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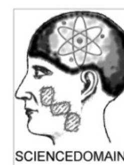

Molecular Epidemiology of Verticillium Wilt of Olive in Southern and Central Tunisia: Evidence of Host Adaptation Hypothesis

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Authors' contributions

This work was carried out in collaboration between all authors. Author YG designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors EB and MAT managed the analyses of the study. Authors MAT and RG managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: During the last two decades, verticillium wilt of olive has spread to young olive orchards where highly susceptible crops such as potato, watermelon and tomato are cultivated near to olive orchards.

It was therefore hypothesized that there is an adaptation phenomenon that drives the pathogenicity of *V. dahliae* in order to infect a broad range of hosts. Therefore, it will be useful to identify the factors that increase the severity of the pathogen which helps growers to implement the appropriate crop rotation program.

Place and Duration of Study: This work was performed in the Laboratory of Phytopathology at the

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Olive Tree Institute (Tunisia) between December 2013 and March 2015.
Methodology: This study was conducted using 62 isolates of *V. dahliae* recovered from potato, sunflower and olive. Primarily, a cross pathogenicity tests were performed with the three plant hosts using an artificial infection bioassay. Subsequently, all the isolates were compared at the molecular level using twelve SSR markers and the number of genotypes in each group of isolate was determined by assigning a specific SSR haplotype. The relationship between the isolates groups was defined by comparison of SSR genotypes and pathogenicity data obtained for each isolate.
Results: Olive and potato isolates were pathogenic to both crops with similar disease severity. By contrast, only sunflower isolates were unable to infect sunflower plants. SSR analysis revealed that olive group is the most diversified one with 12 different genotypes followed by the sunflower one. When isolates groups were compared, we concluded that potato and olive groups are highly similar at pathogenic and molecular levels whereas sunflower group was genetically different.
Conclusion: Potato and olive isolates were able to infect both crops with similar pathogenicity level which suggest that these isolates have a well-adapted virulence factors to successfully invade their hosts. By contrast, sunflower isolates were unable to induce wilting on olive plants which could be explained by the fact that sunflower is unusually cultivated near to olive orchards and therefore isolates from this host are not adapted to infect the olive.

Keywords: Host adaptation; pathogenic variation; gene diversity; infection bioassay; SSR; verticillium wilt of olive.

1. INTRODUCTION

Verticillium dahliae is a ubiquitous plant-pathogenic fungus that causes severe yield losses on many economically important crops, including vegetables, ornamentals, fruits and particularly olive. Like in many Mediterranean countries, *V. dahliae* causes olive wilt in Tunisia [1-3]. This fungus was first reported in Sfax region (southern Tunisia) and has recently spread to other regions recently described as free from verticillium wilt of olive (VWO), where it causes severe devastation on young olive orchards [4]. An effective management strategy for disease control involved among other factors the use of resistant cultivars and breeding developed hybrids, which in turn requires knowledge about the genetic diversity within natural populations of the pathogen and about the pathogenicity level of its isolates. The severity of VWO attacks depends on the virulence of the isolate that infect the tree. In fact, *V. dahliae* isolates infecting olive can be classified as defoliating (D) and non-defoliating (ND) pathotypes according to their ability to completely defoliate the plant or to only cause wilt and partial or no defoliation [5,6]. The D pathotype is highly virulent and causes severe symptoms as wilt, chlorosis, weight and height reduction. the ND pathotype causes the same symptoms but at moderate levels [7,8]. In addition, pathogenicity of *V. dahliae* isolates and the host of origin were correlated with vegetative compatibility groups (VCGs) [9]. It is considered to be a source of genetic diversity in *V. dahliae*. In fact, some studies support the correlation between VCG assignments of isolates and their differential virulence in a given host and location [10]. In addition, an association between VCG groups and host specificity was reported in many studies. For example, VCG2A and 2B isolates recovered from artichoke growing regions showed to be the most virulent towards this host [11]. However, VCG groups could be insufficient to correlate the genetic diversity with traits such as level of virulence, geographical distribution and host specificity. Therefore, molecular tools are necessary to explain the complex structure of *V. dahliae* populations. Molecular studies of genetic diversity in *V. dahliae* populations were conducted using different molecular markers. In fact, genetic differentiation of *V. dahliae* isolates has been carried out by restriction fragment length polymorphism (RFLP) [12]. Phylogenetic analysis of DNA sequences such as the intergenic spacer (IGS), the internal transcribed spacer (ITS) and some conserved genes have been also used to study the genetic variation in *V. dahliae* [13,14]. Random amplification of polymorphic DNA (RAPD) has been widely used. However, this technique yielded contradictory results [15,16]. The amplified fragment length polymorphism (AFLP) is an alternative method to assess the genetic variation in pathogen populations, which provides a higher resolution than RAPD. This technique was successfully employed in Spain to investigate the genetic variation among isolates from olive, cotton and

