

GENETIC CHARACTERIZATION OF *VERTICILLIUM CHLAMYDOSPORIUM* ISOLATED FROM PAKISTAN USING RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) PRIMERS

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Abstract

This study was undertaken to determine genetic variations, using RAPDs, among *Verticillium chlamydosporium* isolates infecting *Meloidogyne incognita*, a major pest in the tomato growing areas of Khyber Pakhtunkhwa province of Pakistan. Significant differences noted among the isolates from different locations and host plants. Maximum loci detected in VC-5 and VC-6, resulting in the highest polymorphism. Among the RAPD Primers, OPB-16 amplified maximum of 10 loci. Monomorphic loci detected only when OPA-19 was used. The bands amplified ranged between 200~2000 bp. The average genetic distance ranged between 16.6 to 54.0%. A dendrogram, constructed on the amplification pattern of these primers, revealed that the isolates could be divided into two different distinct groups. The data further indicated that the genetic differences among the isolates influenced by the geographical location but not by host plant.

Introduction

Tomato (*Lycopersicon esculentum* Mill), a member of family Solanaceae, is one of the principal vegetable crops of Pakistan, particularly in the northern areas of Khyber Pakhtunkhwa province. Large number of phytopathogenic organisms like bacteria, fungi, viruses and nematodes are responsible for severe yield reduction of tomato crop, both in quantity and quality (Sasser, 1980). Among Nematodes, root knot nematode (*Meloidogyne incognita*) or RKN is the major tomato pest (Cetintas & Yarba, 2010; Hamid *et al.*, 2003; Siddiqui & Shaukat, 2002; Maqbool & Shahina, 2001; Zaki, 2000). Application of chemical nematocides is the shortest way to combat this nematode (Duponnois *et al.*, 2001). However, these chemicals have poor penetration into nematodes eggs and cause environmental problems (Garima *et al.*, 2005; Oka *et al.*, 2000). The use of resistant cultivars is valuable component in RKN management (Ferraz & Mendes, 1992; Messeguer *et al.*, 1991; Roberts & Thomason, 1986) but in field having mix population of *Meloidogyne spp.*, such resistance is of no use. Because of having wide host range, (Castagnone-Sereno, 2002; Trudgill & Blok, 2001) *M. incognita*, cannot be controlled by simple crop rotation. Other cultural practices such as soil solarization and flooding are helpful in terms of nematode control (Ijoyah & Koutatouka, 2009; Kingland, 2001) but their use is restricted to small scale. Physical methods like heat treatment, radiation with gamma rays and UV light are only effective under *in vitro* or green house conditions (Katan, 1981; Bird, 1987; Cartia *et al.*, 1988). With these concerns, an urgent need exists to develop alternative management strategies that can be used to control root knot disease.

Use of microbial antagonists of RKN has received considerable attention (Dickson *et al.*, 1994; Kerry, 1987). Many nematophagous fungi including *Verticillium chlamydosporium* have been proven effective as biological management of RKN. *V. chlamydosporium* is soil inhabitant and has been found to invade eggs, egg masses, and female nematodes. This fungus can survive

(as chlamydospores) as a saprophyte for longer period in soil without host (De-Leij *et al.*, 1993; Zaki & Maqbool, 1993; Kerry *et al.*, 1986).

The fungus, *V. chlamydosporium* has been characterized using a combination of microscopic and colony characteristics. However, based on morphological characteristics, it is quite difficult to distinguish different isolates of this fungus. Moreover, neither standard laboratory bioassays nor pathotyping offer sufficient information to identify the fungus at sub-species level. In recent years, different Polymerase Chain Reaction (PCR) based molecular markers including Random Amplified Polymorphic DNA (RAPD) have proved to be useful tools for studying genetic diversity and monitoring of soil borne fungi (Mc-Donald, 1997; Williams *et al.*, 1993; Hardys *et al.*, 1992). RAPD-PCR has the advantage of being quick and easy, requiring a minute amount of genomic DNA. Furthermore, DNA finger print can be generated with RAPD by using short nucleotide (10-16 nucleotides bases) sequence of arbitrary nature as primers and does not require any prior knowledge of the target site sequence (Williams *et al.*, 1990). The present study was undertaken to determine the genetic variations, using RAPDs, among the various isolates of *V. chlamydosporium* collected from different geographical regions of Khyber Pakhtunkhwa, Pakistan.

