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COMPARATIVE GROWTH, MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF INDIGENOUS *SCLEROTIUM ROLFSII* STRAINS ISOLATED FROM DIFFERENT LOCATIONS OF PAKISTAN

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Abstract

Growth rate of 8 fungal strains of *Sclerotium rolfsii* on potato dextrose agar plates at 28°C ranged from 0.86-1.35 mm hour^{-1}. Strains D4, D7 and D8 were found to be comparatively fast growing and produced greatest number of sclerotia than others. All strains produced round shaped sclerotia with average diameter of 0.5-2.0 mm. Mycelial compatibility reaction on PDA plates showed that strains D5 and D7 as well as D3 and D8 were compatible with each other. Random amplified polymorphic DNA (RAPD) analysis also revealed that strains D5 and D7 shared 94 % similarity while strains D3 and D8 were 83% similar.

Introduction

Sclerotium rolfsii Sacc., is a soil borne plant pathogen causing diseases on a wide range of agricultural and horticultural crops. It has wide geographic diversity and commonly found in the tropics, subtropics and other warm temperate regions especially the Southern United States, Central and South America, West Indies, Southern European countries bordering the Mediterranean, Africa, India, Japan, Philippines and Hawaii (Aycock, 1966). The first report of the fungus pathogenicity dates back to 1892 in connection with tomato blight in Florida (Weber, 1931). The wide host range, prolific growth and ability to produce persistent sclerotia contribute to the large economic losses associated with this pathogen.

The fungus was placed in the form genus *Sclerotium* by Saccardo (1913) as it formed differentiated sclerotia and sterile mycelium. The fungi included in this genus were characterized by production of small tan to dark brown or black spherical sclerotia with internally differentiated rind, cortex and medulla. *S. rolfsii* was reported as the best known member of the genus by Punja & Damiani (1996).

The cultures of *S. rolfsii* originating from various plant species and different geographical regions present wide variation in growth rate, morphological characteristics, mycelial compatibility and also exhibited genetic variability. However, the cultures of *S. rolfsii* can be identified by the size, color and structure of their sclerotia. The objective of this study was to compare the growth behavior of indigenous fungal strains and to study the morphological and genetic similarities and differences among different fungal strains isolated from various locations of Pakistan.

Table 1. Source/location of fungal strains of *sclerotium rolfsii*.

Strains	Source/location
D2	Dera Ismail Khan
D3	Fungus culture collection, University of the Punjab, Lahore.
D4	Dera Ismail Khan
D5	Chakwal
D6	NARC (Farmer Field)
D7	Chakwal
D8	NARC (Research Field)
D9	Dera Ismail Khan

Materials and Methods

Fungal strains used for the present study were obtained from National Agriculture Research Council (NARC), Islamabad and Fungus Culture Collection, University of the Punjab, Lahore. These cultures were originally isolated from leaves and stem of chickpea infected plants from different localities of Pakistan identified as *S. rolfsii* and stored on potato dextrose agar (PDA) slants (Table 1).

The fungal strains were further transferred/sub-cultured by growing on freshly prepared PDA plates. The fresh PDA was prepared following the method described by Sarma *et al.* (2002).

Morphological characterization

Growth behaviors of fungal strains on PDA plates: Ten mm diameter circular discs taken from the margin of an actively growing (3-4 days old) colony was punched out with the help of a self-designed cutter and placed onto the center of the plate with the mycelial side facing downwards, under aseptic conditions. The plates were incubated at 28^{o}C for 7 days.

Morphological characteristics such as: i) growth, ii) mycelium condition, iii) radial colony diameter, iv) development of sclerotia, and v) color, type and location of sclerotia for each strain were noted. The observations were recorded at 12 hours interval up to 3 days and then at 24 hours intervals for 7 days.

Mycelial compatibility/incompatability reaction on PDA plates: Eight fungal strains of *S. rolfsii* were subjected to mycelial compatibility reaction on PDA plates in order to identify the morphological similarities/differences among various strains as described by Punja & Sun (2001). Eight fungal strains in combination of three at a time were tested. A total of 56 combinations were prepared using the statistical formula:

$$^{N}C_{n} = \frac{N!}{(N-n)!\, n!}$$

The PDA plates were marked into three portions. Ten mm diameter mycelial disc of the respective strain was inoculated on each portion of PDA plate. After inoculation, the plates were incubated at 28^{o}C for 15-20 days and examined daily for the development of clearing zone in the region of mycelial contact. All combinations were inoculated in triplicate.

Molecular characterization

RAPD analysis: Genetic similarities and differences among eight strains of *S. rolfsii* was assessed using randomly amplified polymorphic DNA (RAPD) analysis as described by Punja & Sun (2001). DNA was extracted from the mycelial mat of each strain grown on PDA plate for 7days at 28°C. Mycelium (200 mg) was transferred to 1.5 mL microcentrifuge tubes containing liquid nitrogen. Subsequently, DNA was extracted by the method of Punja & Sun (2001).

The mycelium was thoroughly ground into a fine homogenate using pallet pestle mixer. Eight hundred µL of lysis buffer [200 mM Tris pH 8.0; 500 mM NaCl; 100 mM ethylene diaminotetra acetic acid (EDTA) pH 8.0; 2.0 % sodium dodecyle sulphate (SDS); 1% 2-mercaptoethanol] was added to the tubes, mixed well and incubated at 24°C for 30 minutes. Afterwards 400µL extraction buffer (phenol/chloroform/isoamyl alcohol, 25/24/1, v/v/v) was added followed by gentle stirring on a vortex mixer till an emulsion was formed. The emulsion was centrifuged at 6000 g for 5 minutes in a microcentrifuge (Eppendorf, Germany) at room temperature. An aliquot of the upper aqueous layer (600 µL) was collected, mixed with an equal volume of extraction buffer and re-centrifuged. The upper layer was discarded and to the lower aqueous layer 10 µL of RNase A (10 mg mL^{-1}) was added and the mixture was incubated at 37°C for 30 minutes. The DNA was precipitated by addition of 2.5 volumes of ice cold ethanol (70%) and the tubes were placed at -20°C for overnight. The mixture was centrifuged at 14 000 g for 30 minutes at 4°C, the pellet was collected and suspended in 70% ethanol and re-centrifuged. Finally, the pellet was air-dried for 10-15 minutes, re-suspended in 100 µL of Tris-EDTA (TE) buffer having 10 mM Tris-HCl (pH 8.0) 1 mM EDTA. The DNA concentration was estimated by electrophoresis in 1% Agarose gel containing 0.05% Ethidium bromide. The DNA concentration was estimated with reference to Lambda DNA marker under UV illumination. The DNA concentration was further confirmed spectrophotometrically. The stock solution of DNA was prepared at a final concentration of 1µg mL^{-1} and used at final working concentration of 100 ng μL^{-1} for subsequent RAPD analysis.

