

Assessment of Genetic Variation of Some *Verticillium dahliae* Isolates by RAPD Analysis

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Abstract: Genetic variation among three isolates belonging to *Verticillium dahliae* was studied using Random Amplified Polymorphic DNA (RAPD) markers. Out of 21 tested primers, 11 primers which revealed polymorphic patterns were selected. Highly polymorphic variations were obtained on either the number and/or the molecular weight of produced bands. The primers OPB-17, OPA-09 and OPW-17 showed the ability to create genetic markers to distinguish the tested isolates VD1, VD2 and VD3 of *V. dahliae* respectively, whereas the primer OPA-14 revealed the potential to discriminate and differentiate between the three isolates by generating different genetic markers. The dendrogram based cluster analysis showed that the isolates VD1 and VD3 group together. This study suggests that RAPD analysis can assist the morphological similarity variation between different isolates, collected from the same geographical area.

Key words: Isolates of *Verticillium dahliae* strain • RAPD analysis • Polymorphism and genetic marker

INTRODUCTION

Verticillium wilt represents one of the most important diseases caused by *V. dahliae* affecting a wide host range including economically important crops and ornamental plants [1].

Many evidences refer to significant variability within *V. dahliae* and that it has the potential to contribute in the disease complex involving plant parasitic nematodes with the soilborn phytopathogenic fungus [2], thus new strains evolve overcoming the resistance in commercial cultivars, particularly on cotton [3].

Moreover, strains differ quantitatively or qualitatively in their pathogenicity can be classified as defoliating (D) and non defoliating (ND) pathotypes according to their ability to defoliate the plants [4].

These variations correlate in some cases with other differences such as morphological and physiological traits, as well as vegetative compatibility groups [5]. However, using these methods for characterization are labor intensive, time consuming and not informative in describing genetic diversity within or among pathogenic populations. Therefore, enhanced methods are needed for rapid, reliable and informative results.

The molecular markers provide many techniques to analyze DNA molecules and to facilitate all these processes. Randomly Amplified Polymorphic DNA (RAPD) marker is one of these techniques which proved to have many advantages in characterizing and analyzing the variation within population pathotypes in *V. dahliae* [6,7].

In this study, the variation of genomic DNA was examined using RAPD markers among three local cotton isolates of *V. dahliae* (as genetic study) to complete a previous study about interactions effect between these isolates and nematodes on two resistant Syrian cotton cultivars.

MATERIALS AND METHODS

Isolates of *V. dahliae*: Three isolates of *V. dahliae* were kindly provided by Dr. W. Naffaa and M. Njoum, (Plant protection department/ Faculty of Agriculture /Damascus University).

The three isolates of *V. dahliae* were isolated and collected from cotton field from same geographical area (Skeilbyeh, Hama, Syria), they exhibited a high degree of morphological variation *in vitro* in Potato dextrose agar.

DNA Extraction: Fungal DNA was extracted using CTAB method according to [8] with same adaptations as follows: Fungal aerial mycelium was scraped from a potato dextrose agar plate and ground in liquid nitrogen to a fine powder. CTAB buffer was added to the powder and gently mixed. The samples were kept at 60°C for 30 min and swirled gently every 10 min. Chloroform-isoamyl (24:1) was added and mixed gently. The tubes were centrifuged for 20 min at 4°C at 18000g and the resulting aqueous phase was transferred into a new eppendorf tube. Fungal DNA was precipitated by adding absolute ethanol to the transferred aqueous phase which was left on ice for 20-30 min. followed by centrifugation for 10 min. at 4°C at 18000g. The pellet was washed with 70% ethanol and centrifuged for 3 min at 4°C at 18000g and then dried till the ethanol was completely eliminated. DNA was dissolved in 100 µl TE buffer and stored at -20°C.

RAPD Amplification Reaction: RAPD reactions were carried out using 21 (10-mer) oligonucleotides random primers provided from Operon kits (Operon Technology, Alameda, CA).

Amplification reactions were performed in a total volume (25 µl) consisting of 12.5 µl of PCR master mix (Fermentas, EU) 2.5 µl primer (10 pmol), 2 µl genomic DNA (50 ng) of 8 µl sterile distilled water – The reaction mixture was overlaid with 20 µl of sterile mineral oil.

Amplification program was started with 2 min at 94 °C for initial denaturation followed by 40 cycles of 94 °C for 1 min (denaturation) 36 °C for 1 min (annealing) and 72 °C for 2 min (extension). The final cycle was 72 °C for 1 min (final extension). the RAPD reaction was performed using the thermocycler (Apollo, ATC 401, USA).

The amplification products were separated on 1-5 % agarose gel and visualized under UV light after they were stained with ethidium bromide.

RAPD analysis: Data were scored according to the presence (1) or absence (0) of the band at a particular position. Only major bands were considered for statistical analysis. A dendrogram was constructed by cluster analysis using unweighted paired group method with arithmetic average (UPGMA) based on Jaccard's similarity coefficient.

