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MOLECULAR CHARACTERIZATION OF *RHIZOCTONIA SOLANI* AG-7 CAUSING ROOT ROT ON COTTON CROP IN INDIA

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ABSTRACT

Rhizoctonia solani (Kuhn) is one of the most important cotton crop pathogens. *Rhizoctonia solani* anastomosis groups (AGs) 7 and 4 are proven to be the most common pathogenic fungal strains on cotton crop. AG-7 can cause root rot in plants while damping-off of seedlings is most frequently attributed to AG-4. Sequencing of the ITS (Internal Transcribed Spacer) region of ribosomal DNA was performed on northern Indian isolates in order to determine their relatedness. Sequence analysis using BLAST revealed that rDNA ITS region of MV1 [JX576189.1] was at least 98% identical to that of other AG7 isolates (AY154305.1) on the GenBank database. Bio-molecular evaluation by means of electrophoretic profile of isozyme polyglacturonase and RFLPs of ribosomal DNA internal transcribed spacers were carried out. The isolates shared a common pectic zymogram while sequence analysis of rDNA showed a closer similarity to anastomosis group-7(AG-7). Based on morphological characteristics, hyphal fusion compatibility and sequence analysis, 98% of the isolates collected belonged to AG-7 and 2% of the isolates were AG-4 *R.solani* . This is the first report of *R. solani* AG-7 infecting cotton crop in Northern India.

KEYWORDS: Cotton root rot, *Rhizoctonia solani*, anastomosis group, ITS region,

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INTRODUCTION

The root rot disease of cotton crop caused by the fungus *Rhizoctonia solani* Kuhn (teleomorph *Thanatephorus cucumeris*) is a serious disease of cotton in India especially in the Northern region. It is also reported from Bihar, Gujarat, Andhra Pradesh and Tamil Nadu. Seedling diseases are presently causing great losses to cotton producers in Northern India. Pre- and post-emergence damping-off and root decay can reduce stands by as much as 5—10% and losses of more than 24% have been recorded in Europe and USA[1]. Since the outbreak of the disease in 1935 in Punjab provinces in north India, the disease established endemically in the entire North West Indian states of Punjab, Haryana and Rajasthan causing moderate to severe losses[2]. The root rot disease is one of the most significant but highly complex diseases of cotton caused by *Rhizoctonia solani.* Detailed studies on various aspects of root rot disease have been undertaken by several workers[3, 4]. The disease affects both the *hirsutum* (American cotton) and *arboreum* (Desi cotton) species, being more serious on desi cottons[5]. The complexity and economic importance of this disease has instigated many studies in cotton-growing regions around the world. To date, 13 anastomosis groups (AGs) of *R. solani* are described in various agricultural and Horticultural crops[6]. However there is a strong demand for current information on the *R. solani* disease complex because it has become one of the major limiting factors in cotton production in Northern India. In recent years various molecular techniques have been used for genetic characterization and anastomosis grouping of *R.solani* such as isozyme analysis, random amplified polymorphic DNA (RAPD) analysis [7, 8]. There have also been reports from the Mediterranean and Canada on molecular differentiation of *R. solani*-complex fungi using rDNA-RFLP (Ribosomal DNA restriction fragment length polymorphisms)[9,10]. These techniques make it possible to overcome the difficulties of differentiating *R. solani* genotypes, because they are much more robust than traditional plant pathological techniques. Isolates of *R. solani that* infect cotton crop are predominantly of AG-4 and AG-7[11, 12]. Sequence analysis of the genomic regions encoding the internal transcribed spacers ITS1 and ITS2 is convenient for AG determination [13, 14]. The aims of this work was to characterize AG-7 by describing: (i) its unique anastomosis reaction among AGs of R. solani; (ii) its cultural characteristics on potato dextrose agar (PDA); (iii) its pectic zymogram analysis (iv) determine the AGs of the isolates based upon its rDNA-internal transcribed spacer region length and sequence.