Primer selection for DNA amplification: The GL decamer primers (Gene Link, USA) set A and B were initially screened to detect polymorphism among 8 strains of *S. rolfsii*. The GL Decamer set A indicated higher degree of polymorphism, were selected for the diversity analysis studies. The DNA sequences (5'-3') of all primers in GL decamer set A used for DNA amplification are given in Table 2.

DNA amplification and agarose gel electrophoresis: The RAPD-PCR reaction was carried out in 50µL volume. The PCR reaction contained 1 µL template DNA (100 ng μL^{-1}), 1 µL (4.5 pMol) of respective primers, 1 µL dNTPs (10 mM dNTP stock), 5 µL PCR buffer (Tris HCl buffer), 5 µL Mg Cl_2 (25 mM stock), 2.5 µL gelatin (1%) and taq polymerase 0.5 µL of (5 units μL^{-1}). Deionized water was used to make the total reaction volume up to 50 µl. In order to ensure the reproducibility of the RAPD reaction, appropriate negative controls (without DNA template) were also run.

PCR tubes were placed in thermocycler (Eppendorf, Germany) for DNA amplification using the following temperature profile: DNA was denatured at 94°C for 4 minutes, forty cycles of 94°C for 1 minute, 36°C for 1 minute and 72°C for 2 minutes. Following the cycling, mixture was incubated at 72°C for 10 minutes and then kept at 4°C for 12 hours.

Table 2. RAPD primers and their sequences.

S. No.	Primer name	Primer sequence (5'-3')
1	A-01	CAGGCCCTTC
2	A-02	TGCCGAGCTG
3	A-03	AGTCAGCCAC
4	A-04	AATCGGGCTG
5	A-05	AGGGGTCTTG
6	A-06	GGTCCCTGAC
7	A-07	GAAACGGGTG
8	A-08	GTGACGTAGG
9	A-09	GGGTAACGCC
10	A-10	GTGATCGCAG

On completion of PCR amplification, the tubes were removed from the thermocycler. After adding 5 µL of loading buffer (0.1% bromophenol blue, 0.05% xylene cyanol FF and 30% glycerol), 15 µL of the RAPD product was loaded on 1% agarose gel. Using TE buffer, electrophoresis was performed for 4 hours at 100 volts in an electrophoresis apparatus (BioRad). The gel was stained with Ethidium bromide (0.1%) and photographed under UV illumination.

The fungal isolates compared with each other using their RAPD-PCR profiles and bands of DNA fragments were scored as present (I) and absent (0) for each of the decamer primer used. For the data collection very very faint bands were not included. Genetic similarity matrix was generated on the basis of similarity coefficients. A dendrogram based on the similarity coefficients was constructed by using un-weighted pair group method of arithmetic means (UPGMA) as described by Nei & Lis (1979).

Results and Discussion

Morphological characterization of different strains: Morphological characteristics of 8 fungal strains grown on PDA plates revealed that mycelial growth rate of different strains varied considerably upto three days. Mycelia of most strains showed growth along the surface of the medium that was lying to the base (LTB) while a few strains showed fluffy (F) appearance i.e. mycelia were growing at right angle to the surface of the plate. The whole plate was covered with mycelium within 3 days. Linear /apical growth of mycelia on PDA plates at 28^{o}C ranged from 0.86-1.35 mm hour^{-1} (Table 3). Barnett (1968) observed that growth and branching of *S. rolfsii* filamentous fungi occurred at the apex of mycelium and pointed out that growth was regulated by a delicate balance between cell wall synthesis and degradation. Further, it was noted that two enzymes viz., β 1-3 glucanase and glucane synthetase were responsible for this activity. However, equilibrium of these two enzymes controlled the hyphal growth and branching in *S. rolfsii* as studied by Kritzman *et al*., (1978).

The formation of sclerotia initiated after 72 hours of incubation and continued till 168 hours. Initially, white colored sclerotia were formed. Then their color changed from white to off-white, light brown and dark brown as they attained maturity. After utilization of nutrients, the plates became dry. However, dark brown and black coloured sclerotia survived for longer times. The change in color of sclerotia might also be due to utilization/exhaustion of nutrients.

Table 3. Growth and sclerotia formation by *S. rolfsii* strains on PDA plates during 7 days of incubation at 28°C.

Strain	Radial colony growth (cm) h^{-1}							Mycelium condition	No. of sclerotia $plates^{-1}$			
	12 h	24 h	36 h	48 h	60 h	72 h	Growth rate mm h^{-1}		96 h	120 h	144 h	168 h
D2	-	1.5	2.5	5.9	6.9	9.0	1.15	LTB	+	405	710	950
										W	OW/LB	LB/DB
D3	-	1.8	2.0	5.8	6.3	9.0	1.05	LTB	-	-	80	150
											LB/DB	LB/DB
D4	-	2.0	3.5	6.5	7.2	9.0	1.20	LTB	+	625	830	1210
										W	OW/LB	LB/DB
D5	-	1.5	2.1	4.7	6.1	9.0	1.01	LTB	-	91	134	169
										W	W	LB/DB
D6	-	1.4	2.2	4.8	5.5	9.0	0.91	LTB	+	230	490	610
										W	W/OW/LB	OW/LB
D7	-	1.7	2.7	6.0	7.8	9.0	1.30	F	-	270	390	485
										W	W	W/OW
D8	-	2.4	4.1	5.4	8.1	9.0	1.35	F	+	415	560	700
										W	OW	OW/LB
D9	-	1.4	2.7	4.9	5.9	9.0	0.98	LTB	+	333	390	620
										W	W/OW/LB	LB/DB

F= Fluffy; LTB= Lying to base; W =White; OW = off white; LB = Light brown; DB=Dark brown, - = Nil growth.