Materials and Methods

Collection of *Verticillium chlamydosporium* isolates: To obtain isolates of the fungus, rhizosphere soil samples from a total of 35 nematode-infested localities collected including 7 locations from Peshawar, 6 from Mardan, 7 from Dargai (Malakand) and 15 from Swat, Pakistan. Five soil samples per field and 5 fields per locality used for this purpose. Soil samples of each field were thoroughly mixed to make a composite sample, labeled and stored at 4°C until used. *Verticillium chlamydosporium* isolated from soil using a semi-selective medium of De-Leij *et al.*, (1993). The fungus identified using the identification key of Barnett & Hunter (1998). Pure cultures of the fungus

maintained on fresh medium and stored 4°C until used. Based upon their better anti-nematode properties (data not shown), 6 isolates of the bio-control fungus selected for further studies.

Mycelial growth of *Verticillium chlamyosporium* isolates: The 6 different isolates of *V. chlamyosporium* from different localities of Khyber Pakhtunkhwa grown on the semi-selective medium broth (Kerry *et al.*, 1993). Small blocks (4-5cm) from hyphal tip culture (10 days-old) of *V. chlamyosporium* added to flasks containing the selective broth, sealed with parafilm and incubated at 25°C for 7-10 days on a continuous shaker at 120 rpm. Mycelial mat removed by passing the culture through sterile Whatman (0.2 µm) filter paper (Kerry *et al.*, 1986).

Extraction of DNA: RAPD-PCR conducted at the Institute of Biotechnology and Genetic engineering (IBGE), The University of Agriculture, Peshawar-Pakistan. Total genomic DNA extracted by the procedure of Stewart & Via (1993) CTAB (hexadecyltrimethylammonium bromide) method with little modification. The mycelial mat was taken, put on sterilized aluminum film and surface dried in laminar flow unit. Approximately 100mg of mycelial mat collected, transferred to sterilized Eppendorf tube, submerged in 500µl of absolute ethanol and kept at room temperature for 1 hour (Sharma *et al.*, 2003). Alcohol allowed to evaporate and the mat crushed with a plastic micro-pestle specially designed to grind small samples in Eppendorf tube. The homogenized mycelial tissue transferred to pre-warmed 2 X CTAB DNA extraction buffer (100 mM Tris-HCl, pH 8.5; 20 mM EDTA, pH 8; 1.4 M NaCl, 2% CTAB and 2 µl.ml⁻¹ β-mercaptoethanol), incubated for 1 h in a 60°C water bath, with occasional gentle swirling. To the homogenate the 300µl of chloroform-isoamylalcohol (24:1) added and gently swirled by hand for 15 min. The aqueous phase transferred to another tube after centrifugation at 14,000 rpm for 15 min. The DNA precipitated by adding absolute alcohol, incubating overnight and centrifuging at 14,000 rpm for 10 min. The pellet washed with 70% alcohol and dissolved in T10E1 buffer (pH 8). Two microliter of the DNA separated on 0.8% agarose gel for quantification and checking its integrity.

PCR amplifications: For the optimization of RAPD-PCR reaction, various buffer components, concentrations of genomic DNA of the fungal isolates and primers applied at different thermal conditions (data not shown).

