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RAPD BASED CHARACTERIZATION OF CHICKPEA ISOLATES OF *SCLEROTIUM ROLFSII*

ABIDA AKRAM[1*], PAKEEZA AMBER[1], SHEIKH MUHAMMAD IQBAL[2], RAHMATULLAH QURESHI[1], ARSHAD JAVAID[3] AND SAEED MUKHTAR[1]

[1]Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan
[2]Department of Microbiology, University of Huripur, Pakistan
[3]Institute of Agricultural Sciences, University of the Punjab, Lahore, Pakistan
[*]Corresponding author's e-mail: abidauaar@hotmail.com, abidaakram@uaar.edu.pk

Abstract

Collar rot disease caused by the fungus *Sclerotium rolfsii* Sacc., results in heavy economic losses to chickpea (*Cicer arietinum* L.) crop every year. Previous work on this devastating fungus reported just its morphological and physiological aspects, but little is known at molecular level. Present research was focused on estimating the genetic variation among 12 strains of *S. rolfsii* isolated from infected chickpea plants through Random Amplification of Polymorphic DNA (RAPD) analysis. Fungal strains were obtained from Crop Sciences Institute, National Agricultural Research Centre (NARC), Islamabad. Out of 40 random decamer primers studied, 6 gave the clear polymorphic amplification pattern in terms of band number. A total of 52 loci were traced by these primers and 100% polymorphism was obtained. The value of similarity matrix ranged from 55.8–88.5%. Strain AM-04 and AM-06 shared maximum of 88.5% similarity while AM-07 and AM-09 were 55.8% similar. Similarity of the remaining genotypes was between 55.7–84.6%. Cluster composition results indicated that AM-07 was the most diverse from the rest of genotypes which showing genetic distance of 35.7%. On the whole, all the strains were 64.3% similar to each other and only 35.7% variation existed among them.

Key words: Chickpea, *Cicer arietinum*, Collar rot, Polymorphism, RAPD, *Sclerotium rolfsii.*

Introduction

Collar rot is one of the destructive and fast spreading fungal diseases of chickpea caused by *Sclerotium rolfsii* Sacc. (Javaid & Khan, 2016). This devastating soil-borne fungal plant pathogen has a wide host range (Le *et al.,* 2012; Remesal *et al.,* 2012; Javaid & Iqbal 2014). Under favourable environmental conditions, significant losses in chickpea yield have been reported due to this pathogen (Akram *et al.,* 2008; Gopalakrishnan *et al.,* 2010; Singh & Gaur, 2017). Mathur & Sinha (1970) have reported up to 95% seedling mortality in chickpea due to *S. rolfsii* infection. Disease caused by this pathogen also lead to heavy losses in vegetable crop yield especially during the wet season (May & October) when weather conditions are favorable for both crop production and for the growth and dissemination of the sclerotia of the pathogen (Okereke & Wokocha, 2007). For control of such distressing plant pathogens, the most cost-effective and feasible measure is the selection of resistant crop cultivars (Akram *et al.,* 2008).

Studies of variability within the population in a geographical region are important because these also document the changes occurring in the population (Sarma *et al.,* 2002). Given the tools of molecular biology, investigators now routinely explore micro evolutionary patterns in plant-fungal associations (Molina *et al.,* 2005; Ahmad *et al.,* 2014). Advances in techniques of molecular biology make it possible to develop large numbers of highly informative DNA markers to identify genetic polymorphism and to characterize populations of plant pathogens (Bardakci, 2001). During the last decade, Randomly amplified polymorphic DNA (RAPD) technique has been one of the most commonly used PCR based molecular markers. To assess levels of genetic diversity as well as phylogenetic relationships within and between species and to identify particular races, molecular markers may be used (Cilliers, 2000). The analysis of DNA products generated through randomly amplified polymorphic DNA (RAPD) has provided information on variation (Kerssies *et al.,* 1997) and segregation of genetic traits among strains (Bergmans *et al.,* 1993). In RAPD-PCR a small sample of the DNA selectively amplifies a million fold producing a surplus quantity of concentrated DNA. This DNA then can be separated into bands of different molecular weight DNAs through agarose gel electrophoresis. Banding patterns from -a population or an individual can then be used as a fingerprint which distinguishes it from genetic materials of other types (Ruiz *et al.,* 2000). Differentiation among species of fungus through morphological and physiological tools is time consuming, labour intensive and not reliable. Molecular biology techniques like RAPD-PCR overcome all the limitations. RAPD-PCR technique is a useful tool for differentiating between species either alternatively or complementary to methods based upon morphological and pathological characteristics (El-Fadly *et al.,* 2008). RAPD technique was the most discriminatory among PCR-RFLP and southern blot. RAPD technique is much cheaper than the other two techniques and less time consuming (Smith *et al.,* 2002).

Keeping in view the significance of chickpea and the threat of *S. rolfsii* to chickpea, the present study was carried out to assess the genetic variability among the different strains of *S. rolfsii* using RAPD.

Materials and Methods

Isolation of pathogen: The present study was carried out during 2009-10 at Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan. The cultures of 12 strains of *S. rolfsii* were isolated from stem sections of infected chickpea plants with visible symptoms of collar rot disease and further sub-cultured in Crop Protection Laboratory, Crop Science Institute, National Agricultural Research Centre (NARC), Islamabad, Pakistan.

The diseased samples were surface sterilized with clorox for one minute, washed three times with sterilized distilled water, dried on blotting paper and inoculated on autoclaved chickpea seed meal extract agar medium (dextrose 20 g, agar 20 g, chickpea seed meal extract 1 L) in 9-cm Petri plates under aseptic conditions. Plates were incubated at 25±2°C under for 7 days diffused light.

The isolated fungal strains were further sub-cultured by growing on freshly prepared chickpea seed meal extract agar medium. With the help of sterilized cutter, 10 mm diameter circular discs were punched out from the margins of actively growing colonies and placed onto the centre of medium plates with mycelial side facing downwards. Inoculation was done in replication of four and each plate was labeled for future identification. These plates were placed in incubator at 25±2°C under diffused light. After about 3-5 days sclerotial formation started. Initially the colour of sclerotia was white which turned brown with the passage of time. After 25 days, Petri plates were completely filled with mature sclerotia. Pure cultures were obtained by inoculating the media plates with 2-3 sclerotia of respective strains and incubated at 25±2°C for 7 days. Mycelia from these pure cultures were used for growing sclerotia for each strain.