The color and arrangement of sclerotia on agar plates were different for different strains (Fig. 1). The sclerotia of strain D2 and D4 were arranged as peripheral ring on the plate. Initially, white colored sclerotia were produced which later on transformed into light brown and then dark brown. It was observed that these strains were not only fast growing but also produced maximum number of sclerotia on PDA plates i.e., approximately 1210 and 950 per plate, respectively. In case of strains D7 and D8 there was heavy vegetative growth showing fluffy appearance of mycelia and the sclerotia were embedded within the mycelium. The white/off-white sclerotia of strain D8 formed a ring in the centre of the plate while, the sclerotia of strain D7 were off-white/light brown in color and scattered on the plates. Strain D9 produced a ring of off-white to light brown sclerotia in the centre of the plate whereas sclerotia of D6 were arranged as peripheral ring. Strain D5 produced off-white/light brown sclerotia scattered throughout the plate. Strain D3 produced minimum number of sclerotia on agar plates i.e., approximately 150 per plate. Strain D3 was found to be comparatively slow growing and the production and arrangement of sclerotia was entirely different from other strains. These were produced in the form of bunches only at the site of inoculation with marginal mycelial growth (Fig. 1).

During the present study, it was observed that the sclerotia of these strains were mostly round in shape. The sclerotial diameter of 9 fungal strains ranged from 0.5-2.0 mm. However, the average sclerotial diameter of 1.0 ± 0.2 mm at 20^{o}C was recorded by Punja & Damiani (1996). Production of small, spherical and tan to dark brown and black colored sclerotia were also reported by Zarani & Christias (1997) and Sarma *et al.*, (2002). The number of sclerotia produced by different strains was different and ranged from 91-1210 per plate (Table 3). In another study done by Punja & Damiani (1996) reported that greatest number of sclerotia (1043 ± 340) was produced by *S. rolfsii* on PDA plates at an incubation temperature of 35^{o}C.

It was further observed in our studies that strains with heavy mycelial growth produced more number of sclerotia. Therefore, strains D4, D7 and D8 were found to be comparatively fast growing, producing more number of sclerotia on agar plates. These findings were consistent with the earlier investigations made by Wheeler & Sharan (1965) and Zoberi (1980) that media which supported extensive growth also produced greatest number of sclerotia.

Moreover, sclerotia of some strains showed shiny appearance due to presence of gummy material on their surface. The presence of gummy material on the surface of sclerotia might be due to the production of extracellular polysaccharides by these strains. Flieger *et al*., (2003) reported that filamentous fungi were very promising producer of β D-glucan as the hyphal cell wall and extracellular matrix contain more than 75% polysaccharides.

Earlier studies on sclerotia formation by *S. rolfsii* revealed that a large number of factors were responsible for production of sclerotia such as some nutritional and non nutritional factors (Trevethick & Cooke, 1971), nutrient depletion (Hadar *et al*., 1983), restriction of growth by a physical barrier (Wheeler & Waller, 1965), as well as by imposing a step down in the level of nitrogen following active growth (Punja, 1986).

In the present study, it was observed that if a contaminant (bacterial or fungal) came into contact with *S. rolfsii* strain on agar plate, a number of sclerotia developed around the contaminant and blocked its further growth and proliferation as shown in Fig. 2**.** However, by producing large number of sclerotia, the fungal cells were protected. Therefore, some times sclerotia might be developed as a part of defense mechanism.

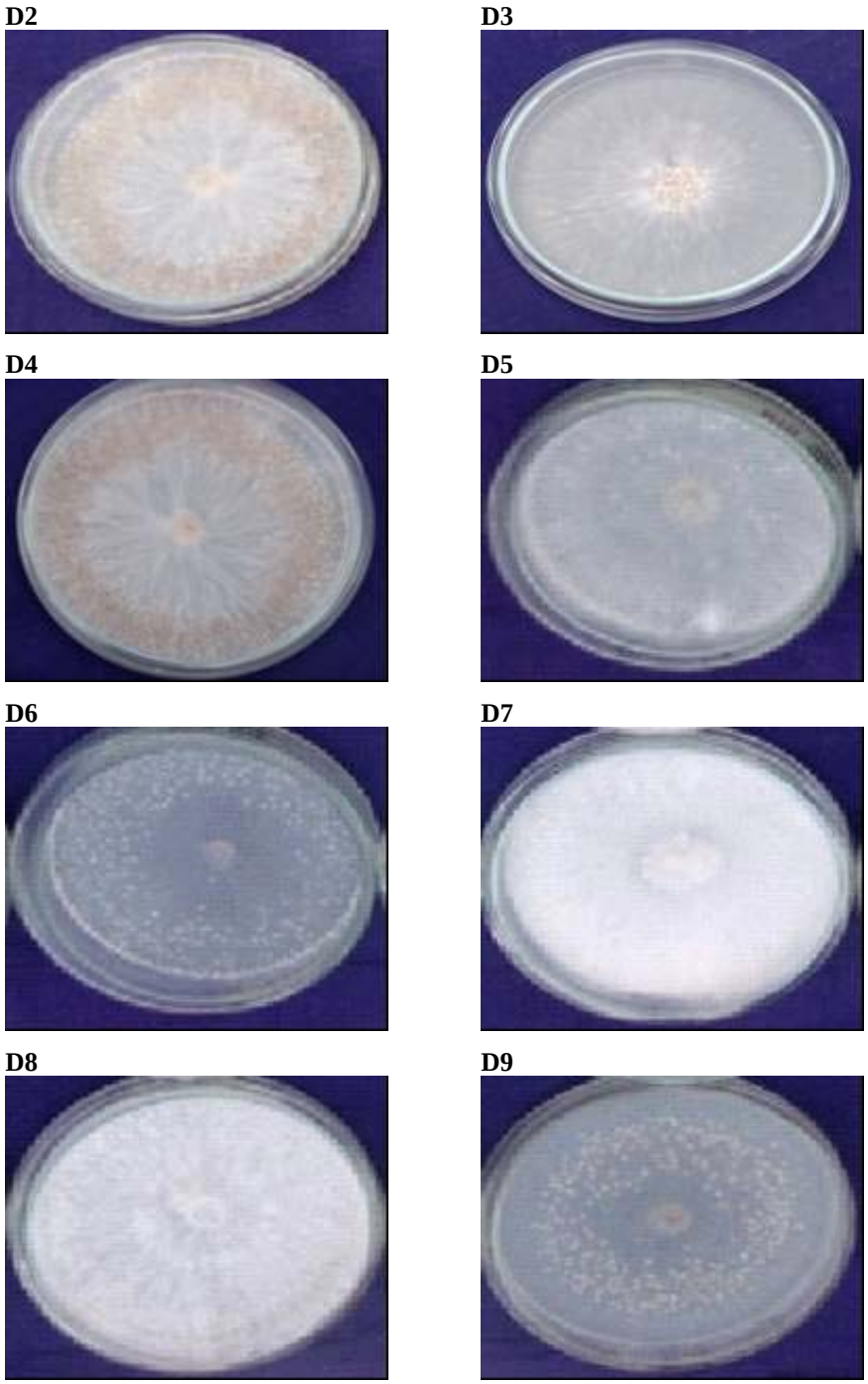

Fig. 1. Growth of eight fungal strains on PDA plates after 20 days of incubation at 28°C.