Amplifications performed in 20µl reaction volume in 0.2ml PCR tubes. The reaction mixture included 2.0µl KCl buffer, 0.2µl each of ATPs, CTPs, GTPs and TTPs, 3.0 µl MgCl₂, 1µl RAPD Primer, 1µl Gemonic DNA and 2 Units of Taq Polymerase. The volume adjusted to 20µl with sterilized de-ionized water. The thermocycler programmed for an initial denaturation step of 4 min at 95°C, followed by 35 cycles of 1 min at 94°C, 2 min at 38°C and extension carried out at 72°C for 2 minutes. After 35 cycles a final extension step of 72°C for 7 minutes included. After PCR amplification, the amplified products separated in 1.5% agarose gel (w/v), run in 1X TBE buffer and visualized with ethidium bromide. Samples electrophoresed at 100 volts and photographs taken with a UVP Polaroid camera.

Data analysis: Data scored from good quality photographs. Amplified fragments scored by starting from top of the lane to its bottom. Amplification profiles of all the isolates compared with each other. The Isolates were clustered on the basis of presence (1) or absence (0) of a band. Genetic distances were calculated using “Unweighted Pair Group of Arithmetic Means” (UPGMA) procedure (Nei & Li, 1979). The following formula used for genetic distance determination.

$$GD = 1 - dx / (dx + dy - dxy)$$

where GD= Genetic distance between two genotypes.

Dxy =Total number of common loci (bands) in two genotypes.

Dx = Total number of loci (bands) in genotype 1.

Dy = Total number of loci (bands) in genotype 2.

Results

The PCR amplification profile obtained using the different RAPD Primers OPA-06, OPB-16, OPA-19, OPB-10 and OPB-14 presented in Fig. 1. The primers OPA-06, OPB16 and OPA19 amplified the DNA from all the six isolates while no amplification noted for VC-4 using primer OPB-10 and VC-1 and VC-2 while using the primer OPB-14. Five RAPD primers, thus amplifying an average of 16.00 loci per isolate and 19.2 loci for each primer (Table 1), detected the 96 loci. The RAPD profile analyzed visually and the obtained finger prints were reproducible within the particular isolate under the similar controlled conditions. The reproducibility of the fingerprinting pattern confirmed by amplifying VC-1 in ten different reactions using the primer OPA-06.

Table 1. DNA Polymorphism (%) found among isolates of *Verticillium chlamyosporium* using RAPD.

Isolates	Total bands	Polymorphic bands	Monomorphic bands	Percent polymorphism
VC-1	17	15	2	88.24
VC-2	15	13	2	86.67
VC-3	16	14	2	87.50
VC-4	12	10	2	83.33
VC-5	18	16	2	88.89
VC-6	18	16	2	88.89