Mass culture of sclerotia: Twenty five grams of wheat straw were soaked overnight in 2% sucrose solution. Water was removed so that straw retain only moisture. Two grams of straw were transferred to each 500 mL flasks and autoclaved at 121°C for 20 min and cooled inside laminar flow.

Sterilized wheat straw was inoculated with 10 mm mycelial discs of the respective strains of *S. rolfsii* taken from the margin of actively growing cultures using a sterilized cutter and incubated at 25±2°C under diffuse light for 5 days. Afterward flasks were placed in thermo scientific plant growth environmental chamber at 28±2°C for 23 days. Inoculation was done in replication of four. After 28 days incubation, mature brown sclerotia were collected, air dried for one day, labeled and stored in eppendorf tubes at 4°C for further analysis.

DNA extraction: DNA was extracted under optimized conditions as described by Punja & Sun (2001) from fresh sclerotia of each fungal strain. Sclerotia (200 mg) were taken in autoclaved pestle and mortar and crushed to fine paste using 800 µL lysis buffer (200 mM Tris Base pH 8.0, 100 mM EDTA pH 8.0, 500 mM NaCl, 2% SDS, 1% 2-mercaptoethanol). The homogenate was transferred to 1.5 mL eppendorf tubes with the help of micropipette and incubated at 24ºC for 30 minutes in water bath (I 800-A, Irmeco GmbH, Germany). Chloroform: isoamylalcohol (24:1 v/v) equal to the volume of homogenate was added to eppendorf tube containing homogenate. After vortexing, the mixture was centrifuged at 12000 rpm for 20 minutes in a microcentrifuge (Eppendorf, Germany) at room temperature. The supernatant was transferred to a another eppendorf tube. The DNA was precipitated by addition of an equal volume (600 µL) of ice chilled isopropanol and mixed by gently inverting up and down. The tubes were placed at 4ºC for 10 min and then centrifuged at 12000 rpm for 10 min at temperature 4ºC. The supernatant was discarded and DNA was collected as a pellet. The pellet was air dried and washed with 200 µL of 70% ethanol and centrifuged at 12000 rpm for 5 min at 4ºC. Finally, the pellet was air dried for 15 minutes by inverting it on blotting paper. The pellet was dissolved in 100 µL of TE buffer (1M Tris HCl pH 8.0, 0.5 M EDTA pH 8.0). To remove RNA, the DNA samples were treated with 7 µL of RNase A (10 mg ml^{-1}, #EN0531, Lot: 00022967, Fermentas) and the mixture was incubated for 30 minutes in a water bath maintained at 37°C.

The DNA quality was checked by electrophoresis in 1% agarose gel. Concentration of DNA was estimated spectrophotometrically. A 1:1 dilution of DNA was made for subsequent RAPD analysis. DNA samples were stored at –80°C in ultrafreezer (Sanyo).

Polymerase chain reaction: The 40 GL (Gene Link) decamer (10 oligonucleotide) primers set A and B were initially screened to detect polymorphism among 12 strains of *S. rolfsii*. The primer, which indicated higher degree of polymorphism was further selected for the diversity analysis studies.

Optimization of PCR conditions: The volume of RAPD-PCR reaction mixture was 20 µL. The PCR reaction contained 1.2 µL template DNA (50 ng μl^{-1}), 2 µL (6 pMol) of respective primers, 0.4 µL dNTPs (25 mM dNTP stock), 2 µL PCR buffer, 1.6 µL $MgCl_2$ (25 mM), and taq polymerase 0.2 µL (5 units μL^{-1}). Deionized water was used to make the total reaction volume up to 20 µL.

Amplification conditions: The assembled reaction was kept in programmable Thermal Cycler (Eppendorf AG 22331, Hamburg, Germany). Fragments were amplified from the genomic DNA. For RAPD analysis, DNA amplification was done using the following temperature profile: denaturation at 94°C for 5 minutes, followed by 40 cycles each consisting of a denaturation step at 94°C for 1 minute, annealing step at 36°C for 1 minute and an extension step at 72°C for 2 minutes. After the last cycle, 10 minutes were given to the extension step at 72ºC and then held at 4ºC for 12 hours.

Agarose gel electrophoresis: On completion of PCR amplification, the PCR tubes were removed from the thermal cycler A. After the reaction, DNA was analyzed through gel electrophoresis (Plate 2 B). After adding 3 µL of loading buffer (0.1% bromophenol blue, 0.05% xylene cyanol FF and 30% glycerol), 10 µL of the PCR product was loaded on 1.5% agarose gel. Using TBE buffer, electrophoresis was performed for 45 min at 100 volts in an

electrophoresis apparatus (Biorad). After staining the get with ethidium bromide, photography was done under UV illumination. Using gel documentation system and computer program (Gel Doc XR, Quantity One), UV light was used to visualize DNA fragments. In all cases genetic ruler, 1 kb DNA Ladder (Catalogue # SMO313, Lot: 00018968, concentration: 0.1 µg uL^{-1} Fermentas), was used for quantification and sizing of wide range double stranded DNA fragments on agarose gel. For direct loading on gel, the ladder was premixed with 6X loading dye.

Statistical analysis: For scoring RAPD data for analysis, ethidium bromide stained agarose gel photographs were used. For statistical analysis, each band was considered as a single locus/allele. The fungal isolates were compared with each other using their RAPD-PCR profiles and bands of the DNA fragments. The presence of a band was scored as 1 and absence as 0 for each of the primers used. Bands having the same mobility were considered as identical fragments. PCR bands positions were compared with molecular weight standards. Software package Gelcompar II version 4.602 Applied Maths Inc. USA was used for analysis. After gel images processing, all pair wise similarity values were calculated using a similarity coefficient. The genetic similarity matrix was generated on the basis of similarity coefficients. A dendrogram based on the similarity coefficients was constructed using UPGMA (unweighted pair group method with arithmetic mean) clustering algorithm (Nei & Li, 1979).

