http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 8 (9): 1233-1236, 2005
ISSN 1028-8880
© 2005 Asian Network for Scientific Information

Molecular Characterization of *Verticillium dahliae* Isolates in Southern Turkey

[1]S. Dervis, [1]S. Kurt and [2]M. Bicici
[1]Department of Plant Protection, Faculty of Agriculture, Mustafa Kemal University, 31040, Hatay, Turkey
[2]Department of Plant Protection, Faculty of Agriculture, Cukurova University, 01330, Adana, Turkey

Abstract: PCR products amplified from genomic DNA of the 21 pathogenic *V. dahliae* isolates from both different provinces and Vegetative Compatibility Groups (VCGs) were characterized by RAPD analysis. Of 20 primers tested, five primers produced polymorphic amplification patterns. Phylogenetic analysis indicated two distinct clusters. The first cluster contained 8 isolates belonging to VCG2B and one isolate belonging to VCG4B. The second cluster contained 8 isolates belonging to VCG2A and one isolate belonging to VCG2B of *V. dahliae*. RAPD-PCR analysis was mostly suitable to differentiate *V. dahliae* isolates based on VCGs or subgroups. Because all VCG2B isolates from cotton except one were identified as members of cluster one and all VCG2A isolates belonged to the second cluster. Supporting this conclusion, one isolate belonging to VCG4B and the Heterokaryon Self-incompatible (HSI) isolate, never included in both clusters.

Key words: *Verticillium dahliae*, vegetative compatibility groups, RAPD-PCR

INTRODUCTION

Verticillium Wilt (VW) disease, caused by the soilborne fungal vascular-wilt pathogen *Verticillium dahliae* Kleb., is one of the most important diseases of numerous host plants throughout many parts of the world. The disease has become a major constraint in Turkey's cotton production industry[1] as in most major cotton-producing areas of the world[2] in recent decades.

V. dahliae isolates infecting cotton can be classified as Defoliating (D) and Nondefoliating (ND) pathotypes according to their ability to defoliate the plant. Highly virulent pathotype (T-1 defoliating) and mild virulent pathotype (SS-4, nondefoliating) of *V. dahliae* have had serious effects on diminishing cotton yields and economic returns[3]. Therefore, the identification of pathotypes in the populations of *V. dahliae* is of crucial importance for the resistance breeding of the disease, some other control measures and biology, pathogenicity and genetic relatedness of representative isolates of the pathogen.

Cotton isolates of *V. dahliae* can be characterized as to pathotype by means of morphological and physiological traits as well as by vegetative compatibility groups of the pathogen and virulence tests on cotton cultivars[4]. However, typing isolates by the aforementioned procedures can be cumbersome and uninformative when dealing with host specificity, genetic diversity within, or relationships among population of the pathogen[5]. Therefore, improved methods are needed for rapid, reliable, specific and informative characterization of D and ND *V. dahliae* isolates. More recently, Polymerase Chain Reaction (Pcr)-mediated Random Amplified Polymorphic DNA (RAPD) and restriction fragment length polymorphisms (RFLPs) technique have allowed the characterization of soilborne plant-pathogenic fungi [6-12].

Studies using the RAPD and specific PCR analyses revealed that banding patterns obtained by arbitrary single primers differentiated *V. dahliae* isolates as Defoliating (D) or Nondefoliating (ND)[5]. In addition, different RFLP profiles and RAPD bands were detected among the D and ND *V. dahliae* pathotypes belonging different vegetative compatibility groups VCGs[5,8,13]. No similar information is available on the relationships among various traits of *V. dahliae* isolates in Turkey.

This study was conducted to analyze the genetic variation in *V. dahliae* isolates from different VCGs and different locations by RAPD-PCR.