artichoke and its association to other phenotypic traits such as virulence, VCG and geographic origin. Microsatellites markers, especially Simple Sequence Repeat (SSR) were recently used to study the genetic diversity in *V. dahliae* populations from different hosts. In this context, several polymorphic SSR markers have been previously developed. These markers have been successfully correlated to the host of origin and VCG groups of isolates [17].

Despite the extent and importance of olive production in Tunisia and the threat caused by verticillium wilt, local populations of *V. dahliae* have not been studied from a molecular point of view. Determining the status of such diversity is essential to select suitable resistant olive cultivars for specific growing regions, and to monitor changes in the structure of the pathogen populations that represent a threat to olive cropping in Tunisia and other Mediterranean countries. In the present study, we used SSR markers to gain insight into the pathogenic and genetic structure of the Tunisian *V. dahliae* population. In a survey conducted during the period of 2011-2013 we sampled 120 olive orchards in the main olive-growing regions in Tunisia and isolated a total of 42 *V. dahliae* isolates from infected olive trees. To compare these isolates with other ones from other countries, we included 10 isolates from Canada, which were collected from potato and sunflower.

2. MATERIALS AND METHODS

2.1 Isolates Origin

We tested sixty two isolates of *V. dahliae*, including ten from sunflower, and ten from potato. These isolates were previously recovered from samples of potato and sunflower collected in Manitoba fields. The forty two remaining isolates were recovered from diseased olive trees in various olive growing regions of Tunisia (Fig. 1). Prior to the experiments, isolates were identified based on colony morphology and microscopic characteristics of conidia and microsclerotia production [3]. Subsequently, single spore cultures isolates were prepared and retained for subsequent analysis.

2.2 Pathogenicity Tests

Pathogenicity of the isolates was tested on two-year-old olive trees cv. Chemlali, the most known cultivar in Tunisia. The experiment was conducted in a green-house under controlled conditions (25℃±2℃; 16/8 h of light/dark period. The conidial suspension of each isolate was prepared from 10 days old cultures on PDA and adjusted to 10^6 conidia/mL. Plant roots were dipped for 1h in the conidial suspension and then transplanted into new polyethylene pots containing a sterile substrate (peat: sand, 1:1 v/v). The experiment was arranged in a completely randomized block with three replicates and three control plants dipped in sterile distilled water (SDW). Disease severity was assessed weekly, starting 15 days after inoculation. A scale from zero to four was used according to the percentage of affected plant tissue, in which, zero = healthy plant; one = ≤33% affected tissue; two = 34-66% affected tissue; three = 67-99%; four = dead plant). Estimation of the area under disease progress curve (AUDPC) was calculated as described previously by Gharbi et al. [4]. Statistical analysis of variance was performed using SPSS software to determine the variability among the isolates.

Fig. 1. Map showing the eight olive producing locations in Tunisia which 42 strains of *Verticillium dahliae* were collected

2.3 DNA Extraction

V. dahliae isolates were grown on PDA plates at 25℃ for one week. After incubation, mycelia

were scraped off the surface of colonies and ground in liquid nitrogen with mortar and pestle. Then, 100 mg of mycelium were resuspended in 200 µL sterile water. The total volume was extracted by ZR Fungal/Bacterial DNA mini prep D6005 Kit (Zymo Research, Irvine, CA, USA) as recommended by the manufacturer. Extracted DNA was re-suspended in 50 µl of elution buffer and stored at -20℃ until subsequent analysis.