Arithmetic Means (UPGMA) procedure is as follows:

$$GD_{xy} = 1- d_{xy}/d_x+d_y-d_{xy}$$

where,

GD_{xy} = Genetic distance between two genotypes
d_{xy} = Total number of common loci (bands) in two genotypes
d_x = Total number of loci (bands) in genotype 1
d_y = Total number of loci (bands) in genotype 2.

Based on the data of RAPD primers, the bivariant 1-0 data matrix for each fungal strain was used to construct a dendrogram with a computer program "Popgene 3.2" version 1.31 (Yeh *et al.,* 1999).

Results

In the present study, the selected strains of *S. rolfsii* were subjected to molecular characterization using RAPD-PCR. To detect DNA polymorphism a total of 40 GL (Gene Link) decamer primers were screened on 12 strains of *S. rolfsii*. Out of these 40 primers used, only 6 primers viz. OPA-02, OPA-04, OPA-06, OPA-09, OPA-10 and OPA-12 showed clear polymorphic bands. These six primers were chosen to amplify the whole 12 strains. The oligonucleotide sequence of these 6 primers is given in Table 1.

Only clear and intense bands were scored while faint bands against smear background were not considered for the further analysis. The loci were scored as 1 (present) and 0 (absent). Total number of loci/alleles traced by these primers were 52. All the 52 bands were polymorphic and none was detected as monomorphic. The mean percentage of polymorphism among these genotypes was 100%. The size of amplification product ranged from 250 bp to 10,000 bp.

The highest number (26) of scorable bands was obtained with primer OPA-10 while the lowest number (15) was obtained with primer OPA-06. Maximum genotypes (12) were amplified by primer OPA-09 and minimum (9) by primer OPA-06. Different primers showed variation in the number of polymorphic fragments. Primer OPA-09 showed the highest number of polymorphic fragments (11). Primers OPA-2 and OPA-04 showed the lowest number of polymorphic fragments (7).

Genotypes AM-03, AM-05, AM-6, AM-07, AM-08, AM-09, AM-10, AM-11 and AM-12 were amplified with maximum number of primers (6) whereas genotype AM-01 was amplified by only one primer. Gel electrophoresis patterns of OPA-02, OPA-04, OPA-06, OPA-09, OPA-10 and OPA-12 RAPD primers are shown in Figs. 1 to 6, respectively. The efficiency of these primers to amplify the genotypes ranged from 9 genotypes by primers OPA-06, 11 genotypes by primers OPA-02, OPA-04, OPA 10, OPA-12 and 12 genotypes by primers OPA-09.

Fig. 1 represents the amplification profile of 12 samples by primer OPA-02 in which 11 samples gave 21 scorable bands. Sizes of scorable bands ranged from 250 to 10,000 bp. Maximum scorable bands (5) were given by AM-07 and minimum number of scorable band (1) was detected in AM-03, AM-04, AM-06, AM-09, AM-10, AM-11 and AM-12. All the genotypes that showed scorable bands were overall polymorphic. Seven polymorphic bands were detected. AM-07 revealed unique bands at the band size of 350 bp and 1000 bp. Another unique band was given by AM-05 at 4000 bp. Total genomic DNA from AM-01 did not amplify using OPA-02 and hence was not included in the analysis.

Fig. 2 summarizes the amplification profile of 12 samples with primer OPA-04 in which 11 samples gave 20 visible scorable bands. Sizes of scorable bands ranged from 250 to 10,000 bp. Maximum scorable bands (3) were given by AM-03, AM-08 and AM-12 and minimum number of scorable band (1) was detected in AM-02, AM-04, AM-07, AM-09 and AM-10. A total of 7 polymorphic bands were detected. A unique band was observed in AM-03 at 400 bp and in AM-06 at 740 bp. Total genomic DNA from AM-01 did not amplify using OPA-04 and hence not included in the analysis.

Table 1. RAPD primers and the percentage of polymorphic bands.

Count	Primer code	Nucleotide sequence	Size of fragments (bp)	Polymorphic bands	Polymorphism (%)
1	OPA-02	TGCCGAGCTG	250 to 10,000	7	100
2	OPA-04	AATCGGGCTG	250 to 10,000	7	100
3	OPA-06	GGTCCCTGAC	250 to 10,000	8	100
4	OPA-09	GGGTAACGCC	250 to 10,000	11	100
5	OPA-10	GTGATCGCAG	250 to 10,000	9	100
6	OPA-12	TCGGCGATAG	600 to 10,000	10	100

Fig. 3 shows an agarose gel electrophoresis pattern obtained using the primer OPA-06. This primer has amplified a total number of 9 strains of the fungus. Size of scorable bands ranged from 250 to 10,000 bp. A total of 15 scorable bands were obtained. 8 loci were polymorphic. This primer showed maximum bands (4) in genotypes AM-07 and minimum number of bands (1) were detected in AM-03, AM-06, AM-08, AM-09 and AM-10. A unique band was found in AM-05 at the band size of 500 bp. AM-07 gave unique band at 1200 bp. AM-12 showed unique bands at 4000 bp, respectively. Total genomic DNA from remaining 3 genotypes AM-01, AM-02 and AM-04 did not amplified using OPA-06 and hence was not included in the analysis.

Fig. 4 indicates that primer OPA-09 has amplified all the 12 genotypes. Maximum polymorphic fragments were amplified by this primer. Sizes of scorable bands ranged from 250 to 10,000 bp. A total of 21 scorable bands were obtained and 11 loci were polymorphic. Unique band was found in AM-01 at 4000 bp, AM-09 at 1200 bp and AM-12 at 550 bp. Bands of 1000 bp, 2000 bp and 6000 bp were found only in AM-04. The primer has produced maximum bands (4) in AM-04 and minimum bands (1) in AM-01, AM-02, AM-05, AM-06, AM-08, AM-10 and AM-11.