MATERIALS AND METHODS

Isolation of *V. dahliae* from affected plants: Isolates of *V. dahliae* were collected from diseased cotton, watermelon and eggplant plants. For isolations from infected plants, root, stem and petiole tissues exhibiting vascular discoloration were rinsed thoroughly in tap water and air-dried for 5 to 10 minutes. Infected tissues were aseptically cut to dimensions of approx. 5-10 mm long and surface-disinfested in 0.525% NaOCl solution for 2 min,

Corresponding Author: Sibel Dervis, Department of Plant Protection, Faculty of Agriculture, Mustafa Kemal University, 31040, Hatay, Turkey Fax: +90 326 2455832

rinsed twice in sterile distilled water and dried between sterile filter papers. Disinfested tissues were plated on Potato Dextrose Agar (PDA, Difco) and ethanol agar (20 g of agar, 6 mL of 95% ethanol, 1 L of distilled water) amended with streptomycin sulphate at 100 μg mL^{-1} to inhibit bacterial growth. Petri plates were incubated at 23 to 24°C in the dark for 5 to 7 days. After incubation, hyphal plugs of each growing isolate colony were transferred to petri dishes (6 cm diameter) of PDA amended with the same concentrations of antibiotics and incubated in the dark at 23°C for 10 days. Colonies forming microclerotia (ms) were identified as *V. dahliae* according to the taxonomic features of the fungus via microscopic examination. Single-spore isolates of *V. dahliae* were obtained from water agar medium amended with 100 μL mL^{-1} streptomycin sulphate and maintained in vials containing PDA at 4°C.

Pathogenicity tests: Pathogenicity of the 21 isolates was tested by injecting the conidia directly into the xylem tissues of 6-week-old cotton plants of cultivar Coker 310 with a hypodermic needle containing conidial suspension ($3x10^6$ conidia per mL of sterile water) of each isolate of *V. dahliae*. Control plants were treated with sterile distilled water. Plants were grown into a growth chamber at 25-27°C by day and 18-22 °C by night, 75% relative humidity, with 14 h white fluorescent light by Philips 400W lamps. The number of diseased plants was counted 2, 3, 4 and 5 weeks after inoculation. The disease development in individual plants was rated on a 0-4 scale based on the rate of foliage with VW symptoms, where 0 indicated the plant without symptoms and 4 indicated the plant dead.

Random Amplified Polymorphic DNA (RAPD) analysis

Genomic DNA extraction: Methods described by Peever *et al.*[14] were followed to extract genomic DNA from *Verticillium*. Fungal mycelia for DNA extraction were produced in 90 mm glass petri plates filled with 15 mL of potato dextrose broth medium. Plates were incubated in the dark at 24°C for 10 to 15 days, following a dark period the mycelia were harvested, drained on filter paper and placed in 2 mL micro-centrifuge tubes. Mycelia in tubes were immediately frozen at -20°C and lyophilized. Lyophilized mycelium was ground at room temperature with a metal rod and 50 μg of mycelium from each isolate were extracted with 400 μL lysis buffer containing 50 mM EDTA, 100 mM Tris (TE), pH 8, 3% Sodium Dodecyl Sulfate (SDS), 100 μg mL^{-1} proteinase K, 1% sodium bisulphide for 30 to 45 min at 65°C. Mycelium was pelleted by centrifugation at 10.000 x g for 15 min and the supernatant was precipitated with 8 M potassium acetate at -20°C for 15 min. Following centrifugation step, to two rounds of phenol/chloroform/ isoamyl alcohol (25:24:1, vol/vol/vol) extraction and one round of chloroform/isoamyl alcohol (24:1, vol/vol) extraction were added to supernatant. Final supernatant samples were precipitated with 2 volumes of 100% EtOH and 0.5 M NaCl. Pellets were resuspended in 500 μL water, precipitated in 14% polyethylene glycol and 1 M NaCl and resuspended in 50 μL TE buffer (10 mM Tris, 1 mM EDTA). RNA was digested with RNAse A at 20 μg mL^{-1} and 37°C for 3 h. DNA samples were stored at -20°C for further use.