2.4 SSR Analysis

Twelve microsatellites markers previously described by Almany et al. [18]. All SSR sequences and the primers used for amplification were listed in Table 2. Polymerase chain reaction (PCR) assays were conducted to amplify individual loci. Each reaction mixture (25 µL) contained 1 X standard buffer, 2.5 mM of $MgCl_2$, primers at 0.25 µM each, (Table 1) dNTPs at 250 µM each, 1.25 U Taq DNA polymerase (Fermentas) and 10–50 ng template DNA. Reactions were performed in a thermocycler (Biorad), with an initial denaturation step at 94℃ for 4 min, followed by 40 cycles of denaturation at 95℃ for 1 min, annealing at 59℃ for 1 min and elongation at 72℃ for 1 min, plus a final elongation step of 72℃ for 6 min. A common annealing temperature (59℃) was selected for all primer pairs. For polyacrylamide gel electrophoresis (PAGE) allele identification, microsatellite alleles were separated on 6% polyacrylamide gels in 0.5 X Tris-Acetate-EDTA (TAE) buffer. PCR products were stained with ethidium bromide and visualized under UV light. Molecular weights were estimated using a 10-bp ladder (Invitrogen). Polymorphic markers were noted for presence (1) or absence (0) based on the AFLP pattern obtained for each isolate. The obtained binary matrix was used to generate a dendrogram by unweighted pair-group mean arithmetic method analysis (UPGMA) clustering implemented in the NTsys software (Rohlf, 2000).

2.5 Marker Data Analysis

In order to establish the potential of the new microsatellite markers to resolve genetic diversity in *V. dahliae*, genetic diversity parameters were calculated for three collections of *V. dahliae* isolates obtained from olive, potato and sunflower. In addition, the 62 isolates of *V. dahliae* were obtained from different locations.

The degree of polymorphism estimated by gene diversity [19] was calculated for all 62 isolates of *V. dahliae* using POPGEN version 1.32. Subsequently, gene diversity in each of the three

Table 1. Repeat motifs, primer sequences and accession numbers of SSR loci used in this study

Locus	Repeat motif	Primer sequences	Allele size (bp)	Accession number
VD1	$(CCTG)_{29}$	F:CTGTTTCTGCCTTCCCATGT R:AGTACCAGTGAGCGCGATTT	301-337	FJ851470
VD2	$(TCTGGC)_{12}$	F: CACCCTTGACCTCACCTCAT R: GTGGAGAGGCCTTCCTTTCT	333-423	FJ851474
VD3	$(CACGCCCT)_8$	F: GGTTCATGGTGTCCTCGAAT R:AGCAAGGTGAGCTTTGGAGA	277-333	FJ851480
VD4	$(CTG)_{18}$	F:GCCTGGTAGCGGTAGAGAGC R: TCCTCGAAAAGGGTGTCATC	322-379	FJ851483
VD7	$(AG)_{44}$	F:GGTGAGGCCAGACAGAAGAG R: CGCGCATGGAGTCATACATA	312-340	GQ160902
VD8	$(GAT)_{31}$	F: AAGCCCATCGGATGATATTG R: GATCTTGCGACGTGTTCTGA	332-401	FJ851489
VD9	$(GATGCGT)_{10}$	F: TGCTAGGTGCTGTCTCATGC R: TTCGCTCCAGAATCCCATAC	323-393	FJ851494
VD10	$(TTGC)_{28}$	F: GGCGAGACAATCGTCATTCT R:GCGCGAGATGAAGAACAACT	313-369	FJ851499
VD11	$(GTCTGCCT)_8$	F: TAGGTACCGAAGTGGGTTGC R: CCAGGCGTCTTTCTTGTTTC	278-358	FJ851504
VD12	$(TTTC)_{17}$	F: TAGAATTTTCGGGACGCTGT R: AGCTGCATCGTTTTCTGACC	305-361	FJ851508
VD26	$(GCAGAGAG)_8$	F: CTCTCGCACAGCAAGATTGA R: CGAGGCTTTGAGCTCTGACT	293-317	FJ851511
VD27	$(CAATGCCTCG)_7$	F:TGTCTACATGATCGCCGTGT R:GCACCTGTATGACGACAGGA	341-401	FJ851514

isolate collections (Olive, potato and sunflower) was calculated using the same software. Analysis of the molecular variance (AMOVA) within and among *V. dahliae* populations according to their host of origin was performed using ARLEQUIN v. 3.1 [20]. Therefore, a genetic differentiation coefficient (GST) for the isolate collections from olive, sunflower and potato was calculated as: GST = (HT - HS) ⁄ HT, where HS = the average of the estimated gene diversities of each of the three populations, and HT = the estimated gene diversity of the total population. The total number of unique genotypes and the frequency of the most common genotype in each isolate collection were calculated using MULTILOCUS v 1.3. Multilocus genotypes were assigned to each isolate in the three *V. dahliae* isolate collections described above (olive, potato and sunflower), by combining alleles sizes across the twelve polymorphic SSR markers.