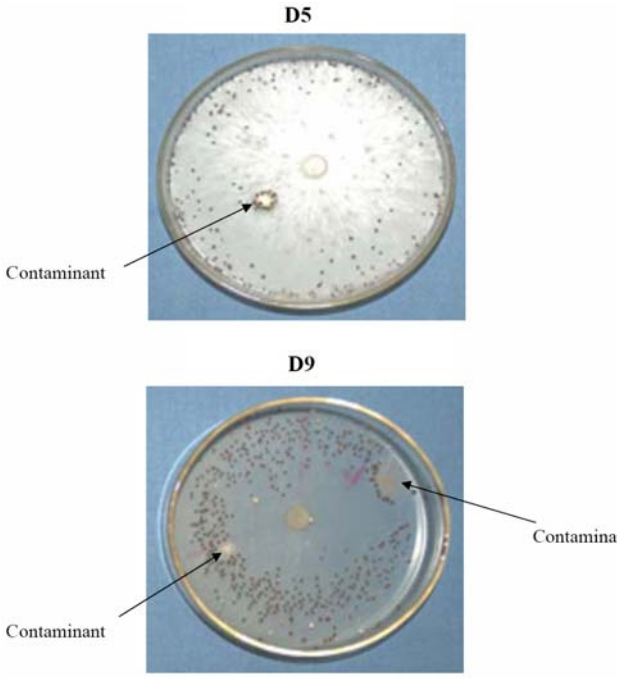

Fig. 2. Production of sclerotia by *S. rolfsii* strains D5 and D9 in response to contaminants.

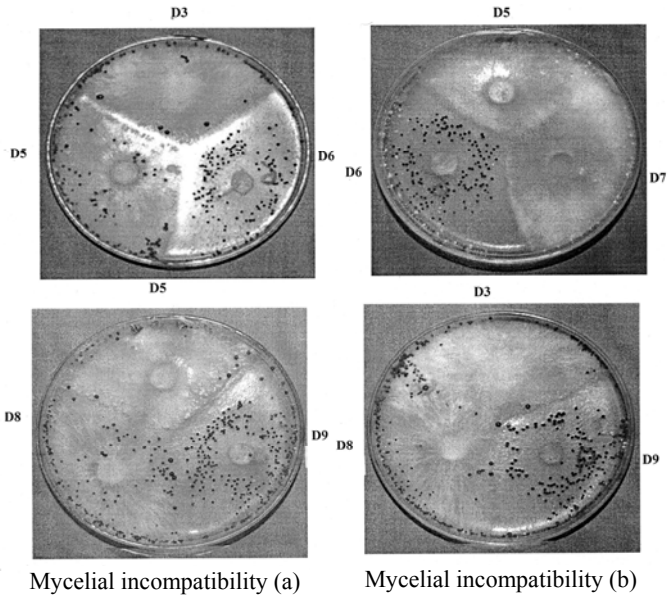

Fig. 3. Comparison of (a) incompatible reaction (top and bottom) with (b) compatible reaction (top and bottom) among different strains of *S. rolfsii* on PDA plates after 20 days of incubation at 28^{o}C.

Mycelial compatability/in compatability among different strains: After inoculation and growth of three different strains on one plate, it was observed that as the colony size increased, their mycelia came in contact with each other. Initially, intermingling of the mycelia of two incompatible strains took place but later on lysis of mycelia of two strains and development of a clear zone was observed at the region of interaction (mycelial contact). The mycelial incompatibility reaction is shown in Fig. 3a (top and bottom). It was observed that strain D3, D5 and D6 while growing on the same plate developed clearing zone between strain D3 and D5, D3 and D6 as well as between D5 and D6 (Fig. 3a, top). However, sometimes production of sclerotia by incompatible strains was also observed along the sides of the clearing zone as shown in Fig. 3a (bottom). Strain D5, D8 and D9, when grown on the same plate, developed clearing zone between strains D5, D8 and D8, D9 as well as between D5, D9. It was further observed that strains D5, D8 and D9 also produced sclerotia along both sides of the clearing zone. Therefore, strains D5, D8 and D9 also showed incompatible reaction between themselves. On the other hand, when two compatible strains came in contact with each other, mycelia of both strains intermingled and sclerotia were also produced at the region of interaction, however, development of clearing zone was not observed. For example, when strains D5, D7 and D6 came in contact with each other, the mycelia of D5 and D7 intermingled without the development of clearing zone as shown in Fig. 3b (top). However, D6 on contact with D5 and D7 developed clearing zone at the region of interaction and sclerotia were also developed. Therefore, strain D6 showed mycelial incompatibility with strain D5 and D7 while strains D5 and D7 showed mycelial compatibility between them. Similarly, strains D3 and D8 showed compatible reaction between each other whereas D9 showed incompatible reaction with strain D3 and D8 as shown in Fig. 3b (bottom). Therefore, the study revealed that mycelial compatibility/incompatibility reaction could be used to distinguish morphologically different strains belonging to the same species. Mycelial compatibility/ incompatibility reaction by different species was also reported by Punja & Sun (2001) and Sarma *et al.,* (2002).

Among the 56 combinations, 12 combinations showed mycelial compatibility between two strains. On the basis of mycelial compatibility behavior two pairs i.e. D3 D8 and D5 D7, were found similar to each other. It was further observed that strains D3 and D8 when paired with all other strains showed mycelial compatibility between them. Similarly, strains D5 and D7 also showed mycelial compatibility when paired with other strains. However, the remaining strains showed incompatible reaction.