Fig. 5 represents the amplification profile of 12 samples using primer OPA-10 in which 11 samples gave 26 visible bands. Maximum scorable (26) bands were obtained by this primer and 9 loci were polymorphic. Size of scorable bands ranged from 250 to 10,000 bp. Maximum scorable bands (5) were detected for the genotypes AM-08 and AM-10 and minimum band (1) was obtained in genotypes AM-03, AM-05 and AM-11. Unique bands were found in AM-08 at the band size of 300bp and 350 bp. AM-10 revealed unique bands at 1300bp and 1500 bp. Another unique band was found in AM-12 at 1000 bp. Total genomic DNA from remaining 1 sample AM-01 did not amplify using OPA-10 and hence was not included in the analysis.

Fig. 6 depicts the amplification profile of 12 samples using primer OPA-12 out of which 11 samples gave 25 visible bands and 10 loci were polymorphic. Size of scorable bands ranged from 600 to 10,000bp. Maximum scorable bands (3) were detected for the genotypes AM-06, AM-07, AM-09, AM-10, AM-11 and AM-12 and minimum band (1) was obtained by genotypes AM-02, AM-03 and AM-08. A unique band was found in AM-05 at 740 bp. AM-07 gave unique band at 1200 bp. Unique bands were also found in AM-08 and AM-12 at 500 bp and 2500 bp respectively. Total genomic DNA from remaining 1 sample AM-01 did not amplify using OPA-12 and hence was not included in the analysis.

A similarity matrix was generated by RAPD amplification data to estimate genetic diversity and relatedness among 12 selected fungal genotypes. Similarity matrix is presented in Table 2. The value of similarity coefficient of selected fungal genotypes ranged from 0.558 (55.8%) to 0.885 (88.5%). Genotypes which showed maximum similarity of 88.5% were AM-04 and AM-06. On the other hand minimum similarity of 55.8% indicated high diversity in genotypes AM-07 with AM-09. The similarities of remaining genotypes lie between the range of 55.8% and 88.5%. Genotypes showing above 80% similarity were AM-01 with AM-02 having 84.6% similarity. AM-02 with AM-04, AM-05 and AM-06 having 82.7% similarities. AM-03 with AM-04 and AM-06 having 82.7% similarities. AM-03 with AM-01 and AM-02 having 80.8% similarities. AM-05 with AM-06 having 80.8% similarity. AM-11 with AM-04, AM-05, AM-06, AM-10 and AM-12 having 80.8% similarities.

Dendrogram shows the precise analysis of genetic distance by doing the grouping of genotypes on the bases of similarities and differences. Bivariate data 1 and 0 were used to estimate genetic distances (GD) between the genotypes by using UPGMA. The dendrogram of RAPD based genetic diversity evaluation of *S. rolfsii* clearly indicated four main clusters A, B, C and D. Dendrogram drawn for the genetic distances is shown in the Fig. 7.

Cluster A included a total of 2 genotypes viz. AM-07 and AM-08, which are the most diverse genotypes from the remaining genotypes showing maximum genetic distance. Due to large genetic distance both genotypes in cluster A are subdivided into two subclusters A1 and subcluster A2. Within A1 subcluster, genotype AM-07 is present. AM-07 had maximum genetic distance with rest of the strains in cluster B, C and D. Genotype AM-07 is genetically diverse strain showing a genetic distance of 0.357 (35.7%) with the remaining 11 genotypes. Subcluster A2 included genotype AM-08 having a genetic distance of 0.343 (34.3%) with the remaining genotypes.

Cluster B included a total of 3 genotypes viz. AM-12, AM-10 and AM-09. Within this cluster, maximum genetic distance is shown by genotype AM-12 which is 0.256 (25.6%). This genotype is diverse from other two genotypes in cluster B. AM-10 and AM-09 had a genetic distance of 0.21 (21%) with each other. Cluster C included 2 genotypes viz. AM-05 and AM-11. Both the genotypes shared genetic distance of 0.191 (19.1%).

Cluster D included 5 genotypes viz.AM-01, AM-02, AM-03, AM-04 and AM-06. This cluster is subdivided into two subclusters D1 and D2. Subcluster D1 included AM-03, AM-04 and AM-06. Genotype AM-03 had a genetic distance 0.175 (17.5%) with other two genotypes in this subcluster. Genotypes AM-04 and AM-06 shared a genetic distance of 0.115 (11.5%). AM-04 and AM-06 are closely related strains among all the other genotypes as they shared least genetic distance. Subcluster D2 included genotypes AM-01 and AM-02 having genetic distance 0.155 (15.5%).

Discussion

Often differences among the fungal strains cannot be revealed with sufficient reliability by classical taxonomic and morphological characters. To detect polymorphisms in fungi, various molecular techniques such as RAPD-PCR have been successfully used (Mahmoud *et al.,* 2012; Kordalewska *et al.,* 2015; Yang *et al.,* 2017). In the present study, molecular evaluation of the selected strains of *S. rolfsii* suggested that the allelic diversity of this germplasm can be used for improving chickpea crops against infection of *S. rolfsii* strains. So molecular evaluation is helpful to reveal the pathogenic characteristics of this fungus (Kokub *et al.,* 2007). The results obtained under the present study confirmed the efficiency of RAPD-PCR technique for determination and estimation of genetic similarities and differences among fungal strains used in this study. Thus the RAPD analysis was found to be an informative DNA marker system to assess genetic relatedness and diversity among different strains (Tanwir *et al.,* 2007).