RAPD primers and amplification conditions: Twenty random primers from Operon primer Kit A (Operon Technology, Alameda, CA) were screened with DNA of all isolates of *V. dahliae* from both different provinces and Vegetative Compatibility Groups (VCGs) tested by pathogenicity assays. Amplification reactions were performed in a total volume of 25 μL, containing master mix, H_2O, 50 μM primer, 1 μL of genomic DNA. Negative controls (no template DNA) were performed in all experiments to test for contamination. Amplification procedures were performed with a DNA Thermal Cycler programmed as follows: initial 2 min melt at 93°C followed by 44 cycles of 92°C for 1 min (melt), 37°C for 1 min (anneal) and 72°C for 2 min (extension). The final cycle was 72°C for 10 min (21). PCR products were separated in 1% agarose gels with ethidium bromide and photographed under UV lights. A profile comparison for each primer was conducted on the basis of the presence versus absence (1/0) of RAPD products of the same length. Bands of the same length were scored as identical[6] and a dendrogram was constructed from the similarity coefficient data by using PAST Ver.1.28 software.

RESULTS AND DISCUSSION

Twenty *V. dahliae* isolates (18 isolates from cotton and 2 from cotton watermelon) assigned to Vegetative Compatibility Groupings (VCGs) in previous study[15] and one additional isolate from eggplant were used for pathogenicity and RAPD analysis studies (Table 1).

Disease assays by these isolates were performed to verify their pathogenicity on cotton cv. Coker 310, which is susceptible to this wilt pathogen. All isolates were pathogenic at the level of 1.3-3.6 disease index (Table 1). There were no symptoms on the control plants.

Five of the 20 Operon primers (Table 2) produced a total of 44 polymorphic bands from 21 pathogenic *V. dahliae* isolates, which were reproducible in repeated amplifications pattern. Based on UPGMA analysis of these RAPD banding patterns, 21 *V. dahliae*

Table 1: Isolates of *Verticillium dahliae* listed by their host, geographic origin, Vegetative Compatibility Group (VCG), RAPD group and disease index values

Isolate	Original host	Geographic origin	VCG	RAPD group[y]	Disease index[w]
Ch09 (15)	Cotton	Hatay	2B[x]	I	3.4
W2 (16)	Watermelon	Mersin	2B[x]	I	2.9
Ch17 (17)	Cotton	Hatay	2B[x]	I	3.1
Ch05 (5)	Cotton	Hatay	2B[x]	I	2.5
Ch06 (2)	Cotton	Hatay	2B[x]	I	2.3
Ch25 (8)	Cotton	Hatay	2B[x]	I	2.5
Cm2 (4)	Cotton	K.Maras	2B[x]	I	3.3
Cko3 (1)	Cotton	Adana	2B[x]	I	1.8
E2 (3)	Eggplant	Hatay	4B	I	2.3
Ch16 (6)	Cotton	Hatay	2A[x]	II	1.6
Cy6 (11)	Cotton	Adana	2A[x]	II	1.5
Cko6 (12)	Cotton	Adana	2B[x]	II	1.6
Ch13 (18)	Cotton	Hatay	2A[x]	II	2.3
Ch02 (9)	Cotton	Hatay	2A[x]	II	2.8
Ch14 (10)	Cotton	Hatay	2A[x]	II	2.5
Cy3 (19)	Cotton	Adana	2A[x]	II	1.5
Ch08 (14)	Cotton	Hatay	2A[x]	II	2.4
Ch10 (21)	Cotton	Hatay	2A[x]	II	2.6
Cko1 (7)	Cotton	Adana	4B[x]	-	1.3
W1 (13)	Watermelon	Mersin	2B[x]	-	3.1
Ch07 (20)	Cotton	Hatay	HSI[XZ]	-	3.6

[w]Mean of 15 replicates on a scale of 0-4: 0 = plant healthy, 1 = one cotyledon dead, 2 = cotyledons dead, 3 = cotyledons dead and necrosis more than one leaf, 4 = plant dead,
[x]Vegetative compatibility groups in *Verticillium dahliae* isolates from cotton reported in previous study[15]
[y]As determined by random amplified polymorphic DNA polymerase chain reaction, [z]HSI= Heterokaryon Self Incompatible

Table 2: Code and sequences of the 5 primers tested, with number of amplified and polymorphic DNA fragments obtained with each primer in RAPD experiments