The earlier workers also observed that when mycelia of different isolates belonging to the same species confront with one another, either on agar media or a suitable growth substrate, a distinct zone of demarcation (barrage or aversion zone) was developed between the colonies. Recognition of non-self from self is the underlying basis of the incompatible reaction (Punja & Sun, 1997). Mycelial compatibility reaction was also used by Sarma *et al.*, (2002) **t**o study variability and relatedness among fungal species belonging to different geographical regions**.**

Molecular characterization

RAPD analysis: DNA of 8 fungal strains of *S. rolfsii* was amplified using 10 random decamer primers. Out of the 10 primers used, 9 could detect polymorphism while one primer (A-10) produced monomorphic pattern. Primer A-01 amplified a total of 17 bands corresponding to 2500-250 base pairs (bp). Out of these, 12 were polymorphic. The polymorphic bands have been indicated by stars as shown in Fig. 4. Primer A-02 amplified 6 bands out of which 4 were polymorphic (Fig. 5). Primer A-03 amplified 13

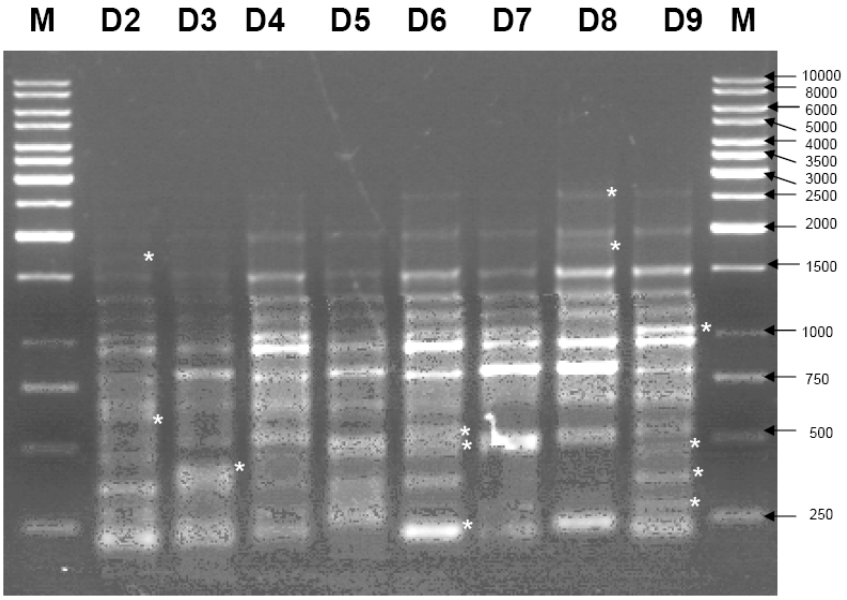

Fig. 4. RAPD of eight fungal strains using A- 01 primer.
Left-right Lane 1:10, 1kb DNA marker (M). Lane 2-9: Randomly amplified DNA of fungal strains.
Polymorphic bands are pointed as *

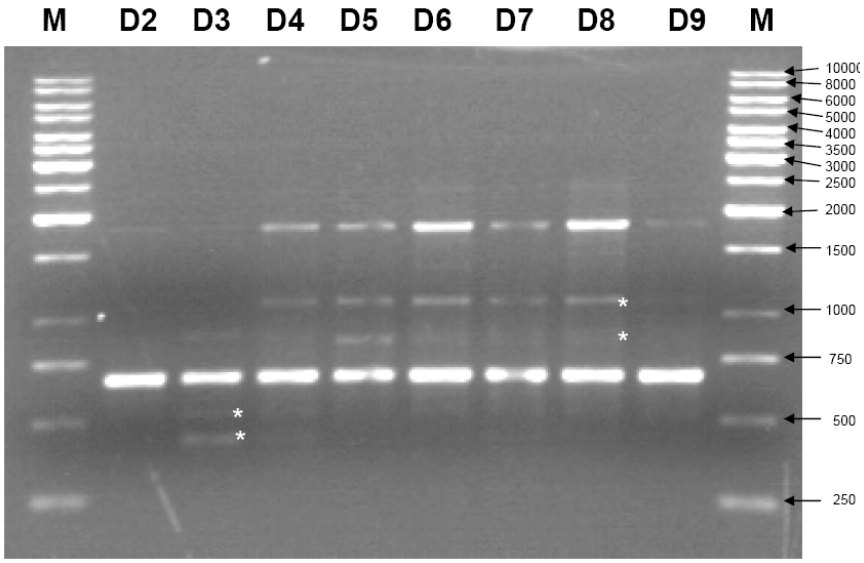

Fig. 5. RAPD of eight fungal strains using A- 02 primer.
Left right Lane 1: 10, 1kb DNA marker (M). Lane 2-9: Randomly amplified DNA of fungal strains.
Polymorphic bands are pointed as *

bands, out of which 4 were polymorphic. One amplification product approximately corresponding a to 700 base pairs (bp) was observed in strains D2, D4, D6 and D9 while one band corresponding to 500 bp was amplified only in strain D8. Based on this band, primer A-03 could be used to differentiate strain D8 from other strains as shown in Fig. 6. Random decamer primer A-04 amplified total of 11 bands out of which 4 were polymorphic. However, one band corresponding to 250 bp was present in all strains except D8. The banding pattern is shown in Fig. 7. Primer A -05 amplified 13 bands, 6 of them were polymorphic. One band corresponding to about 300 bp was observed only in D6 which could be used to differentiate strain D6 from other strains on the basis of this particular band as shown in Fig. 8. Moreover, one band corresponding to about 800 bp was absent in strain D3, D5, D6 and D8. Primer A-06 amplified a profile of 15 bands, out of which 13 were polymorphic. By using this primer, one amplicon corresponding to about 700 bp was observed only in strain D3 as shown in Fig. 9. Therefore, random decamer A-06 can be used to differentiate D3 from other strains. Primer A-07 amplified 12 bands, out of that 8 were polymorphic. This primer amplified one band corresponding to 2500 bp which was specific for D8. The banding pattern is shown in Fig. 10. Primer A -08 produced a profile of 13 bands containing 10 polymorphic bands. This primer amplified D6 and D8 specific bands, therefore, primer A-08 could be used for identification of these strains (Fig. 11). Maximum of 6 bands could be scored with Primer A -09, out of which 3 were polymorphic. Absence of amplification product corresponding to about 600 bp in strain D6 indicated genetic variation of this strain from others as shown in Fig. 12. Random decamer primer A-10 amplified 10 bands which showed almost monomorphic banding pattern for all strains investigated for genetic variation except one polymorphic band for strain D3 corresponding to about 800 bp was amplified by this primer as shown in Fig. 13.

Collectively 116 bands were produced, with an average of 11.6 bands per primer. Out of these amplified products 64 (55.17%) were polymorphic. Maximum number of bands i.e., 91 was amplified by the fungal strain D4 followed by 85 by strains D2 and D5 each, while minimum number of bands i.e., 79 and 81 were amplified by the fungal strains D9 and D6, respectively.