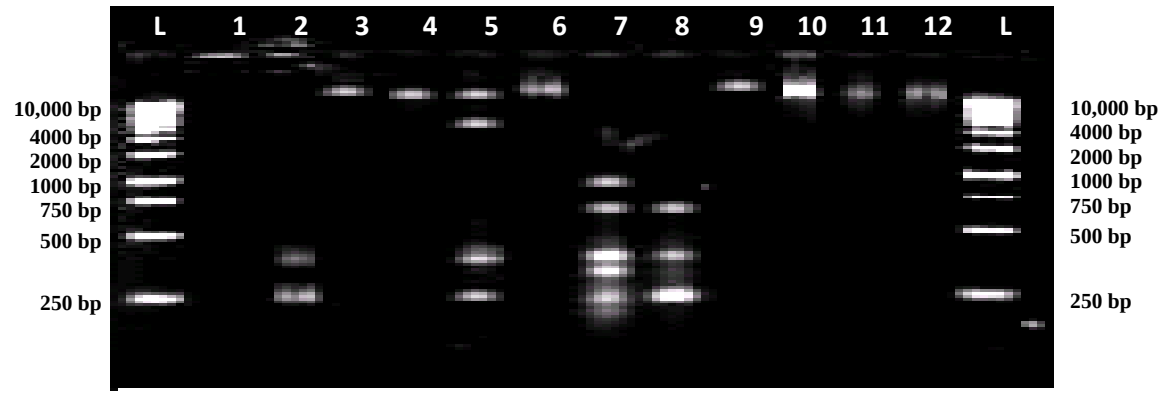

Fig. 1. RAPD amplification pattern using primer OPA-02 (TGCCGAGCTG).

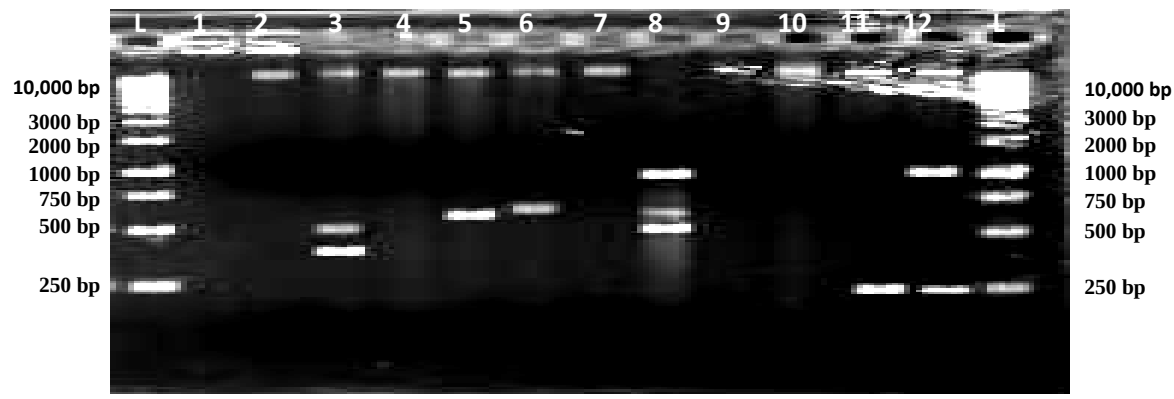

Fig. 2. RAPD amplification pattern using primer OPA-04 (AATCGGGCTG).

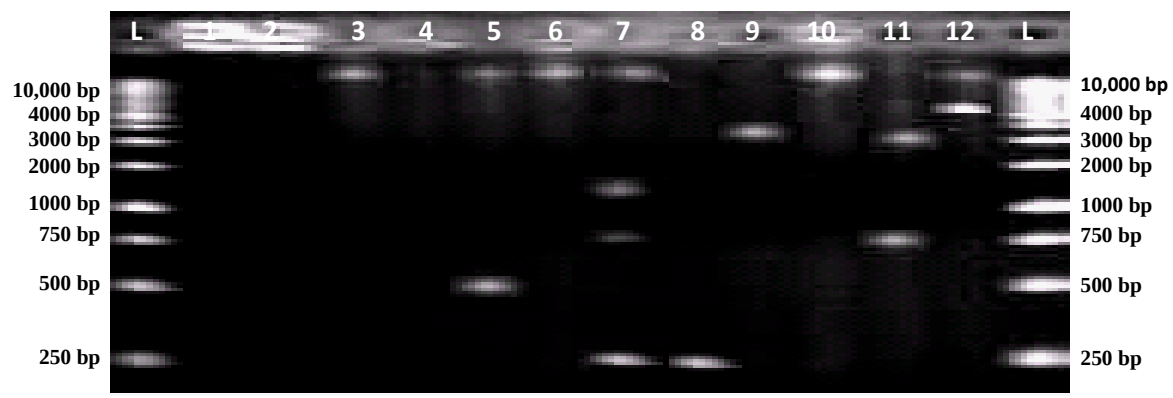

Fig. 3. RAPD amplification pattern using primer OPA-06 (GGTCCCTGAC) numbering from 1 to 12 corresponds to 12 experimental fungal strains from AM-01 to AM-12.

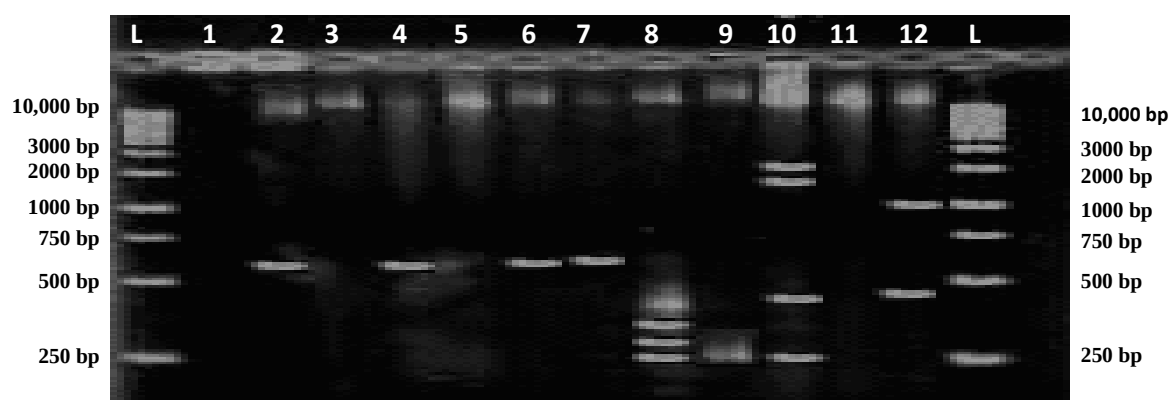

Fig. 4. RAPD amplification pattern using the OPA-09 (GGGTAACGCC).