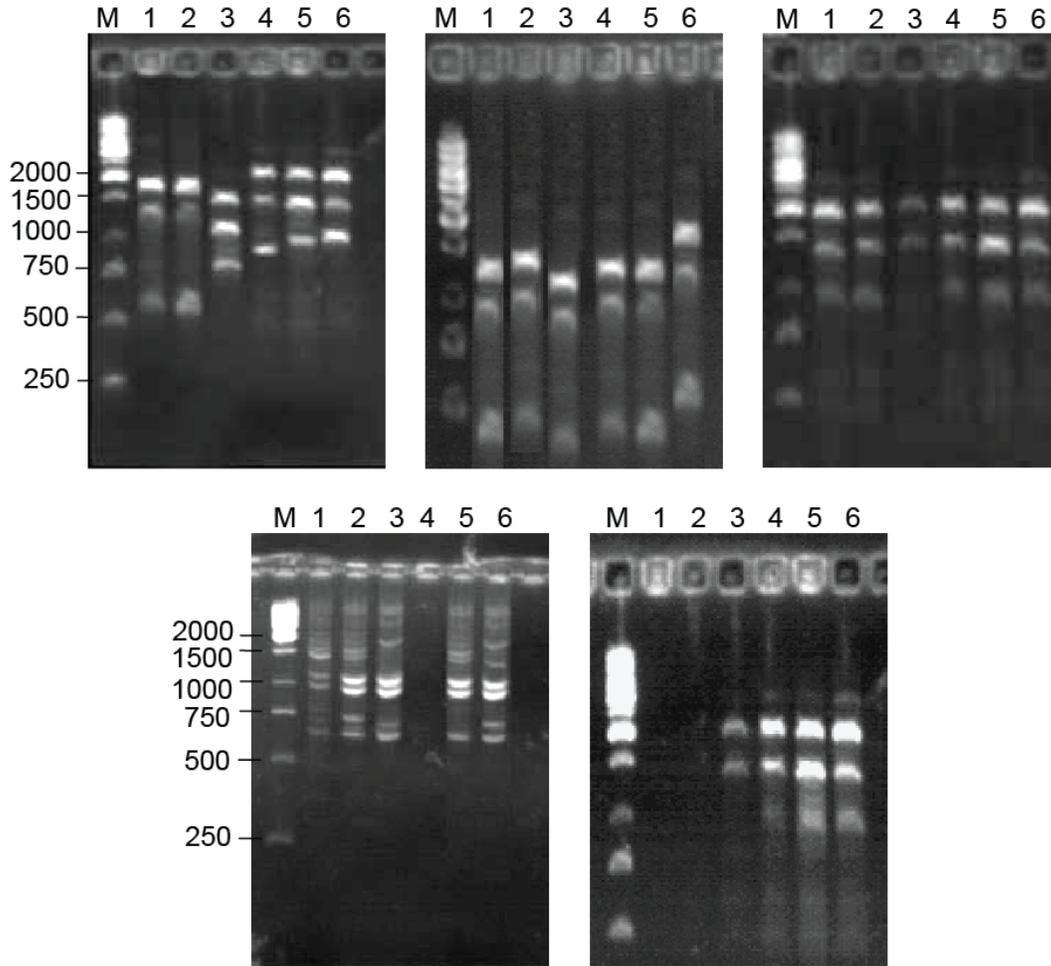


Fig. 1. DNA amplification pattern of the *Verticillium chlamydosporium* isolates from different locations of Khyber Pakhtunkhwa using random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) primers OPA-06 (Top Left), OPB-16 (Top Middle), OPA-19 (Top right), OPB-10 (Bottom Left) and OPB-14 (Bottom Right), M= Molecular size marker (1 kb Ladder), 1 = VC-1, 2 = VC-2, 3 = VC-3, 4 = VC-4, 5 = VC-5, 6 = VC-6.

Maximum number of 18 loci amplified in VC-5 and VC-6 (Table 1). Only 2 loci were monomorphic and maximum polymorphism of 88.89% observed in VC-5 and VC-6. Similarly, maximum of 10 loci were amplified by the primer OPB-16 (Table 2). RAPD Primer OPA-19 produced only monomorphic bands (Table 2).

The RAPD primer OPA-06, thus amplifying an average of 2.83 loci per isolate (Fig. 1a), amplified the 17 loci. The bands amplified ranged between 600-2000 bp. The banding pattern revealed that the maximum genetic distance of 100% was present among 6 isolates (VC-1/VC-4, VC-1/VC-5, VC-1/VC-6, VC-2/VC-3, VC-2/VC-

4, VC-2/VC-5 and VC-2/VC-6) while no genetic distance noted among VC-5/VC-6. The banding pattern of genotypes obtained using primer OPB-16 (Fig. 1b) showed various level of genetic polymorphism at DNA level. The bands amplified ranged between 200-1300 bp. The 22 alleles were amplified in 6 genotypes giving an average of 4.00 alleles per genotypes. Genetic distance estimated for all the possible combinations ranged from 0% to 100%. Maximum genetic distance (100%) observed between VC-2/VC-6 and VC-3/VC-6 while VC-4/VC-5 were similar at DNA level.

Table 2. Comparison of random primers for the production of RAPD polymorphic bands.

