-PCR, primers AB (3-2), AA2M2, L45, AS4, As15, As15inv, Fok1, HE 45, AS-19 and L 21, were tested (Table 2). The UP primers were used individually and in pairwise combinations (a total of 21 combinations).

Statistical analysis

The primers that produced reproducible and scorable amplifications were used in the analysis. Binary matrices were analyzed by NTSYS—PC (Version 2.02; Exeter Biological Software,

Setauket, NY). Jaccard's coefficients were clustered to generate a dendrogram-using SHAN clustering programme selecting unweighted paired group method with arithmetic averages (UPGMA) (12).

Table 1 Isolates of R. solani AG 7 used in the present study

Isolate No.	Name of the Isolate	Place of Collection (State Name)	Plant Parts used for Isolation
MV1	R. solani	CCSHAU, Hisar (Haryana)	Cotton root
MV2	R. solani	Suratgarh (Rajasthan)	Cotton root
MV3	R. solani	SFCI, Suratgarh (Rajasthan)	Cotton root
MV4	R. solani	KVK, Rohtak (Haryana)	Cotton root
MV5	R. solani	Bhiwani (Haryana)	Cotton root
MV6	R. solani	CICR, Sirsa (Haryana)	Cotton root
MV7	R. solani	KVK, Bhatinda (Punjab)	Cotton root
MV8	R. solani	CAZRI (Rajasthan)	Cotton root
MV9	R. solani	PAU, Faridkot (Punjab)	Cotton root
MV10	R. solani	Suratgarh (Rajasthan)	Cotton root
MV11	R. solani	IARI, New Delhi (Delhi)	Cotton root
MV12	R. solani	Sirsa (Haryana)	Cotton root
MV13	R. solani	New Delhi (Delhi)	Cotton root
MV14	R. solani	CICR, RS, Sirsa (Haryana)	Cotton root
MV15	R. solani	Hisar (Haryana)	Cotton root
MV16	R. solani	Agricultural Research station, Durgapura (Raj.)	Cotton root
MV17	R. solani	Tohana (Haryana)	Cotton root
MV18	R. solani	RAU, Sriganganagar (Rajasthan)	Cotton root
MV19	R. solani	KVK, Faridkot (Punjab)	Cotton root
MV20	R. solani	Rewari (Haryana)	Cotton root
MV21	R. solani	Rohtak (Haryana)	Cotton root
MV22	R. solani	Sangrur (Punjab)	Cotton root
MV23	R. solani	Fatehabad (Haryana)	Cotton root
MV24	R. solani	MTCC No: 4633, Chandigarh, (Haryana)	Culture on PDA
MV25	R. solani	MTCC No: 4634, Chandigarh (Haryana)	Culture on PDA

Table 2 Sequences of oligonucleotide primers used for UP-PCR amplification of R. solani AG7

UP-PCR Primer	Sequence (5' to 3')	GC content (%)	Polymorphic bands/ isolate	Percentage polymorphis	Band Mol. weight (bp)
HE 45	GTAAAACGAGGCCAGT	50	4	36.4	1600-211
L15/AS19	GAGGGTGGCGGCTAG	73.3	19	43.2	2341-337
AS19	GAGGGTGGCGGCTAG	73.3	0	---	---
AB (3-2)	TAAGGGCGGTGCCAGT	62.5	6	66.6	950-250
AS4	TGTGGGCGCTCGACAC	68.7	5	45.4	1850-514
AS15	GGCTAAGCGGTCGTTAC	58.8	7	77.7	1680-468
AS15inv	CATTGCTGGCGAATCGG	58.8	6	60.0	1700-210
AA2M2	CTGCGACCCAGAGCGG	75	13	86.6	1720-220
Fok1	GGATGACCCACCTCCTAC	61.1	5	41.6	1950-300
L21	GGATCCGAGGGTGGCGGTTCT	66.6	6	54.5	1700-300
L45	GTAAAACGACGGCCAGT	52.9	13	88.0	1950-393

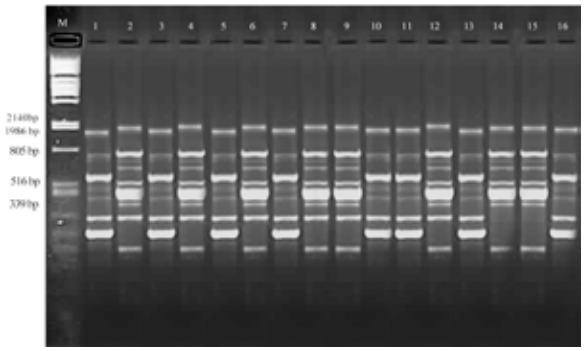

Figure 1. UP-PCR banding profiles for R. solani AG7 generated with universal primer AA2M2 Lanes 1–16 (MV1 to MV15) isolates of R. solani, respectively Lanes M: molecular weight marker (lambda phage DNA digested with PstI).