MATERIALS AND METHODS

Collection of cotton root samples

A total of 200 isolates of *Rhizoctonia solani* were collected from cotton plants grown in fields distributed across semi-arid tropic region of the country in the year of 2008, 2009 and 2010. A Regional division of cotton production in Northern India was taken into account in the sampling, with higher numbers of isolates collected from areas with more intensive production.

Isolation of Fungal Strain

Fungal strains of *R. solani* were isolated from infected cotton roots. Infected cotton roots were cut into pieces of 5 cm and surface disinfected in 0.1% $HgCl_2$ for 30 sec and rinsed three times in sterile distilled H_2O.Then dipped in 70% alcohol for 1 min and once again ringed with sterile distilled H_2O. Pieces of sterilized root were placed in petri dishes (five segments per plate) containing an isolation medium, i.e. selective media (modified Ko & Hora medium). Cultures were incubated for 2 to 3 days at 28°C in the dark. As the growth appeared, individual fungal colonies possessing the typical characteristics of *Rhizoctonia solani* were hyphal tipped and transferred to Potato Dextrose Agar (PDA medium, HiMedia Laboratories Limited, Mumbai, India) at 28°C for five days. After an incubation period, colonies were purified and identified as *Rhizoctonia solani*. The isolate was deposited in the Microbial Type Culture Collection (MTCC) at Institute of Microbial Technology,

IMTECH, Chandigarh, India and the MTCC number given to this culture was 9993. In the present study two isolates of *Rhizoctonia solani* were also included as reference strains having MTCC nos: 4633 and 4634.

Growth Media Composition

The selective media used for the isolation of *R. solani* from root rot samples was similar to the media as described by Ko & Hora (1971)[15], with minor modifications. It contained 1g of K_2HPO_4, 0.5g of $MgSO_4.7H_2O$, (0.5g) KCl, (0.01g) of $FeSO_4.7H_2O$, (0.2g) of $NaNO_2$, (0.05g) of chloramphenicol, (20g) of agar and 1,000ml distilled water. After sterilization in autoclave at 121^{o}C/20 min, wait until the medium cools down to 50^{o}C, then (0.2532g) of the fungicide metalaxyl (Ridomil 2E: 25%) and (0.05g) of streptomycin were added.

Cultural Characteristics

Hyphal diameter and the number of nuclei per cell were determined by growing each isolate on PDA at 25 to 28°C. The Mycelium was stained with aniline blue dye and examined under microscope at ×400. For counting nuclei,vegetative hyphae of *Rhizoctonia solani* was stained with aniline blue[16].

Anastomosis reactions between strains of R. solani

Hyphal anastomosis grouping (AG) was conducted by pairing unknown isolates with known tester strains according to standard procedures[17]. For each isolate, two replicate slides were examined. Anastomosis reactions were determined by calculating the frequency of each reaction type observed in five fields of view and the isolate classified by the most frequent reaction observed.

DNA Isolation

DNA was extracted from the mycelia of *R. solani* AG-7 according to the method described by Keijer *et al*. (1996) [18] with minor modifications. Lyophilized mycelium (0.2 g) was macerated in liquid nitrogen using a mortar and pestle. The ground mycelium was suspended for 10 min at room temperature in 3 ml of extraction buffer (200 mM Tris, pH 8.5, 250 mM NaCl, 25 mM EDTA, and 0.5% sodium dodecyl sulfate). DNA extraction was done by using equal volumes of Tris-HCl-saturated phenol (pH 8.0)-chloroform-isoamyl alcohol (25:24:1). The nucleic acid was precipitated with 0.1 volume of 3 M sodium acetate (pH 5.5), and 0.7 volume of isopropanol. The final DNA pellet was dissolved in 50 µl of TE buffer.