RESULTS AND DISCUSSION

RAPD analysis was performed for the three *V. dahliae* isolates using 21 random primers in order to determine the genetic variation between isolates. Amplification products were obtained from only 11 primers (Table 1). This number of amplified primers is generally considered to be sufficient to study the genetic variation and also for further examinations [9] other researchers used less number of primers to analyze the variation among isolates on the DNA level of *V. dahliae* [7,10].

The obtained results showed good degree of polymorphism among the tested isolates. Figure (1) shows the RAPD amplified fragments in the three *V. dahliae* isolates using 11 primers.

Polymorphisms may be scored as the number and/ or the molecular weight of the amplified fragments. The number of amplified fragments varied between 1 (OPA- 09) to 11 (OPB-18) with a mean of six bands per primer. Furthermore, analysis of 11 random primers in this study generated 55 bands, 53 bands of which were polymorphic, (4.8 polymorphic bands per primer). This fact reflects mainly the variation of primer sequence and/ or individual genotypes [11].

The amplified bands were also different in their molecular weights, their range was between 181bp (OPB-17) and 3311 bp (OPZ-02). This reflects length differences among the sites of primer sequences that are complementary with the genome of tested isolates [12].

Moreover, variant specific primers bands were detected among the three isolates for example primer OPB-15 revealed a RAPD marker with isolate VD1 with a band size 950 bp and primer OPA-09 generated a another marker with isolate VD2 with a band size 1584 bp. Primer OPW-17 distinguished the isolates VD3 by three genetic markers with 1000, 630 and 500 bp. Differential primer (OPA-14) was able to discriminate the three isolates from each other by three different genetic markers with 1400, 830 and 400 bp for isolates VD1, VD2 and VD3 respectively.

The polymorphic fragments produce by RAPD analysis were used for the development of chromosome, genome, species, or isolates specific markers [13] and to estimate the degree of similarity between individuals, populations and species [14].

Table 1: The amplification products of 11 selected primers by RAPD analysis

Primer name	Band size (bp)	<i>V. dahliae</i> isolates			Primer name	Band size (bp)	<i>V. dahliae</i> isolates		
		VD1	VD2	VD3			VD1	VD2	VD3
OPA-09	1584	0	1	0	OPJ-04	2290	0	1	0
OPA-11	2818	0	1	0	OPJ-04	1380	0	1	0
	1096	0	0	1		1096	0	0	1
	1000	0	1	1		1000	0	1	0
	851	1	0	0		912	0	0	1
	794	0	1	0		870	0	1	0
OPA-14	1380	0	0	1	OPW-17	630	0	1	0
	831	0	1	0		1000	0	0	1
	363	1	0	0		831	0	1	0
OPB-12	2290	0	1	0	OPZ-02	630	0	0	1
	1202	0	1	0		524	0	0	1
	1000	0	1	1		3311	1	0	0
	758	0	1	0		1659	1	0	0
OPB-15	410	1	0	0	OPZ-02	1258	1	0	0
	1445	0	1	0		1230	0	0	1
	954	1	0	0		831	0	1	1
OPB-17	2630	0	1	0	OPZ-19	691	0	1	0
	870	0	1	0		575	0	1	1
	660	0	1	0		1621	1	0	0
OPB-18	182	1	1	1	OPZ-19	1096	0	1	1
	2754	1	1	0		870	0	1	0
	1995	0	1	0		724	0	1	1
	1584	0	1	0		660	0	1	0
	1202	0	0	1		524	0	1	1
	1096	1	0	1		-----		-----	
	831	0	0	1		Total Amplified bands	Total Polymorphic bands		
	794	1	1	0		-----		-----	
630	0	1	1	55	53				
549	1	1	1	-----		-----			
416	0	0	1	-----		-----			
331	0	0	1	-----		-----			

Table 2: RAPD variant specific markers for the three *V. dahliae* using 11 primers

Primer	Genetic marker (bp)	Isolates
OPB-15	950	VD1
OPA-09	1584	VD2
OPW-17	1000, 630, 500	VD3
OPA-14	1400, 830, 400	VD1,VD2,VD3 respectively

RAPD amplification was also used to determine relationships among the isolates. A dendrogram was constructed by cluster analysis using RAPD- based genetic distance present in Fig.(2). The obtained results showed that isolates VD1 and VD3 clustered together.

In this study RAPD analysis came in agreement and assisted strongly the previously obtained results (Naffaa and Najoum, Un publish data), in which the researcher has shown that although the three tested isolates of *V.dahliae* belong to non defoliating types, giving mild effects when infecting two resistant local Syrian cotton cultivars, but a significant morphological variation was observed among these isolates in response to complex diseases when studying their interaction with plant parasitic nematodes.