Data, in the form of one (1) or zero (0), based on the presence or absence of a particular band, was used for the estimation of similarity matrix (Table 4) to calculate genetic divergence and relatedness among *Sclerotium rolfsii* strains isolated from various localities of Pakistan. Genetically most similar strains were D5 and D7 (94.44%) and D3 and D8 (83.52%) similar while the most distant strains were D2 and D7 (81.69% similar).

Cluster analysis (dendogram) as shown in Fig. 14 prepared by using Nei & Li's coefficients, showed that fungal strains of *S. rolfsii* could be clustered into two groups. Cluster I comprising of D3 and D8 showed similarity of 83.52% while cluster II contained 6 strains i.e., D2, D4, D5, D6, D7 and D9 with similarity range of 83.75 to 94.44%. The two groups joined at a similarity level of 81.26%. Dendrogram depicted that in group I genetically, most similar isolates were D5 and D7 (94.44%) however, strains D5, D7 and D4 were 91.17% similar whereas D5, D7, D4 and D9 shared similarity of 88.70%. The dandogram further depicted that D5, D7, D4, D9 and D6 were 89.56% similar while D5, D7, D4, D9, D6 and D2 were 83.75% similar. On the whole, all strains were 81.26% similar to each other and only 18.74% variation existed among them. It was also confirmed by RAPD analysis that strains D5 and D7 were 94.44% similar while strains D3 and D8 were 83.52% similar.

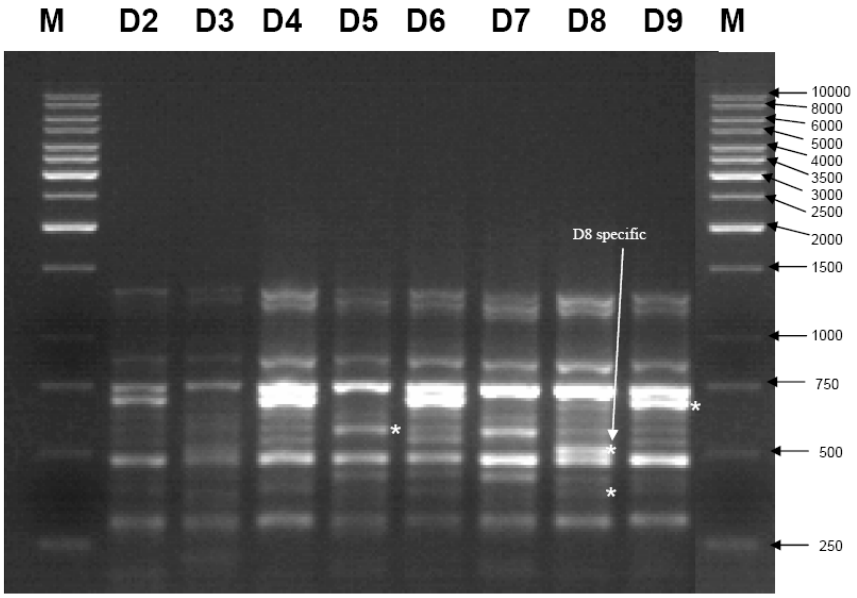

Fig. 6. RAPD of eight fungal strains using A- 03 primer.
Left-right Lane 1: 10, 1kb DNA marker (M). Lane 2-9: Randomly amplified DNA of fungal strains.
Polymorphic bands are pointed as *

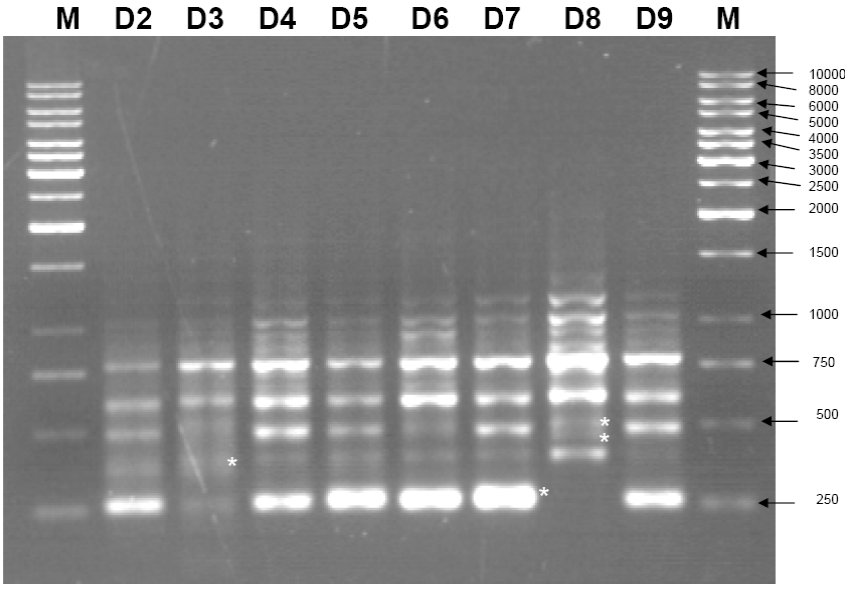

Fig. 7. RAPD of eight fungal strains using A- 04 primer.
Left-right Lane 1: 10, 1kb DNA marker (M). Lane 2-9: Randomly amplified DNA of fungal strains.
Polymorphic bands are pointed as *

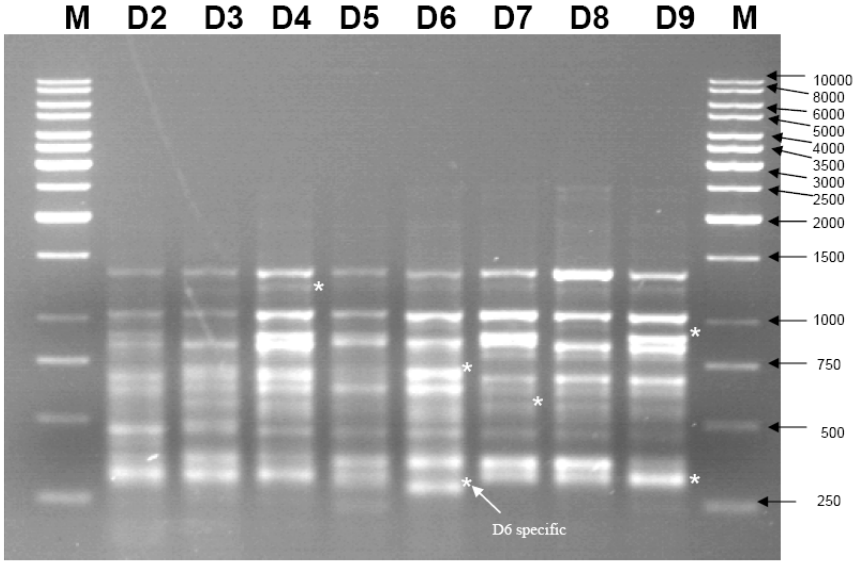

Fig. 8. RAPD of eight fungal strains using A- 05 primer.
Left-right Lane 1: 10, 1kb DNA marker (M). Lane 2-9: Randomly amplified DNA of fungal strains.
Polymorphic bands are pointed as *