3. RESULTS

3.1 Pathogenicity Tests

The pathogenicity of 62 *V. dahliae* isolates on the cultivar Chemlali was assessed (Table 2). For any given isolate, disease scores were not significantly different between replicate inoculated plants ($P > 0.05$). Disease scores were used to rank isolates as highly, moderately or weakly aggressive towards olive. In fact, some of the olive isolates such as VDO1 from the region of Sousse, VDO10 from the region of Monastir, VDO14 from the region of Zaghouane, induced 65 to 100% of disease (including death) in the bioassay conducted at 25℃, whereas VDO08 from the region of Sfax and VDO7 from the region of Kairouan, were less aggressives. No symptoms of wilting were observed when isolates from sunflower were inoculated to olive plants. In contrast, isolates from potato were able to induce wilt on olive plants. Nevertheless, they are significantly less pathogenic than isolates from olive ($P < 0.05$). By contrast, when sunflower isolates were inoculated to olive plants, no wilt symptoms have been observed over the time of experiment, which indicates that sunflower isolates were nonpathogenic on olive. Sunflower isolates were low pathogenic towards potato and AUDPC values recorded in this crop ranged between 8 and 17.33%.

3.2 SSR Analysis

Analysis of the 12 microsatellite markers sequences showed a total of 37 alleles for the 62 *V. dahliae* isolates studied (Table 3). The loci displayed from two to seven alleles, and more than two alleles were identified for the 12 loci. The size of alleles based on capillary electrophoresis concurred with that estimated from PAGE, and differences of two or three nucleotides between alleles were resolved clearly (Fig. 2). Gene diversity (H) ranged from 0.09 to 0.66 among loci (Table 3). The highest gene diversity was displayed by loci VD1, VD2 and VD27 (Table 3).

The number of alleles observed per locus for the *V. dahliae* isolates from olive, sunflower and potato ranged from two to seven. Gene diversity in the olive isolate group was greater than that from potato and sunflower (Table 3). The AMOVA analysis showed that variability between collections from olive and potato was 30% of the genetic variation among the isolates ($P < 0.0001$) was attributable to variation between the two groups and 70% was within the two host groups. Differentiation among the sunflower and potato isolates (GST=0.63 at $P<0.001$) was significant as indicated by the randomization test, the genetic differentiation between sunflower and olive isolates was very high (GST = 0.72 at $P < 0.001$). Comparison of olive isolates with those infecting potato demonstrated that the isolates of two groups were genetically similar with a genetic differentiation coefficient of GST= 0.23 at $P < 0.001$.

The number of multilocus genotypes in the three studied *V. dahliae* collections ranged from five to 12 (Table 4). In the olive, sunflower and potato collections, a total of 18, 9 and 8 multilocus genotypes, respectively. The most common genotype of *V. dahliae* was detected 12 times within the olive isolates (Table 4). Genotypic diversity in the olive and sunflower groups of *V. dahliae* was greater than that observed in the potato group (Table 4). Genotypic diversities were significantly greater ($P<0.001$) than 0 in all three isolate collections studied. The dendrogram produced from SSR data by UPGMA analysis indicated the presence of three major clusters. SSR I contained most of the isolates infecting olive and five potato isolates, SSR II consisted of the remaining olive and potato isolates, whereas SSR III was consisted of those infecting sunflower and only two isolates from olive. The three groups showed 47% similarity (Fig. 3), while isolates within a particular group were 74 to 100% similar.