PCR amplification of rDNA

Internal transcribed spacer 1 (ITS1) and ITS2 regions, including the ribosomal 5·8S RNA gene, were amplified using the universal primers ITS-1(5'-TCCGTAGGTGAACCTGCG G-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al*., 1990) [19]. The amplification was carried out in a 25-µl PCR master mixture that contained the DNA template (50 ng of purified DNA), 1X polymerase chain reaction (PCR) buffer (10 mM Tris- HCl, pH 8.3, 50 mM KCl, and 1.5 mM MgCl2) (Banglore Genei, Banglore), 1.5 mM of MgCl2, 0.5 µM of primers ITS1 and ITS4, 0.2 mM deoxynucleoside triphosphates (dNTPs), and 1 unit of *Taq* polymerase (Invitrogen). The amplification was carried out: Initial denaturation at 94°C for 5 min; followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec, and extension at 72°C for 1 min; with the final extension at 72°C for 10 min. Amplification products were electrophoretically separated through a 1.5% agarose gel (SRL) at 100 V with TAE buffer for 1 hr.

DNA sequencing

PCR products were purified with QIAquick PCR purification kit, (Qiagen, USA) and the purified PCR products were sequenced at IMTECH (Chandigarh), India.

ITS-rDNA nucleotide sequence comparisons.

Identification to the anastomosis group level was done as a comparison of 5.8S and ITS2 rDNA sequence similarity of more than 96% with other strain sequence of *R. solani* in the GenBank database. To perform molecular ITS sequence analysis, reference sequences required for comparison were downloaded from the EMBL database using the site http:// www.ncbi.nlm.nih.gov/Genbank(Table.1). All the

sequences of ITS- rDNA were aligned using the multiple sequence alignment program CLUSTAL W. The aligned sequences were then checked for gaps manually, arranged in a block of 600 bp in each row. The nucleotide sequences of fungal ribosomal DNAs were aligned and compared with those in the GenBank database using the BioEdit Sequence Alignment Editor 5.0.6 and the CLUSTAL W 1.82 programmes.

Table 1
Ribosomal DNA-ITS sequences of Rhizoctonia solani isolates recovered from the GenBank (National Centre for Biotechnology Information-NCBI).

Anastomosis group (AG)	Isolate	Origin	Host	GenBank Accession Number of ITS sequences
AG-1	DC32 (Verbena B)	USA	*Verbena X hybrida*	AJ868442
AG-1-ID	Thanatephorus cucumerisBV61-6		Cotton	EF197804.1
AG-2-2	Thanatephorus cucumerisCR9	USA	*Phaseolus vulgaris* cv.MUS181	DQ452128.1
AG-3	Thanatephorus cucumeris 11	India	cotton	DQ408294
AG-4HG-I	Rhizoctonia solaniNPC-03-1	China	Swiss chard	EF679777
AG-4 HG-III	A8B	Greece	Cotton	FJ480887
AG-5	HL-20-2	China	Potato	JQ946296.1
AG-6	Rh182	Italy	Apple	JF519844.1
AG-7	P4B3	Greece	Cotton & Tobacco	FJ480892.1
AG-7	P20B	Greece	Cotton	FJ480891
AG-7	76Rs	Japan	Soil	AF354096
AG-7	Thanatephorus cucumeris	Brazil	Soil	AY154305.1
AG-7	K9B	Greece	cotton	FJ480895.1
AG-7	MV1	India	Cotton	JX576189
AG-8	F201	Australia	Sugarbeet	FJ492109.3
AG-8	Thanatephorus cucumeris	Brazil	Spinach	AY154303
ND[c*]	Rs clone4	India	Cotton	DQ339103
ND[c*]	Rs12	India	Cotton	DQ223780
ND[c*]	Rs18	India	Cotton	DQ223781
AG-9	TPV12M	Japan	Soil	AB000046
AG-10	ZG9	Australia	Barley	AF354071
AG-10	Rh100278	USA	Chickpea	DQ356408

**** c denotes Anastomosis Group***

Determination of anastomosis groups (AGs)

The ITS1 (218 nucleotides, *nt*), 5·8S *rDNA* (155 *nt*) and ITS2 (233 *nt*) regions were amplified by PCR as a single amplicon. PCR products were sequenced and used to identify anastomosis groups. Sequences of the various anastomosis groups reference strains (Table 2) from database were used for comparison in this study. Five randomly selected isolates designated to AG-7 based on sequence analysis were tested for anastomosis reaction with the AG-2, AG-3 and AG-7 reference strains. Fusion of the hyphae of the AG-7 tester strain, but not the tester strains of AG-2, AG-3 and AG-4 with the hyphae of the Indian isolates confirmed their AG-7 designation.