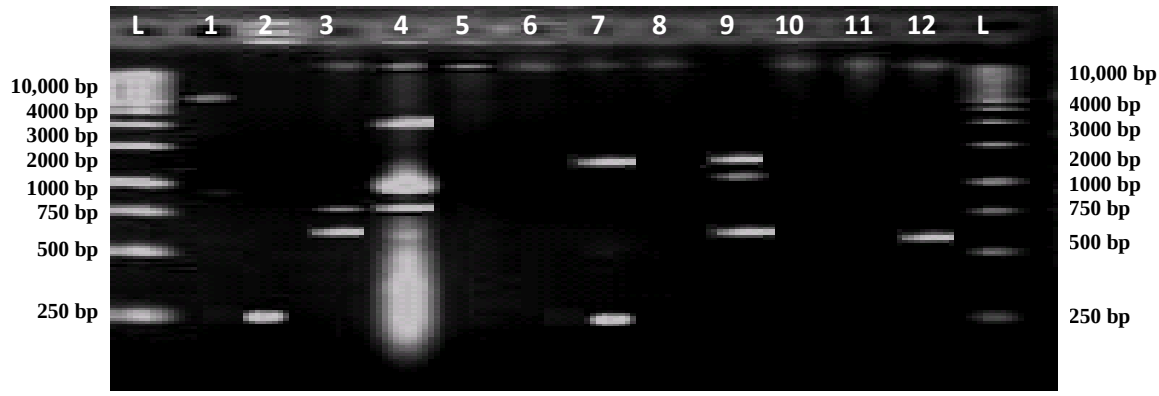

Fig. 5. RAPD amplification pattern using primer OPA-10 (GTGATCGCAG).

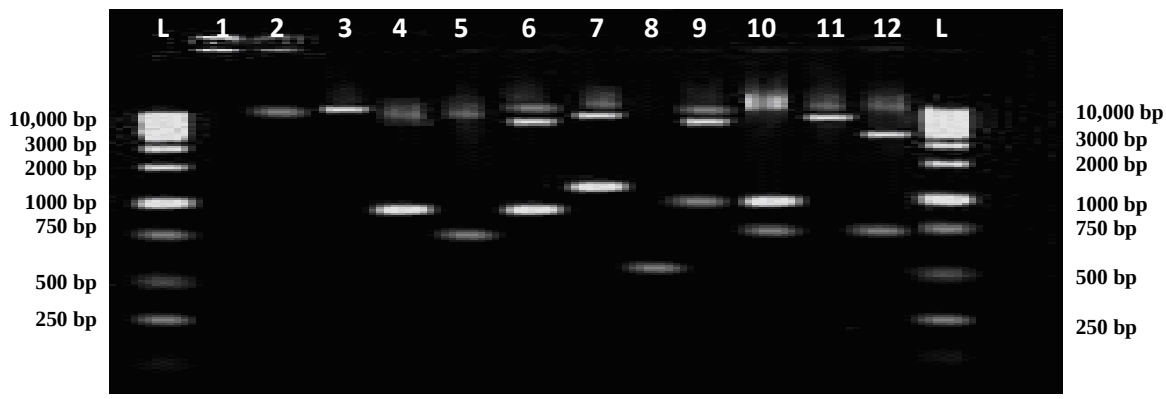

Fig. 6. RAPD amplification pattern using primer OPA-12 (TCGGCGATAG) numbering from 1 to 12 corresponds to 12 experimental fungal strains from AM-01 to AM-12.