Table 2. Total AUDPC calculated based on disease severity on potato, sunflower and olive plants inoculated with *V. dahliae* isolates recovered from olive, sunflower and potato

Host	Isolates														
	VDO1	VDO10	VDO14	VDO7	VDO8	VDP1	VDP3	VDP4	VDP6	VDP9	VDS2	VDS3	VDS5	VDS7	VDS10
Olive	87.15±2.25	91.50±1.80	65.50±0.86	60.10±1.26	52.83±2.02	84.35±3.45	86.45±4.26	73.35±3.55	56.66± 2.88	62.15±1.87	00.00	00.00	00.00	00.00	00.00
Potato	225.5±7.75	245.35±9.60	143.15±7.45	135.45±6.90	92.70±9.10	212.45±8.33	239.15±6.66	146.35±8.25	145.33±7.66	95.15±8.23	17.35±0.45	13.33± 0.35	18.25±0.38	11.15±0.66	08.33±0.36
Sun-flower	64.75±2.25	66.33±1.66	52.47±7.75	52.55±1.65	57.25±2.15	66.75±3.23	63.15±2.23	58.35±2.66	60.33±8.23	54.66±3.39	178.66±4.56	155.39±5.66	152.47±5.75	158.65±6.66	144.25±2.15

Table 3. Allele number, Gene diversity and Polymorphism Information content calculated for each SSR locus in different plant hosts

SSR	Allele number			Gene diversity			Polymorphism information content (PIC)		
	Sunflower	Potato	Olive	Sunflower	Potato	Olive	Sunflower	Potato	Olive
VD1	2	2	7	0.23	0.34	0.66	0.19	0.29	0.61
VD2	2	2	3	0.22	0.19	0.43	0.18	0.14	0.39
VD3	2	2	3	0.11	0.17	0.32	0.08	0.13	0.28
VD4	3	2	2	0.34	0.13	0.15	0.29	0.09	0.11
VD7	5	2	3	0.62	0.11	0.32	0.58	0.08	0.28
VD8	2	4	2	0.12	0.42	0.09	0.08	0.38	0.06
VD9	2	2	3	0.08	0.17	0.34	0.04	0.13	0.30
VD10	2	3	3	0.11	0.14	0.29	0.07	0.10	0.25
VD11	3	3	1	0.29	0.31	0.04	0.26	0.28	0.03
VD12	3	4	3	0.24	0.39	0.27	0.20	0.35	0.23
VD26	2	2	3	0.14	0.16	0.26	0.11	0.12	0.22
VD27	3	3	4	0.26	0.23	0.41	0.21	0.19	0.37

Table 4. Genotype of *V. dahliae* isolates obtained by amplification of twelve polymorphic SSR markers