Code	Sequence 5' to 3'	Amplified fragments	Polymorphic fragments
OPA-02	TGCCGAGCTG	5	4
OPA-04	AATCGGGCTG	5	3
OPA-13	CAGCACCCAC	6	2
OPA-15	TTCCGAACCC	10	10
OPA-19	CAAACGTCGG	25	25

isolates were differentiated into two distinct RAPD groups (Fig. 1). Polymorphism in amplification of genomic DNA was observed among *V. dahliae* isolates belonging to different VCGs and VC subgroups. The first group included the isolates belonging to VCG2B (Isolates 15, 16, 17, 5, 2, 8, 4 and 1) except isolate 3 from eggplant belonging to VCG4B. The second group included 8 isolates assigned to VCG2A (Isolates 6, 11, 18, 9, 10, 19, 14 and 21) and one isolate belonging to VCG2B (Isolate 12) of *V. dahliae*. The watermelon isolate 13, the isolate 7 from cotton belonging to VCG4B and the Heterokaryon Self-incompatible (HSI) isolate 20 from cotton, never included any clusters. As indicated in Table 1, one group contained isolates from Hatay, Mersin, Kahramanmaras and Adana provinces and the second group contained isolates from Hatay and Adana provinces. Isolates from Hatay and Adana were placed in both groups.

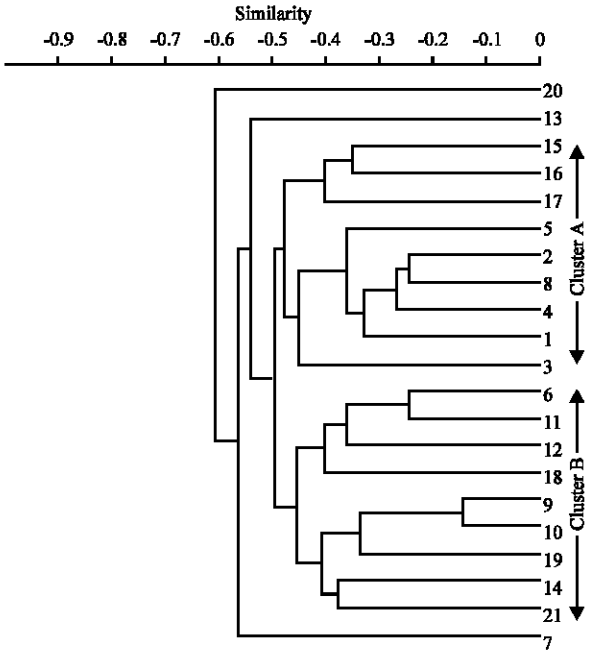

Fig. 1: Dendrogram derived from cluster analysis (UPGMA) of RAPD data obtained with five primers of 21 *V. dahliae* isolates, using software PAST 1.28. Top scale is percentage similarity based on the Jaccard similarity coefficient

Additionally, one isolate from Hatay, one from Adana and one from Mersin were clustered separately from the two major groups. Evidently, no relation was found between geographic distribution and the grouping of the isolates based on RAPD-PCR. However, a number of isolates must be tested to demonstrate the relationship between *V. dahliae* isolates both from different geographical origins and from different host. In the previous study[15], we had emphasized that the subgroup 2B of VCG2 was not strongly connected to subgroup 2A of the same VCG since local isolates of VCG2A were not even weakly compatible with the local isolates of VG2B. Thus, present results confirm the previous research reports. Because all VCG2B isolates except one were identified as members of cluster one and all VCG2A isolates belonged to the second cluster. Therefore, the results from RAPD analysis on 21 isolates of *V. dahliae* suggest that the clustering of isolates based on shared RAPD loci corresponds to vegetative compatibility. This is contradicting with previous reports[10,11,16] on cotton, reporting that RAPD patterns were not associated with VCGs or pathogenicity in *V. dahliae*. These results might be influenced by several factors including the source and procedure used for DNA isolation, the occurrence of

contaminants, etc. In other phytopathogenic fungi, RAPD analyses have proved useful for detecting genomic polymorphism directly related to host specialization[6,10,11,14,17].

Consequently, the results suggest that the genome analysis by RAPD technique seems to be a desirable molecular technique in phylogenetic studies of *V. dahliae* populations.