Restriction enzyme analysis

The amplified ITS1-5.8S-ITS2 region was digested with restriction endonucleases, viz. *HaeIII, AluI, HincII*, and *MspI* as used by Schneider *et al*., (1997)[20]. Seven µl of the PCR products was digested with one units of each restriction enzyme for approximately 2 h at 37°C. The digestion products were resolved in 2.5% agarose gels for about 60min at 200 V.

Pectic zymograms

Pectate lyase enzymes were induced by growing R. *solani* AG7 isolates in flask, each containing 5 ml of culture medium with minor modifications as used by Sweetingham *et al.,* (1986)[21] The culture medium contained 2.64 g $(NH4)_2SO4$, 0.34 g K_2HPO4, 1.14 g$MgSO_4.7H_2O$, 10 g citrus pectin (Sigma P

9135 with 84% galacturonic acid and amethoxy content of 9.4%), 0.01 g $CaCl_2$ and volume made upto 1litre using distilled water. The pH of the medium was adjusted to 5.5 with NaOH. After 5–14 days of incubation at 23 ^{0}C in the dark, culture fluids were stored in a reaction tube and at –20 ^{0}C.

Pectate lyase purification

R.solani culture fluid was harvested by centrifugation of a 1-liter culture. To the supernatant, $(NH4)_2SO_4$ was added to 60% saturation with stirring, and the mixture was kept on ice for 1 h. The precipitate was removed by centrifugation, and the supernatant was 85% saturated with $(NH4)_2SO_4$. The mixture was kept on ice for another 1 hour. The precipitate collected by centrifugation was dissolved in H_2O, dialyzed against H_2O. The enzyme was stored at -20^{0}C for future use. Native polyacrylamidegel-electrophoresis, modified after Laemmli, (1970)[22] was done using a Biorad mini Protean II gelsystem. The separating gel contained 10% (w/v) acrylamide-bisacrylamide (36.5:1) and 0.1% (w/v) citrus pectin in 1.5 M Tris-HCl (pH 8.8). To facilitate polymerization, 3% Ammoniumpersulphate (APS) and 0.035% TEMED (6 M N,N,N',N'-tetramethylethylenediamine) were added. The stacking gel contained 3% (w/v) acrylamidebisacrylamide (36.5:1), 0.1% citrus pectin in 0.5 M Tris-HCl (pH 6.8), 3% APS and 0.035% TEMED. Aliquots containing 10 mg total protein were mixed with 5 µl sample buffer (0.1 M Tris/HCl pH 6.8, 18%v/v glycerol, 0.01%w/v bromophenol blue) loaded into wells. Electrophoresis was conducted in running buffer (250mM Tris–HCl, pH 8.3, 1.92 M glycine), for 8 h at 250 V, and cooled to 5 ^{0}C. After electrophoresis, gels were incubated in 0.1 M DL–malic acid Sigma (M-0875) for 90 min and then stained with 0.02 % (w/v) ruthenium red (Sigma R 2751) for 4 h at room temperature. Gels were de-stained in 3mM Na_2CO_3 overnight at room temperature before being photographed over a light box. On each gel *R. solani* isolate MV1 was used as a reference.