These results confirm that the variation in morphological traits may reflect strong variation on the genomic level. In addition, different responses may be related to specific individual traits. Moreover, the results

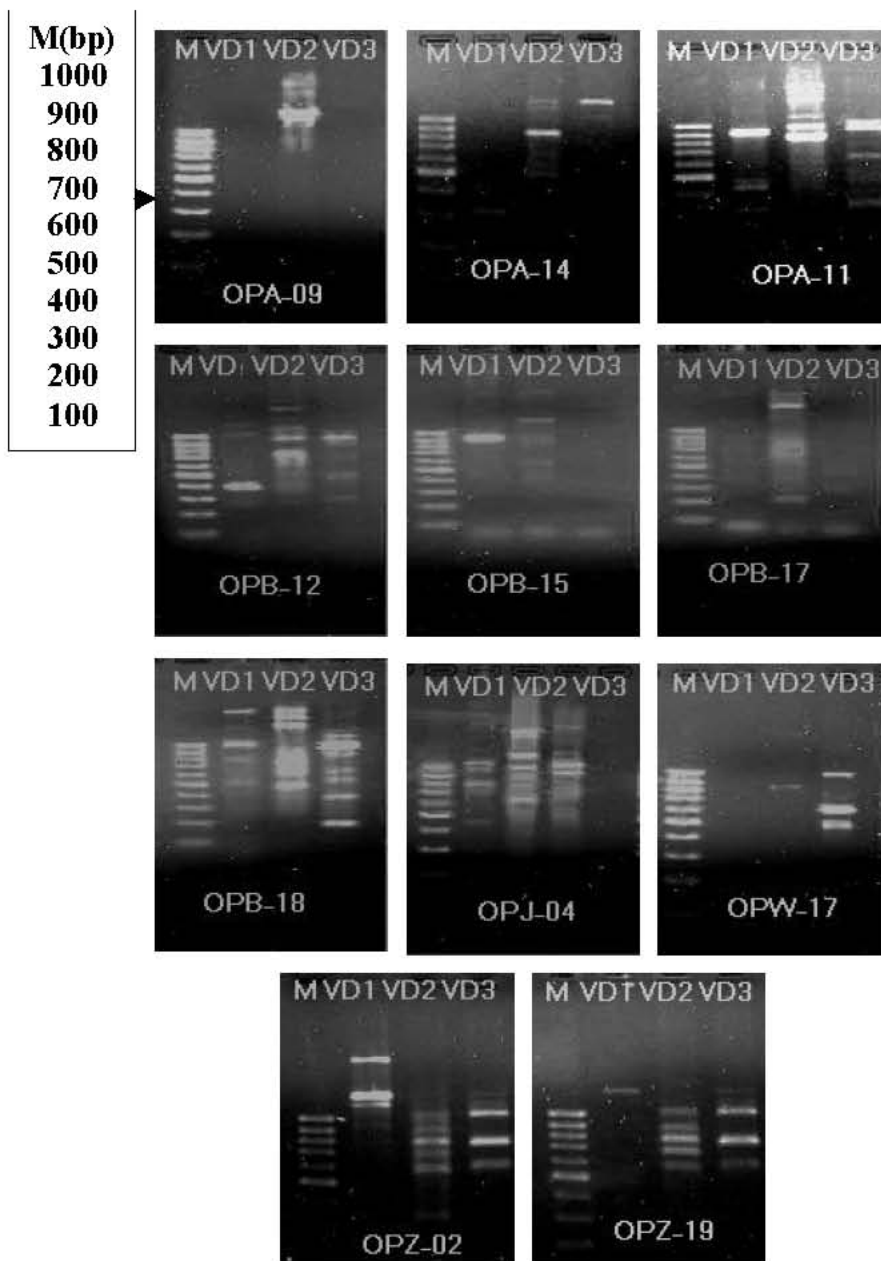


Fig. 1: RAPD amplification products of VD1, VD2 and VD3 isolates using the 11 primers.
 M: DNA ladder with ten fragments in bp.
 VD1, VD2 and VD3 represent the three isolates of *V. dahliae*

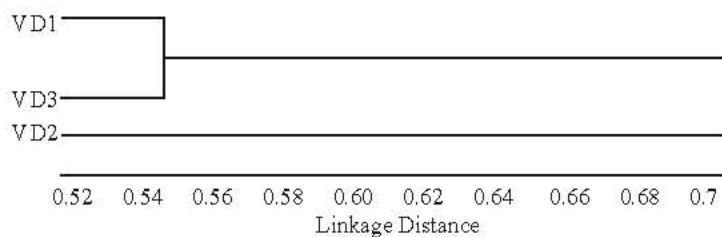


Fig. 2: Cluster analysis of RAPD data using 11 primers

confirm that different fungal variants, isolated from the same geographical area, may differ in their effect and interaction against host plants. Therefore, RAPD markers successfully proved their ability to evaluate the genetic variation of pathogens, in relation to variations of the host plants [15,16].

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