Isolates	*V. dahliae* genotypes											
	VD1	**VD2**	**VD3**	**VD4**	**VD7**	**VD8**	**VD9**	**VD10**	**VD11**	**VD12**	**VD26**	**VD27**
	Olive isolates											
VDO1	29	10	6	18	38	29	6	25	6	15	8	33
VDO2	29	10	6	18	38	29	6	24	6	15	8	33
VDO3	27	08	6	15	36	27	6	25	6	14	6	29
VDO4	27	08	6	15	36	27	6	22	6	14	6	29
VDO5	28	07	5	15	33	27	5	22	6	14	5	28
VDO6	24	08	5	15	31	27	5	24	6	14	5	27
VDO7	24	08	5	15	31	27	5	22	6	14	5	27
VDO8	29	10	6	18	38	29	6	22	6	15	8	33
VDO9	27	08	6	15	36	27	6	22	6	15	8	27
VDO10	29	10	6	18	38	29	6	22	6	17	8	33
VDO11	29	10	6	18	38	29	6	24	6	17	8	33
VDO12	29	10	6	18	38	29	6	24	6	15	8	27
VDO13	24	08	5	15	31	27	5	24	6	15	6	27
VDO14	24	08	5	15	31	27	5	24	6	15	5	27
VDO15	24	08	5	15	31	27	5	24	6	15	5	27
VDO16	24	08	5	15	31	27	5	24	6	15	5	27
VDO17	24	08	5	15	31	27	5	24	6	15	5	27
VDO18	24	08	5	15	31	27	5	24	6	15	5	27
VDO19	27	08	7	15	36	29	7	25	6	15	6	29
VDO20	27	08	7	15	36	29	7	25	6	15	6	29
VDO21	29	10	6	18	38	29	6	25	6	15	6	33
VDO22	29	10	6	18	38	29	6	25	6	15	6	33
VDO23	28	07	5	15	33	29	5	25	6	15	5	28
VDO24	24	08	5	15	31	27	5	25	6	15	5	27
VDO25	24	08	5	15	31	27	5	25	6	15	5	27
VDO26	27	08	7	15	36	27	7	25	6	15	8	27
VDO27	27	08	7	15	36	27	7	25	6	15	8	27
VDO28	27	08	7	15	36	27	7	25	6	15	8	27
VDO29	29	10	6	18	38	29	6	25	6	14	6	33
VDO30	29	10	6	18	38	29	6	25	6	14	6	33
VDO31	25	08	6	15	31	27	6	24	6	15	6	27
VDO32	25	08	6	15	31	27	6	24	6	15	6	27
VDO33	27	08	6	15	36	27	6	24	6	15	6	27
VDO34	29	10	6	18	38	29	6	24	6	17	6	33
VDO35	27	08	7	15	36	27	7	24	6	15	5	27
VDO36	27	08	7	15	36	27	7	25	6	15	5	27
VDO37	24	08	5	15	31	27	5	24	6	15	5	27
VDO38	23	08	5	15	31	27	5	24	6	15	5	27
VDO39	23	08	5	15	31	27	5	24	6	15	5	27
VDO40	23	08	5	15	31	27	5	24	6	15	5	27
VDO41	25	08	6	15	31	27	6	24	6	15	6	27
VDO42	29	10	6	18	38	29	6	25	6	17	6	33
	Potato isolates											
VDP1	27	08	7	15	36	31	7	23	8	15	7	28
VDP2	27	08	7	15	36	28	7	21	7	15	7	30
VDP3	27	08	7	15	36	28	7	21	8	15	7	30
VDP4	27	08	7	15	36	30	7	23	7	16	7	28
VDP5	29	10	6	18	38	29	6	22	6	18	6	31
VDP6	29	10	6	18	38	30	6	22	6	18	6	31
VDP7	29	10	6	18	38	30	6	22	6	18	6	28
VDP8	27	08	7	15	36	31	7	22	7	15	7	30
VDP9	27	08	7	15	36	31	7	21	7	14	7	30
VDP10	29	10	6	18	38	29	6	22	6	14	6	31

Isolates	*V. dahliae* genotypes											
	VD1	**VD2**	**VD3**	**VD4**	**VD7**	**VD8**	**VD9**	**VD10**	**VD11**	**VD12**	**VD26**	**VD27**
Sunflower isolates												
VDT1	24	08	05	13	31	28	05	24	08	13	05	30
VDT2	24	08	05	16	31	28	05	24	08	16	05	30
VDT3	23	07	07	15	32	25	07	23	07	15	07	28
VDT4	23	07	07	15	32	25	07	23	07	15	07	28
VDT5	23	07	07	15	32	25	07	23	07	15	07	28
VDT6	24	08	05	13	29	28	05	24	05	13	05	31
VDT7	24	08	05	13	27	28	05	24	05	13	05	31
VDT8	24	08	05	16	27	28	05	24	05	16	05	31
VDT9	23	07	07	15	30	27	07	23	07	15	07	28
VDT10	23	07	07	15	31	27	07	23	07	15	07	28