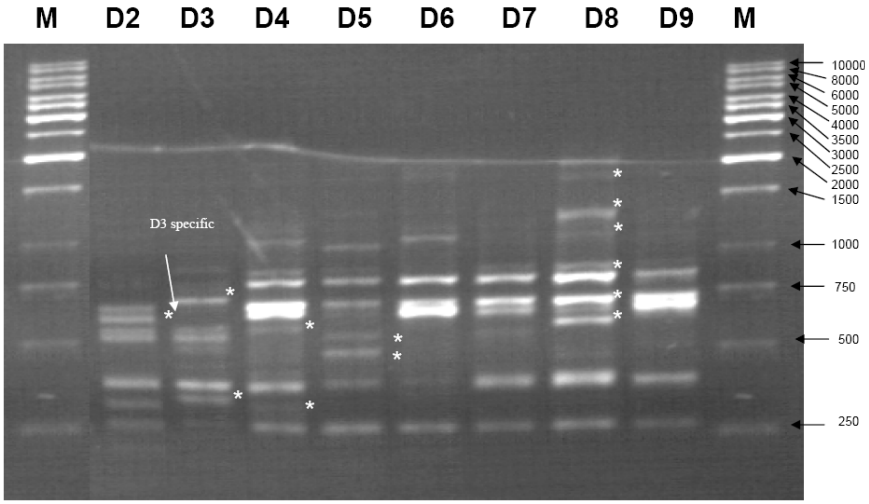

Fig. 9. RAPD of eight fungal strains using A- 06 primer.
Left-right Lane 1: 10,1kb DNA marker (M). Lane 2-9: Randomly amplified DNA of fungal strains.
Polymorphic bands are pointed as *

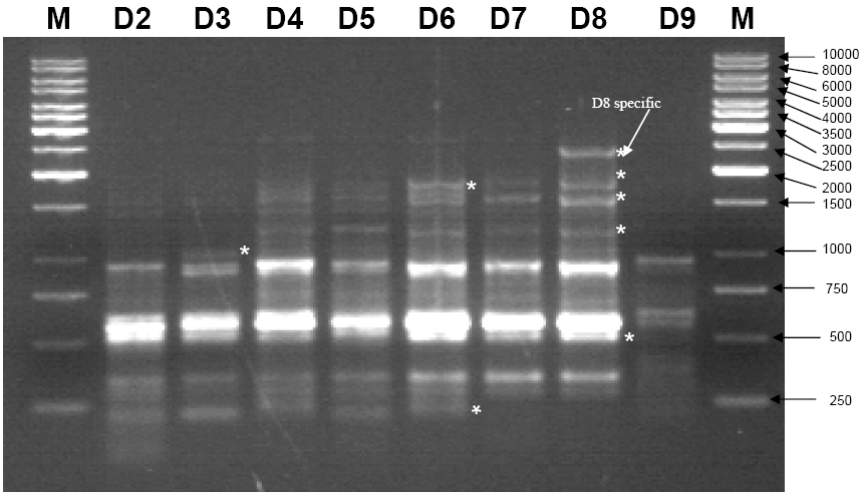

Fig. 10. RAPD of eight fungal strains using A- 07 primer.
Left-right Lane 1: 10, 1kb DNA marker (M). Lane 2-9: Randomly amplified DNA of fungal strains. Polymorphic bands are pointed as *

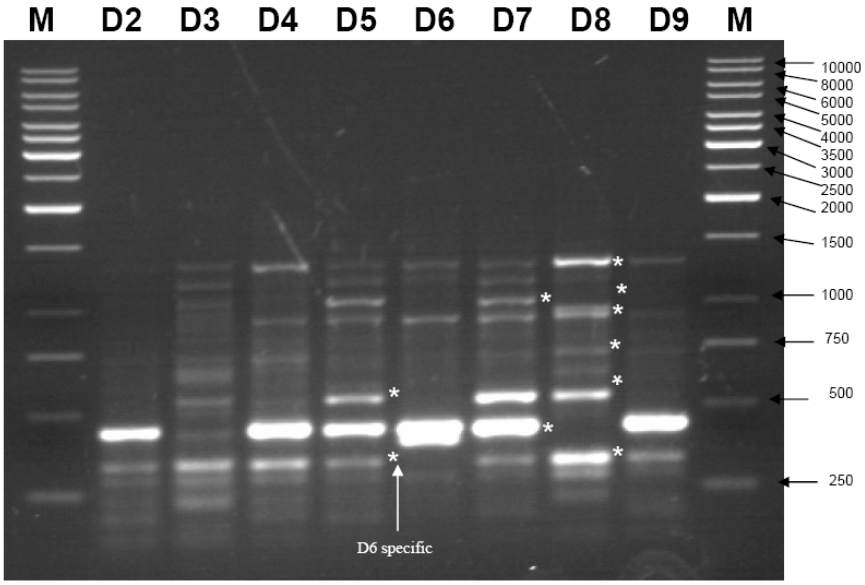

Fig. 11. RAPD of eight fungal strains using A- 08 primer.
Left- right Lane 1: 10, 1kb DNA marker (M). Lane 2-9: Randomly amplified DNA of fungal strains. Polymorphic bands are pointed as *