Primers name	Total bands	Band sizes	Polymorphic bands	Monomorphic bands	Percent polymorphism
OPA-06	9	600 ~ 2000	9	0	100
OPB-16	10	200 ~ 1300	10	0	100
OPA-19	3	650 ~ 1500	1	2	33
OPB-10	7	~ 2000	7	0	100
OPB-14	3	580 ~ 2000	3	0	100

Genetic polymorphism observed among the isolates from different locations using Primer OPA-19 (Fig. 1c). The bands amplified ranged between 650-1500 bp. The 17 alleles were amplified, giving an average of 2.83 alleles per isolate. Range of genetic distance 0.00 to 33%. Maximum of 33% genetic distance observed in VC-1/VC-3, VC-2/VC-3, VC-3/VC-4, VC-3/VC-5 and VC-3/VC-6. However, no genetic differences at DNA level were noted among the other isolates using this primer. The Fig. 1d showed banding pattern of genotypes using Primer OPB-10. The genomic DNA of one isolate (VC-4) was not amplified and hence not included in the analysis. The bands amplified ranged between 580-2000 bp. A total of 28 loci (bands) were amplified giving average of 4.67 loci per isolate, making this primer the most polymorphic. The range of genetic distances observed was between 0.00 to 57%. VC-3/VC-5 showed maximum genetic distance of 57% while minimum polymorphism detected between VC-1/VC-2.

The banding profile obtained using RAPD Primer OPB-14 amplified a total of 10 alleles in 4 isolates (Fig. 1e) giving an average of 2.5 loci per genotype. Genomic DNA from 2 isolates (VC-1 and VC-2) were not amplified and were not included in the analysis. The bands amplified ranged between 700-2000 bp. The range of genetic distance observed was between 0 to 33%. Maximum genetic distance (33%) observed in VC-3/VC-5, VC-4/VC-5 and VC-4/VC-6 while VC-3/VC-4 and VC-5/VC-6 were genetically similar at DNA level using this primer.

The average genetic distances based on five RAPD primers ranged from, 16.6-54.0% (Table 3). Comparison among isolates revealed that highest genetic dissimilarities (54.0%) found between VC-3/VC-5 closely

followed by VC-3/VC-6 and VC-2/VC-3 with 52.4% and 48.4% dissimilarity, respectively. Furthermore, the lowest genetic distance of 16.6% noted in VC-4/VC-5. A dendrogram constructed by using Unweighted Pair Groups of Arithmetic Means (UPGMA) (Fig. 2) which distinguished the isolates into 2 major groups. Group I contained VC-1, VC-2 and VC-3 whereas VC-4, VC-5 and VC-6 clustered into Group II. The isolates VC-1, collected from Sardar Gari, and VC-2, collected from Pabbi, closely resembled each other and fell in Group I. The reason for this close resemblance might be that Pabbi and Sardar Gari situated at a distance of 10km from each other and there is no geographical barrier between these 2 locations. VC-3, collected from Mardan, was genetically distinct from VC-1 and VC-2. The reason for this genetic difference might be that Mardan is situated at some distance from the above named 2 locations and also it is physically separated from the 2 locations by river Kabul. Similarly, VC-4 and VC-5 were isolated from Shergarh and Sakhakot, respectively and closely clustered in Group II. However, VC-6, isolated from Barikot, a geographically distinct area also fell in Group II. Thus it is evident from the data that the RAPD primers had clearly distinguished the *V. chlamyosporium* isolates from the different geographical locations.

Table 3. Average genetic distance (% age) among six isolates of *Verticillium chlamyosporium* using RAPD.

	VC-1	VC-2	VC-3	VC-4	VC-5
VC-2	23.4				
VC-3	39.6	48.4			
VC-4	28.0	28.0	36.0		
VC-5	31.2	31.2	54.0	16.6	
VC-6	22.8	45.6	52.4	33.8	25.6