RESULTS & DISCUSSION

Isolate identification and characterization

The 5.8S region of the rDNA of the six tested isolates of *R. solani* AG-7 was identical in length and sequence to that of isolates representing all other AGs of *R. solani* [23.] The ITS 1 region began 31 positions from the 5' end of primer ITS 1 and was 205 bp long. ITS 2 began 391 positions from the 5' end of primer ITS 4 and was 228 bp long. The ITS 1 regions of the six isolates of AG-7 evaluated were identical, whereas similarity in the ITS 2 region ranged from 99 to 100% (0 to 3 nucleotide differences). Identification of *Rhizoctonia* species has always presented a challenge because of their ill-defined taxonomy. Although identification of isolates as *R. solani* is largely dependent on vegetative characters, members of this group are known to be associated with a *Thanatephorus* teleomorph, or sexual stage. In most *R. solani* AG and subgroups, the asexual stage often is viewed as the predominant stage in their life history, although the sexual stage of many *R. solani* AG (e.g., AG1 through 5) is frequently observed in agricultural fields. *R. solani* anastomosis group 7 (AG-7), the most common anastomosis group associated with roots during the study, is the most common seedling pathogen on cotton crop in Haryana. In Previous studies, isolates of *Rhizoctonia* spp. CAG-5 and *R. solani* AG-4 were predominantly found to be pathogenic on cotton crop in Georgia[24]. In Spain, Melero-Vara and Jiménez-Diaz (1990)[25] found that *R. solani* AG-4 was the primary agent of cotton seedling damping-off and was associated with severe necrosis on root and/or hypocotyl.

Arrangement of ITS region

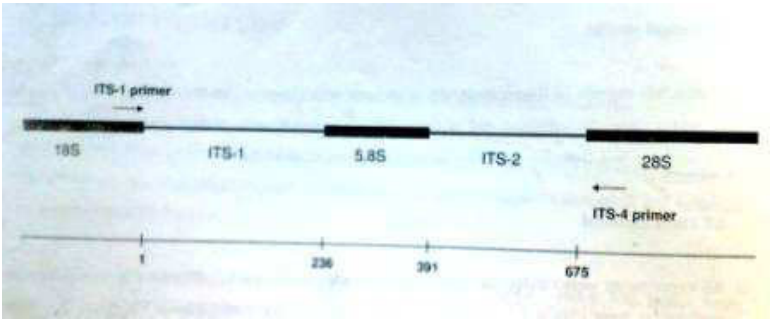

Figure 1
Schematic positioning of Internal Transcribed Spacer region (ITS) 1 and 2. Nucleotide numbering follows the sequence of R. solani AG-7 (JX576189.1) (unpublished data submitted to NCBI gene bank).

Analysis of rDNA sequences

In this study the *R.solani* isolate from cotton roots was designated MV1 and was assigned to an anastomosis group (AG) by both observation of hyphal fusion and DNA sequencing. Perfect hyphal fusion was observed between MV1 and two other isolates of AG-7 tester strains. Schematic positioning of the Internal Transcribed Spacer region (ITS) 1 and 2 inside genes coding ribosomal subunits; 18S, 5.8S and 28S. Primer ITS-1 is located on the DNA sense strand (3' – 5') of the 18S rRNA gene and the ITS-4 on DNA antisense strand (5' – 3') of the 28S rRNA gene as explained by White *et al.,* (1990)[19]. Nucleotide numbering follows the sequence of *R. solani* AG-7 (JX576189.1) (unpublished data submitted to the NCBI gene bank)(Fig 1). The nucleotide sequence of the rDNA-ITS region of MV1 isolate was 100% identical to the nucleotide sequences of *R. solani* as reported by Monga *et al*., (2008)[3]. BLAST searches revealed that the rDNA-ITS region of MV1 isolate was at least 98% identical to that of AG-7 isolates (AY154305.1) on the GenBank nucleotide sequence database. Partial sequence analysis of rDNA revealed high levels of similarity among the *R.solani* strains studied, the mean level of sequence similarity was 99.3% (Table 1). Genetic heterogenicity between, and within anastomosis groups was evaluated by Fenille *et al*., (2003)[26], using sequence analysis of the internal transcribed spacer (ITS) region of the ribosomal DNA. Comparison of the ITS region is significant not only for the determination of anastomosis groups, but these sequences are also useful for verifying subsets. Kuninaga *et al*., (1997)[23] revealed polymorphism between anastomosis groups (AGs) in the ITS1 and ITS2 sequences of ribosomal DNA, while 5.8s rDNA sequence is completely conservative across all AGs. In this study, the ITS region of *R. solani* isolates, which originated from cotton root with typical symptoms of root rot, was analysed in order to determine sequence variations between the isolates, and to identify the anastomosis group and subset of examined isolates. Recently, most identification of *Rhizoctonia solani*, their AGs and intraspecific groups (ISG, AG subgroups) has been accomplished by analysis of the ribosomal genes encoding region and especially ITS-1 and ITS-2 areas [27, 28].