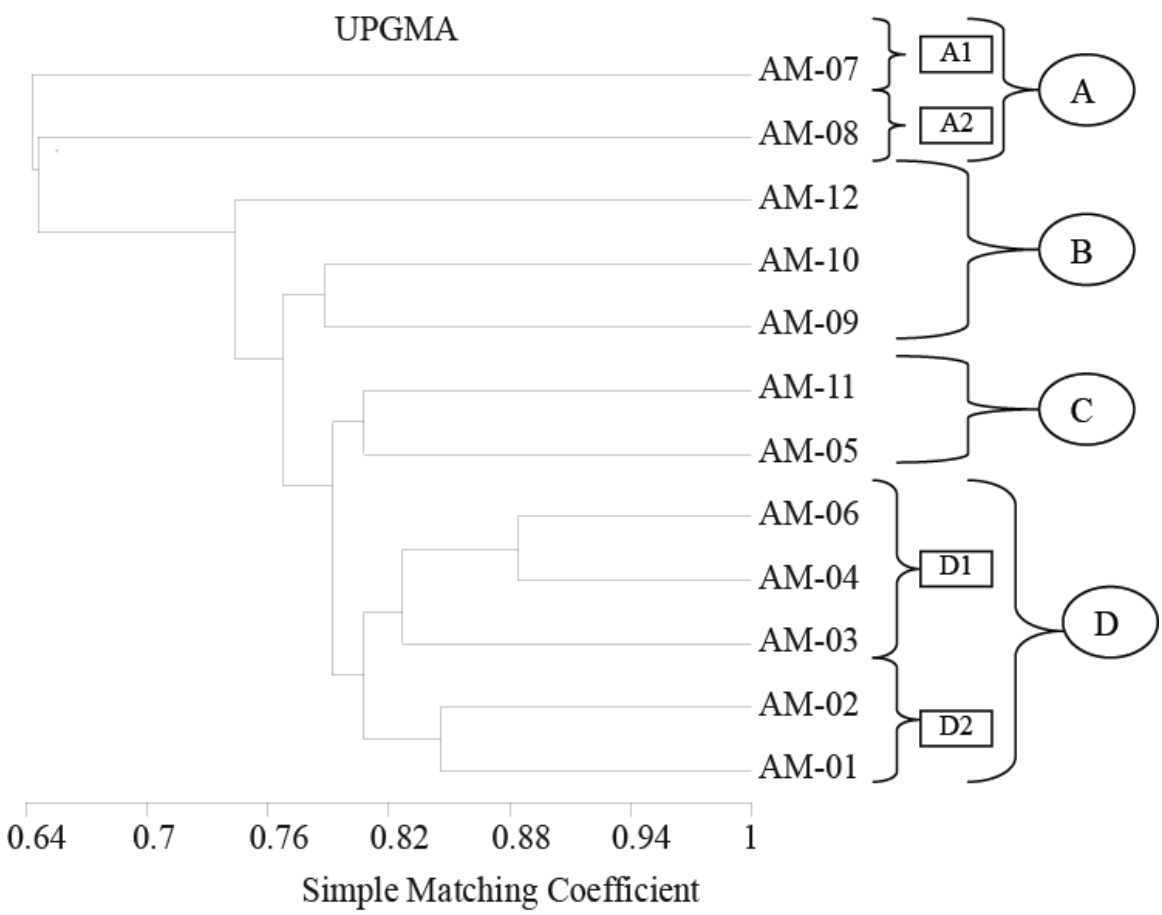

Fig. 7. Dendrogram obtained from RAPD analysis of 12 strains of *S. rolfsii* using Popgene 3.2 version 1.31.

Table 2. Genetic similarity index showing Nei's similarity coefficient among 12 strains of *S. rolfsii* generated by UPGMA analysis.

	AM-01	AM-02	AM-03	AM-04	AM-05	AM-06	AM-07	AM-08	AM-09	AM-10	AM-11	AM-12
AM-01	1											
AM-02	0.846	1										
AM-03	0.808	0.808	1									
AM-04	0.788	0.827	0.827	1								
AM-05	0.750	0.827	0.788	0.769	1							
AM-06	0.788	0.827	0.827	0.885	0.808	1						
AM-07	0.635	0.788	0.635	0.654	0.692	0.692	1					
AM-08	0.712	0.712	0.635	0.615	0.692	0.615	0.615	1				
AM-09	0.769	0.769	0.769	0.788	0.712	0.788	0.558	0.596	1			
AM-10	0.750	0.750	0.750	0.769	0.769	0.769	0.577	0.654	0.788	1		
AM-11	0.788	0.788	0.788	0.808	0.808	0.808	0.654	0.615	0.788	0.808	1	
AM-12	0.712	0.712	0.750	0.731	0.769	0.769	0.577	0.615	0.673	0.769	0.808	1

In the present study, RAPD data analysis of 12 *S. rolfsii* strains revealed 4 main clusters. The cluster composition using RAPD primers revealed that genotypes AM-07 (35.7%) and AM-08 (34.3%) are highly diverse genotypes. While diversity of remaining genotypes was between 11.5% and 25.6%. Collectively 118 bands were produced with an average of 19.6 bands per primer. Hundred percent polymorphism was observed among 12 fungal strains. This revealed that these strains have high genetic diversity and polymorphic RAPD primers resulted in 100% polymorphism.

Genetic similarity matrices indicated that AM-04 and AM-06 are closely related genotypes showing 88.5% similarity with respect to RAPD primers. While AM-07 and AM-09 showed 55.8% similarity and are diverse from each other. Among 12 genotypes, AM-07 was found to be the most diverse in the dendrogram. On the whole all the strains were 64.3% similar to each other and only 35.7% variation existed among them. Kokub *et al.* (2007) worked on the same lines and reported results similar to that obtained in the present research work. The highly polymorphic pattern was obtained among 8 strains of *S. rolfsii* collected from the host chickpea from different locations of Pakistan. It was confirmed by RAPD analysis that on the whole all the strains were 81.26% similar to each other and only 18.74% variation existed among them. The genetic diversity study revealed that the population of *S. rolfsii* showed more diversity and polymorphism among its 132 isolates than 15 isolates of *S. delphnii* (Punja & Sun, 2001). Sources of polymorphism in RAPD assay might be due to deletion, addition or substitution of base within the priming site sequence. Polymorphism in DNA amplified by PCR yield valuable information especially when comparing large groups of strains (Williams *et al*., 1990). This supports the present finding that the population of *S. rolfsii* had high diversity and high level of polymorphism among its strains. In another study, Prasad *et al*. (2010) reported genetic variability among the virulent isolates of *S. rolfsii* using RAPD.

Results obtained in the present study are in agreement with the results reported by Molina *et al.* (2005) that yet isolates obtained from the same habitat have different RAPD patterns, indicating that many populations of this fungus are made up of more than one genet and that few are derived clonally. More divergent isolates originated in the areas with the single crop which suggested that significant heterogeneity and variability at the molecular level can be observed in different isolates of the same *Sclerotium* species affecting the same crop (Paramasivan *et al.*, 2009). It has been reported that a wide diversity among fungal groups can occur within a limited area, within a host or in geographically isolated regions.

References

Ahmad, N., A.S. Mumtaz, A. Ghafoor, A. Ali and M. Nisar. 2014. Marker Assisted Selection (MAS) for chickpea *Fusarium oxysporum* wilt resistant genotypes using PCR based molecular markers. *Mol. Biol. Rep.*, 41: 6755-6762.

Akram, A., S.M. Iqbal, C.A. Rauf and R. Aleem. 2008. Detection of resistant sources for collar rot disease in chickpea germplasm. *Pak. J. Bot.*, 40: 2211-2215.

Bardakci, F. 2001. Random Polymorphic Amplified DNA (RAPD) markers. *Turk. J. Biol.*, 25: 185-196.

Bergmans, V.V., B.F. Bransdwagt, B.J.W. Klooster, C.A.M. Wagemakers and J.A.L. Vankan. 1993. Genetic variation and segregation of DNA polymorphism in *Botrytis cinerea*. *Mycol. Res.*, 97: 1193-1200.

Cilliers, A.J., L. Herselman and Z.A. Pretorius. 2000. Genetic variability within and among mycelial compatibility groups of *S. rolfsii* in South Africa. *Phytopathology*, 90: 1026-1031.

El-Fadly, G.B., M.K. El-Kazzaz, M.A.A. Hassan and G.A.N. El-Kot. 2008. Identification of some *Fusarium* spp. using RAPD-PCR technique. *Egypt. J. Phytopathol.*, 36: 71-80.