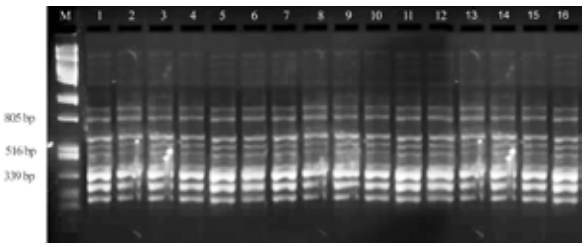

Figure 2. UP-PCR banding profiles for R. solani generated with universal primer L45 Lanes 1–16 (MV1 to MV15) isolates of R. solani, respectively Lanes M: molecular weight marker (lambda phage DNA digested with PstI).

RESULTS AND DISCUSSION

UP-PCR analysis of R. solani isolates

In the present study the diversity of R. solani isolates was evaluated through the production of polymerase chain reaction (PCR) fingerprints by use of semi-random primers i.e. universal primers (UPs) (13). The universally primed PCR (UP-PCR) uses single, 15- to 20-bp long primers that produce multiple amplification products. When compared with internal transcribed spacer (ITS) ribotyping (14) and RAPD (15), UP-PCR had the ability to better resolve the very similar R. solani strains because of the reliable generation of numerous fragments (60–100 per primer) which cover an important portion of the genome. A comparison of the data showed that universally primed PCR based markers are superior to morphological markers in detecting genetic variability among the isolates of R. solani.

Out of the 35 primers initially tested, two primers (AA2M2 and L45) consistently generated reproducible UP-PCR patterns with multiple fragments; therefore they were used for a comparative analysis of all the isolates. The annealing temperature standardized for these two primers was 560C and the MgCl2 concentration standardized for these two primers was 2.5 mM and 3.5 mM, respectively. Amplification products obtained from these two UP primers ranged between 0.3kb to 1.9kb. A combined dendrogram for 25 R. solani isolates was prepared by using similarity coefficients of 30 DNA markers generated by UP primer AA2M2 and L45. The UPGMA dendrogram demonstrated that the isolates of R. solani were grouped into two distinct clusters while the genetic similarity coefficient ranged from 0.27 to 0.83 (Fig. 3). First main cluster consisted of seven isolates while the rest eighteen isolates were grouped into second main cluster. First cluster consisted of seven isolates from states, namely, four isolates from Haryana (MV1, MV5, MV21 and MV17), two isolates from Rajasthan (MV16 and MV10) and one isolate from Punjab (MV7). Second main cluster consisted of eighteen isolates from states, namely two isolates from Delhi (MV11 and MV13), one isolates from Rajasthan (MV2). In second cluster, two isolates from Haryana (MV15 and (MV20) showed 100% similarity coefficient. The goodness of clustering method was assessed by calculating cophenetic correlation coefficient (r=0.7) using COPH modules. The Jaccard's coefficient of similarity or cophenetic correlation coefficient considered a very good fit according to Mantel statistic.

The results obtained from UP-PCR analysis in this study suggested that UP-PCR could be useful in genetic diversity studies

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and identification of various R. solani isolates. Similar results were also observed by several workers (16, 17). Upmanyu et al. (2003) also grouped 18 French bean R. solani isolates collected from different geographical regions of Himachal Pradesh into two anastomosis groups, i.e. AG-1 (AG1-AI and AG1-BI) and AG-4 (18). In conclusion, there was no clear relationship between pathogenicity, host plant and different locations. However, Singh et al., (2002) reported that pathogenic variation is related to the distribution of isolates in different climatic regions and that environment might influence pathogenic variability (19). This study shows that R. solani isolates display a high level of sequence variability using, UP-PCR analysis. UP-PCR, as expected, revealed considerable variability among the isolates belonging to different AGs and different subgroups.

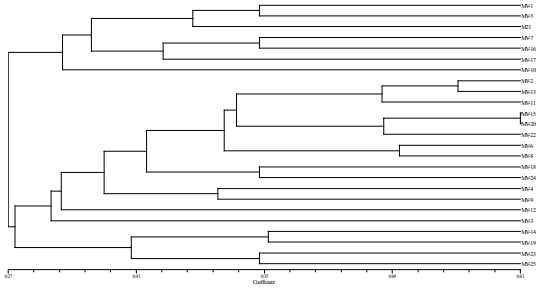

Figure 3.Cluster analysis (unweighted pair groups method using arithmetic averages) of Rhizoctonia solani strains based on DNA fingerprinting using universally primed PCR (UP-PCR).

CONCLUSION

UP-PCR fingerprinting may be used for studies of the genetic variability of other genera and species of fungal pathogens. Our results demonstrate that UP-PCR markers increased our understanding of the biology of this fungus by providing measurements of genetic relatedness and variation within the isolates.

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