ACKNOWLEDGMENT

This research was supported by The Scientific and Technical Research Council of Turkey (TUBITAK).

REFERENCES

1. Kurt, S., S. Dervis and S. Sahinler, 2003. Sensitivity of *Verticillium dahliae* to prochloraz and prochloraz-manganese complex and control of Verticillium wilt of cotton in the field. Crop Protec., 23: 51-55.
2. Bell, A.A., 1992. Verticillium wilt. Cotton diseases (Ed.) R.J. Hillocks. CAB International, Oxon, UK, pp: 87-126.
3. Schnathorst, W.C. and D.E. Mathre, 1966. Cross-protection in cotton with strains of *Verticillium albo-atrum*. Phytopathology, 56: 1204-1209.
4. Daayf, F., M. Nicole and J.P. Geiger, 1995. Differentiation of *Verticillium dahliae* populations on the basis of vegetative compatibility and pathogenicity on cotton. Eur. J. Plant Pathol., 101: 69-79.
5. Pérez-Artés, E., M.D. García-Pedrajas, J. Bejerano-Alcázar and R.M. Jiménez-Díaz, 2000. Differentiation of cotton-defoliation and nondefoliation pathotypes of *Verticillium dahliae* by RAPD and specific PCR analyses. Eur. J. Plant Pathol., 106: 507-517.
6. Assigbetse, K.B., D. Fernandez, M.P. Dubois and J.P. Geiger, 1994. Differentiation of *Fusarium oxysporum* f. sp. *vasinfectum* races on cotton by Random Amplified Polymorphic DNA (RAPD) analysis. Phytopathology, 84: 622-626.
7. Carder, J.H. and D.J. Barbara, 1991. Molecular variation and Restriction Fragment Length Polymorphisms (RFLPs) within and between six species of *Verticillium*. Mycol. Res., 95: 935-942.
8. Dobinson, K.F., M.A. Harrington, M. Omer and R.C. Rowe, 2000. Molecular characterization of vegetative compatibility group 4A and 4B isolates of *Verticillium dahliae* associated with potato early dying. Plant Dis., 84: 1241-1245.
9. Henson, J.M. and R. French, 1993. The polymerase chain reaction and plant disease diagnosis. Ann. Rev. Phytopathol., 31: 81-109.
10. Koike, M., M. Fujita, H. Nagao and S. Ohshima, 1996. Random amplified polymorphic DNA analysis of Japanese isolates of *Verticillium dahliae* and *V. albo-atrum*. Plant Dis., 80: 1224-1227.
11. Messner, R., W. Ibl, M. Schweigkofler, G. Berg and H. Prillinger, 1996. Molecular characterization of the plant pathogen *Verticillium dahliae* Kleb. Using RAPD-PCR and sequencing of the 18s rRNA gene. J. Phytopathol., 14: 347-354.
12. Ramsay, J.R., D.S. Multani and B.R. Lyon, 1996. RAPD-PCR identification of *Verticillium dahliae* isolates with differential pathogenicity to cotton. Aust. J. Agric. Res., 47: 681-693.
13. Korolev, N., J. Katan and T. Katan, 2000. Vegetative compatibility groups of *Verticillium dahliae* in Israel: Their distribution and association with pathogenicity. Phytopathology, 90: 529-36.
14. Peever, T.L., Y. Canihos, L. Olsen, A. Ibanez, Y.C. Liu and L.W. Timmer, 1999. Population genetic structure and host specificity of *Alternaria* sp. causing brown spot of *Minneola tangelo* and rough lemon in Florida. Phytopathology, 89: 851-860.
15. Dervis, S. and M. Bicici, 2005. Vegetative compatibility groups in *Verticillium dahliae* isolates from cotton in Turkey. Phytoparasitica, 33: 157-168.
16. Bhat, R.G. and K.V. Subbarao, 1999. Host range specifity in *Verticillium dahliae*. Phytopathology, 89: 1218-1225.
17. Zeise, K. and A.V. Tiedemann, 2002. Application of RAPD-PCR for virulence type analysis within *Verticillium dahliae* and *V. longisporum*. J. Phytopathol., 150: 557-563.