Gopalakrishnan, S., I.G.K. Kannan, G. Alekhya, S.V. Meesala and D. Kanala. 2010. Efficacy of *Jatropha*, *Annona* and *Parthenium* biowash on *Sclerotium rolfsii*, *Fusarium oxysporum* f. sp *ciceri* and *Macrophomina phaseolina*, pathogens of chickpea and sorghum. *Afr. J. Biotechnol.*, 9: 8048-8057.

Javaid, A, and I.H. Khan. 2016. Management of collar rot disease of chickpea by extracts and soil amendment with dry leaf biomass of *Melia azedarach* L. *Philipp. Agric. Sci.*, 99: 150-155.

Javaid, A. and D. Iqbal. 2014. Management of collar rot of bell pepper (*Capsicum annuum* L.) by extracts and dry biomass of *Coronopus didymus* shoot. *Biol. Agric. Hort.*, 30: 164-172.

Kerssies, A., B.A.I. Vanzessen, C.A.M. Wagemakers and V. Kan. 1997. Variation in the pathogenicity and DNA polymorphism among *B. cinerea* isolates sampled inside and outside a glasshouse. *Plant Dis.*, 18: 781-786.

Kokub, D., F. Azam, A. Hassan, M. Ansar, M.J. Asad and A. Khanum. 2007. Comparative growth, morphological and molecular characterization of indigenous *Sclerotium rolfsii* strains isolated from different locations of Pakistan. *Pak. J. Bot.*, 39: 1849-1866.

Kordalewska, M.E., T. Jagielski and A. Brillowska-Dabrowska. 2015. Typing of *Scopulariopsis* and *Microascus* fungi by Random Amplified Polymorphic DNA (RAPD). *Mycoses*, 58: 185.

Le, C.N., R. Mendes and M. Kruijt. 2012. Genetic and phenotypic diversity of *Sclerotium rolfsii* in groundnut fields in central Vietnam. *Phytopathology*, 96: 389-397.

Mahmoud, M.A., S.A. Al-Sohaibani and A.M.M. Abdelbacki. 2012. Molecular characterization of the pathogenic plant fungus *Rhizoctonia solani* (Ceratobasidiaceae) isolated from Egypt based on protein and PCR-RAPD profiles. *Genet. Mol. Res.*, 11: 3585-3600.

Mathur, S.B. and S. Sinha. 1970. Role of manuring in control of root rot of guar (*Cyamopsis psoraloides* DC.) and wilt of gram (*Cicer arietinum* L.) caused by S*clerotium rolfsii* Sacc. *Mycopathology*, 40: 155-159.

Molina, M.C., T.D. Paula and D.L. James. 2005. Genetic variation in the widespread lichenicolous fungus *Marchandiomyces corallinus. Mycologia*, 97: 454-463.

Nei, N. and W. Li. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. USA*, 76: 5269-5273.

Okereke, V.C. and R.C. Wokocha. 2007. *In vitro* growth of four isolates of *S. rolfsii* Sacc. in the humid tropics. *Afr. J. Biotechnol*., 6: 1879-1881.

Paramasivan, M., S. Mathiyazhagan, S. Mohan, G.S. Ali and M. Karthikeyan. 2009. Molecular variability of *S. rolfsii* in tropical sugarbeet based on restriction fragment length polymorphism (RFLP) of ITS region of ribosomal DNA. *Arch. Phytopathol. Plant Prot.,* 42: 327-333.

Prasad, S.D., S.T. Basha and N.P.G.E. Reddy. 2010. Molecular variability among the isolates of *S. rolfsii* causing stem rot of groundnut by RAPD, ITS-PCR and RFLP. *Eur. Asia J. Biol. Sci.,* 4: 80-87.

Punja, Z.K. and L.J. Sun. 2001. Genetic diversity among mycelial compatibility groups of *S. rolfsii (*teleomorph *Athelia rolfsii)* and *S. delphinii*. *Mycol. Res.*, 105: 537-546.

Remesal, E., R. Jordan-Ramirez and R.M. Jimenez-Diaz. 2012. Mycelial compatibility groups and pathogenic diversity in *Sclerotium rolfsii* populations from sugar beet crops in Mediterranean-type climate regions. *Plant Pathol.*, 61: 739-753.

Ruiz, R.A., D.C. Vacek, P.E. Parker, L.E. Wendel, U. Schaffner, R. Sobhian and R.D. Richard. 2000. Using RAPD-PCR to match natural enemies to their host plant. *Proc. of the X International Symposium on Biol. Control of Weeds*. pp. 289-293.

Sarma, B.K., U.P. Singh and K.P. Singh. 2002. Variability in Indian isolates of *S. rolfsii*. *Mycologia*, 94: 1051-1058.

Singh, S.P. and R. Gaur. 2017. Endophytic *Streptomyces* spp. underscore induction of defense regulatory genes and confers resistance against *Sclerotium rolfsii* in chickpea. *Biol. Control*, 104: 44-56.

Smith, S., F. Cantet, F. Angelini, A. Marais, F. Megraud, E. Bayerdoffer and S. Miehlke. 2002. Discriminatory Power of RAPD, PCR-RFLP and Southern Blot Analyses of *ureCD* or *ureA* gene probes on *Helicobacter pylori* isolates. *Z. Naturforsch.*, 57: 516-521.

Tanwir, A.M., M.A. Iqbal, M.A. Chowdhry, M. Kashif and S.U. Rahman, 2007. DNA marker for leaf rust disease in wheat. *Pak. J. Bot.*, 39: 236-243.

Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucl. Acids Res.*, 18: 6531-6535.

Yang, C.L., X.P. Wu, B.Z. Chen, Deng, Z.E. Chen, Y.Y. Huang and S.S. Jin. 2017. Comparative analysis of genetic polymorphisms among *Monascus* strains by ISSR and RAPD markers. *J. Sci. Food Agric.*, 97: 636-640.

Yeh, F.C., R.C. Yang, T.B.J. Boyle, Z.H. Ye and J.X. Mao. 1999. Popgene 3.2, the User-Friendly Shareware for Population Genetic Analysis. Molecular Biology and Biotechnology Centre. University of Alberta, Edmonton. Alberta, Canada.

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