Amplified ITS region of rDNA R.solani AG-7

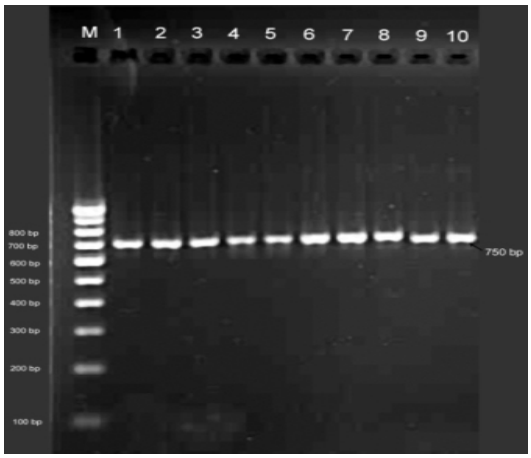

Figure 2

Full length ITS region of rDNA PCR products (750bp) amplified from same anastomosis group AG-7 of Rhizoctonia solani isolates with ITS1/ITS4 primers. Lanes 1-10, DNA amplified from R. solani isolates MV-1 to MV-10; M- Molecular-Weight Markers (100 bp DNA ladder, Promega).

RFLP studies

Amplifications of the ITS region using the primer pair ITS1/ ITS4 resulted in a single PCR product of approximately 750 bp for all *R. solani* AG7 isolates. The restriction digestion profile in electrophoresis gel is shown in fig 2. Similarly, when amplified fragments were digested with restriction enzymes, identical banding patterns were observed for all the isolates. Thus the amplification of ITS region revealed no inter-generic or intra-specific variation. Restriction enzyme *Alu1* showed three bands of approximate sizes of 370 bp and 190 bp (probably a double restriction fragment), *HincII* showed two bands of 520bp and 230pb, *HaeIII* showed a single band of 750 bp indicating that the fragments were not digested and *Msp1* showed two bands of 350bp and 400bp.

PCR-RFLP patterns of the ITS region

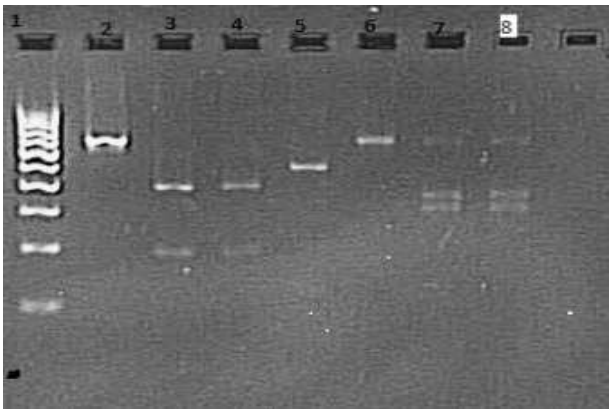

Figure 3

PCR-RFLP patterns from the ITS region of Rhizoctonia solani isolates (lane 2-8) amplified with primer pair ITS1/ITS4 and digested with restriction endonucleases Lane 1. shows the molecular weight marker (100 bp DNA ladder), Lane 2. ITS region 750bp, Lane 3-4. digested with endonuclease Alu1, Lane 5. digested with endonuclease HincII Lane 6. digested with HaeIII, Lane 7-8. digested with Msp1.