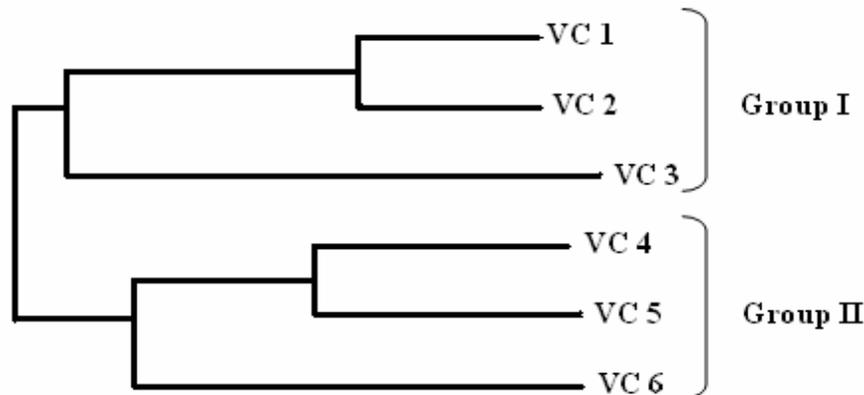


Fig. 2. UPGMA Dendrogram of six isolates of *V. chlamyosporium* constructed for polymorphic loci with five RAPD primers using Pop Gene-32 Computer Program.

Discussion

Use of microbial antagonists in the control of soil-borne phytopathogens including nematodes has received more attention throughout the world (Saifullah, 1996; Dickson *et al.*, 1994; Kerry, 1987; Mulder, 1979). In nature, several nematophagous fungi are effective against nematodes. To develop fungus as biological control agent,

the fungal isolates must be clearly defined, should be virulent to target pathogen, harmless to host plant, persist in soil for longer time and remain stable at sub-species level. *V. chlamyosporium* possesses all the above stated features and is of considerable interest to researchers as a possible biological control agent against root knot nematodes. Species of *Verticillium* are currently identified and taxonomically classified by combination of

morphological and colony characteristics. However, it is almost impossible to distinguish isolates (belonging to the same species) based only on morphological characters. Further, isolates are different greatly in their virulence and production of chlamydospores. Therefore, careful selection must be made before releasing an individual isolate of *V. chlamydosporium* to soil as biological control agent.

Several DNA-based molecular markers; Randomly Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphisms (RFLPs), Simple Sequence Repeats (SSRs) and Amplified Fragment Length Polymorphism (AFLP) have been successfully used to estimate genetic diversity of fungi (Atkins *et al.*, 2003; Morton *et al.*, 2003; Sugimoto *et al.*, 2003). Randomly Amplified Polymorphic DNA (RAPD) is a relatively simple technique and has been commonly used for genetic characterization and identification of individual isolates of fungi (Mauchline *et al.*, 2004). The objective of the present study was to characterize *V. chlamydosporium* isolates, collected from different location of Khyber Pakhtunkhwa, Pakistan, at molecular level. For this purpose 6 different isolates of *V. chlamydosporium* were analyzed using 5 RAPD primers, - OPA-06, OPB-16, OPA-19, OPB-10 and OPB-14. All the tested genotypes in their RAPD assay generated variety of amplification products. Level of genetic polymorphism among the genotypes detected during present study varied from primer to primer.

The *V. chlamydosporium* in the present study isolated from different geographical locations. The isolates VC-1 (collected from Sardar Garhi) and VC-2 (collected from Pabbi) fell in the same cluster. Sardar Garhi and Pabbi lie in close proximity to each other, so the isolates collected from areas were expected to cluster together. Similarly, the isolates from Sakhakot (VC-5) and Shergarh (VC-4) were also clustered together in the dendrogram as these areas are also located close to each other and have similar environmental conditions. Thus, RAPD finger prints generated rightly assigned the fungal isolates from the different regions into distinct groups. The diversity in *V. chlamydosporium*, however, could not be assigned to host preference as isolates obtained from the host fell in different clusters. For example, VC-1, isolated from tomato, fell in the same cluster with VC-2, isolated from okra. Thus, the genetic differences among the isolates of *V. chlamydosporium* may possibly be due to the different geographical locations they were collected rather than the different hosts they were isolated from. This variation of isolates also suggested that RAPD-PCR may be useful tool for genetic diversity studies at sub-species level of fungi and monitoring the individual isolates after release into the soil as biological control agent.

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