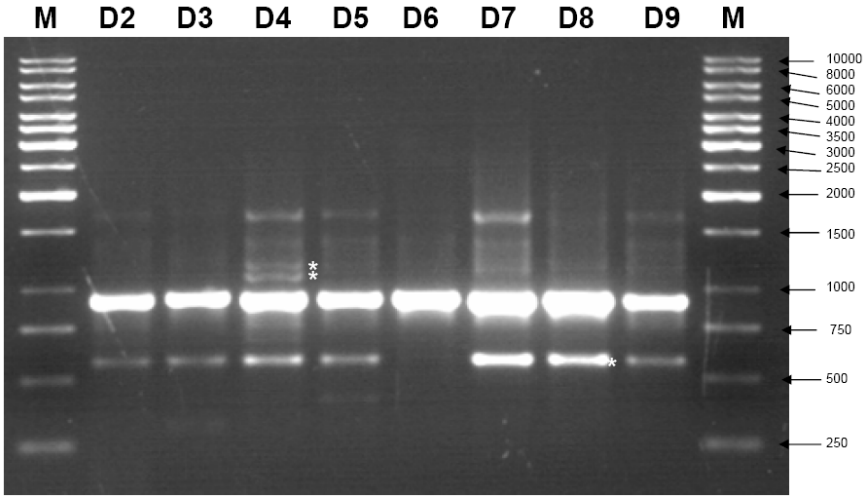

Fig. 12. RAPD of eight fungal strains using A- 09 primer.
Left-right Lane 1: 10, 1kb DNA marker (M). Lane 2-9: Randomly amplified DNA of fungal strains. Polymorphic bands are pointed as *

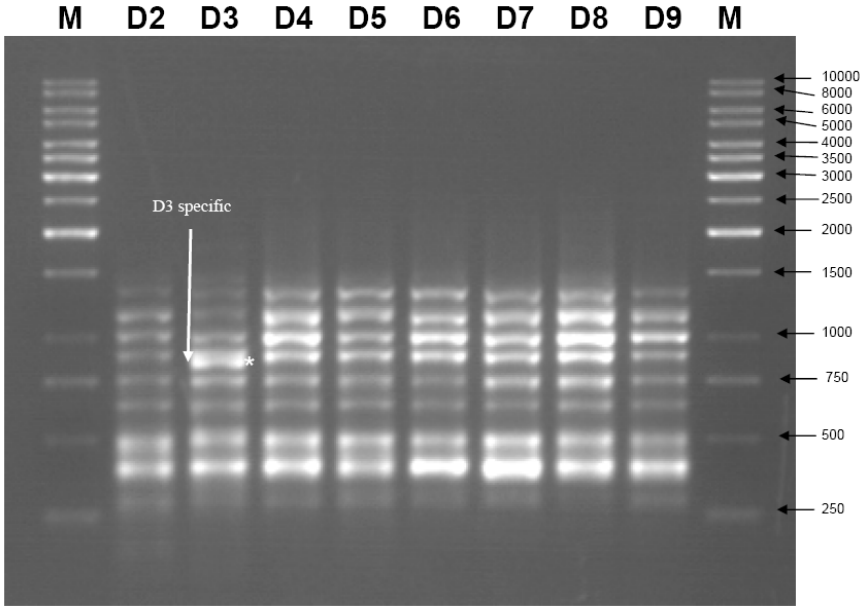

Fig. 13. RAPD of eight fungal strains using A- 10 primer. Left-right Lane1: 10, 1kb DNA marker (M). Lane 2-9: Randomly amplified DNA of fungal strains. Polymorphic bands are pointed as *

Table 4. Similarity matrix for Nei and Li's coefficient for eight fungal strains of *S. rolfsii*.

	D 2	D 3	D 4	D 5	D 6	D 7	D 8	D 9
D 2	1							
D 3	0.8291	1						
D 4	0.8413	0.8459	1					
D 5	0.8519	0.5803	0.8998	1				
D 6	0.8174	0.7759	0.8952	0.8457	1			
D 7	0.8674	0.8312	0.9236	0.9444	0.8592	1		
D 8	0.8169	0.8352	0.8591	0.8809	0.7939	0.8984	1	
D 9	0.8647	0.7968	0.8877	0.8747	0.8440	0.9012	0.8223	1

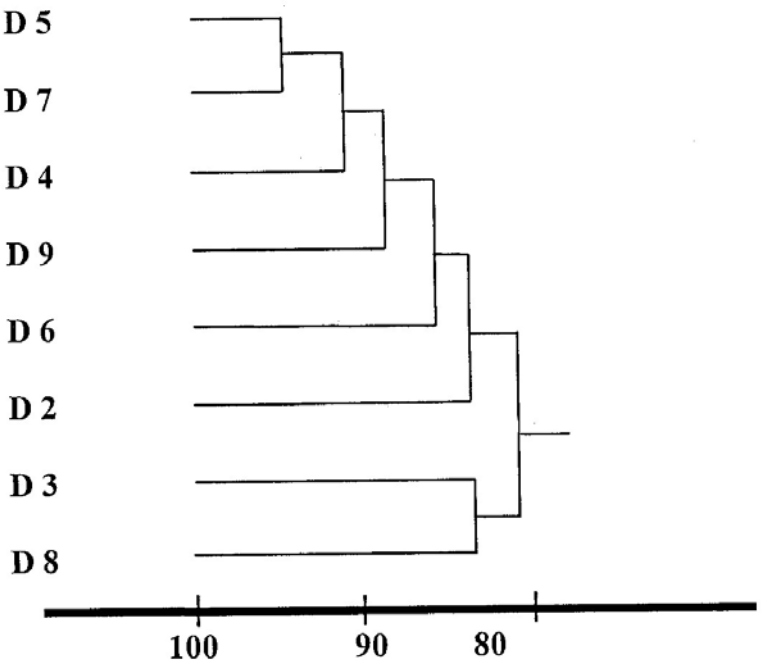

Fig. 14. Dendrogram of eight fungal strains constructed from RAPD data using unweighted pair group method of arithmetic means (UPGMA)

The data obtained under this experiment confirmed the efficiency of RAPD-PCR technique for determination and estimation of genetic similarities and differences among fungal strains collected for the present study. Therefore, RAPD analysis was found to be an informative DNA marker system to assess genetic relatedness and diversity among different strains (Tanwir *et al.*, 2007; Asif *et al.*, 2005).

RAPD-PCR analysis had also been used by other workers to investigate genetic variation among isolates of *S. rolfsii* collected from different geographical regions. Punja & Sun (1997) compared 128 isolates of *S. rolfsii* from 36 host species and 23 geographic regions by means of random amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR) which confirmed that many isolates from the same host belongs to the same mycelial compatibility group (MCG). The variation among 30 isolates of S. *rolfsii*

from different hosts and regions of Brazil was studied by Almeida *et al.*, (2001) by undertaking analysis of genomic DNA through random amplified polymorphic DNA (RAPD–PCR) technique. These techniques confirmed that there was considerable variability among isolates in relation to the number, size and location of sclerotia on the surface of medium.

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