Nucleotide sequence accession numbers

The nucleotide sequences determined in this study have been deposited in the GenBank database under accession numbers JX576189.1 and JX576188.1. Pectic enzyme patterns were studied using a commercially available vertical gel-electrophoresis system and non-denaturing polyacrylamide gels amended with pectin. Anastomosis tester isolates AG 1 to AG 11 revealed different ZG. Twenty five *R. solani* AG 7 isolates belonged to a homogeneous pectic zymogram group. We propose to assign *R. solani* AG 7 isolates pathogenic to cotton and, formed a homogeneous group with 2 distinct ZG. Pectic zymography provides an easy, quick and unambiguous method for routine identification of large numbers of field isolates.

Pectic zymograms

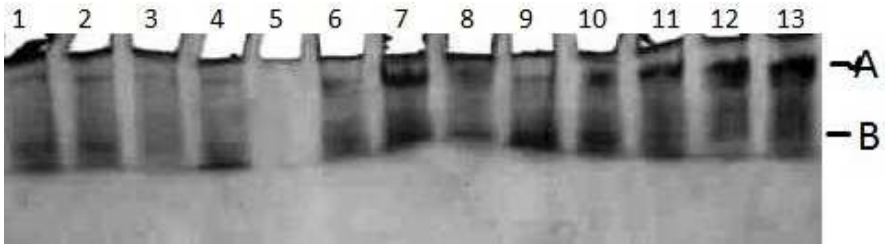

Figure 4
Zymogram analysis within the same anastomosis group of Rhizoctonia solani. The letters A & B represents pectinase activity bands in the Zymogram.

Methods based on RFLP of ribosomal DNA internal transcribed spacers (ITS) and pectic zymograms (ZG) were compared for their use in routine identification of *Rhizoctonia solani* isolates occurring in cotton fields. Twenty five *R.solani* AG7 isolates, pathogenic to cotton, could be distinguished from AG 1-IC, AG 2-2 andAG 4, AG 3 and AG 5 by means of ITS rDNA fragment length and after digestion with *HincII* from AG 4 and AG 5. Digestion of AG7 isolates with *Alu1,* Hae III and *Hinc* II revealed four distinct ITS-rDNA digestion patterns.

Pectic zymograms

The *R. solani* AG7 isolates MV1 to MV13 formed a homogeneous zymogram group (Figure 4). No ZG heterogeneity was observed within the tested *R. solani* AG7 isolates. El-Akkad (1997)[29] found heterogeneity in protein banding patterns among AG-4 isolates of *R. solani*. Cotton isolates shared a common pectic zymogram and the position of isozyme band was same from one isolate to the other. Liu *et al.*, (1990)[30] revealed genetic relationships among isolates of *Rhizoctonia solani* anastomosis group-2 based on isozyme analysis. Bacharis *et al.,* (2010)[31] performed hyphal anastomosis reactions in cotton multinucleate isolates showed that 54 of them belonged in *Rhizoctonia solani* AG-4, 6 in AG-7, (7 isolates), AG-2-1 (1 isolate), and AG-3 (1 isolate). It is concluded that *R. solani* AG-7 is major pathogen of cotton crop, capable of causing extensive damage to the crop especially in cool and moist climatic conditions.

CONCLUSION

Several approaches are currently being used in studies of the taxonomy and epidemiology of plant-pathogenic fungus. Sequence variation in the ribosomal DNA (rDNA) operon has been used for classification of fungal species and for strain discrimination within species. The rRNA genes, commonly used in identification and taxonomic studies, were confirmed in the present study to be particularly appropriate for providing target sequences for molecular detection and research on the dynamics of *Rhizoctonia* populations. As described in the result obtained, the isolates shared a common pectic zymogram while sequence analysis of rDNA showed a closer similarity to anastomosis

group-7(AG-7). Based on morphological characteristics, hyphal fusion compatibility and sequence analysis, 98% of the isolates collected belonged to AG-7 and 2% of the isolates were AG-4 *R.solani*. The significance of this report can be manifested by the fact of it being a first report of this kind

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