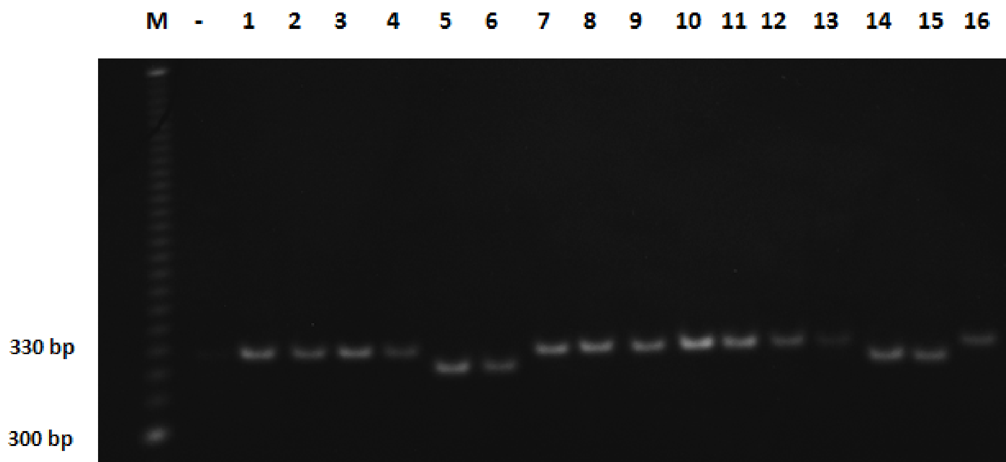

Fig. 2. Amplification products of the VD4 SSR marker resolved in 6% polyacrylamide gel (1-16: *V. dahliae* isolates infecting olive; M: 10 bp DNA ladder, - : negative control)

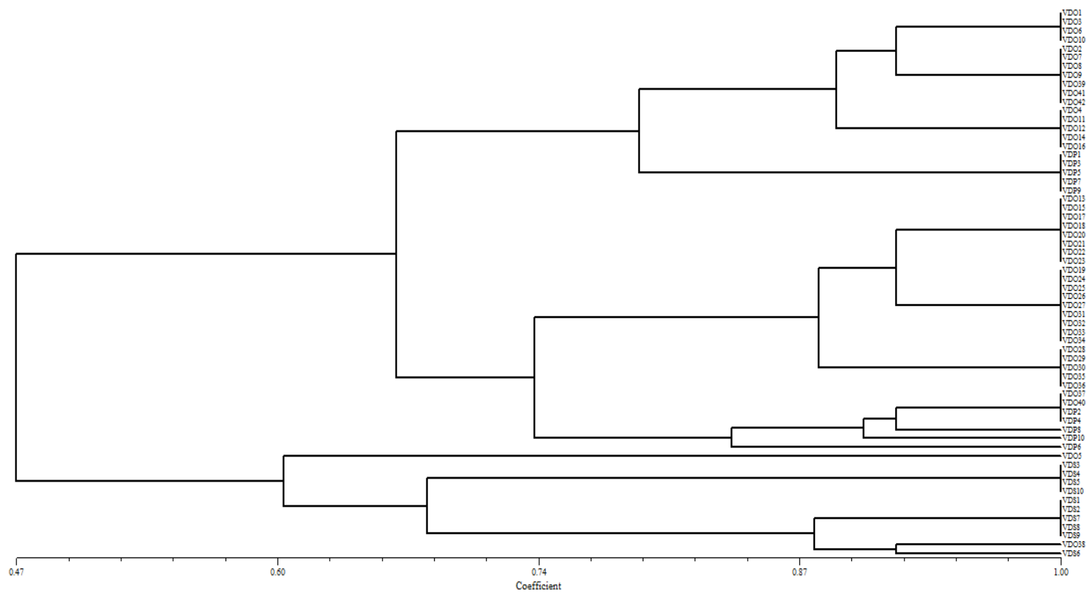

Fig. 3. Dendrogram of 42 *V. dahliae* isolates from olive in Tunisia. Unweighted pair-group method with arithmetic mean (UPGMA) cluster analysis was based on Nei's unbiased genetic distances

4. DISCUSSION

The main goal of this study was to test the suitability of new molecular markers for analysis of genetic diversity in populations of *V. dahliae*. Twelve polymorphic SSR markers were identified to be suitable for genotyping in *V. dahliae*. These markers and which showed high levels of polymorphism among 62 *V. dahliae* isolates included in this study, as revealed by the high gene diversity index. This study also indicated the usefulness of these markers for differentiating three groups of the pathogen infecting different host plants, and collected from orchards located in regions where olive is largely cultivated in presence of susceptible solanaceous crops.

The hypothesis of genetic difference and its relation with the host of origin was confirmed in this study by the occurrence of host specific multilocus genotypes to each of the three *V. dahliae* populations (sunflower, potato and olive). In addition, the relation between the genetic traits and specialization of *V. dahliae* isolates to a well-defined host crop was further supported by the results of cross-pathogenicity tests. In fact, isolates infecting sunflower and olive, which have different multilocus genotypes, were unable to induce wilt symptoms on both hosts [4]. By contrast, isolates from potato which share the same genotype with olive isolates were able to produce wilting symptoms on olive plants. This result could be probably incited by the expansion of olive orchards to occupy lands previously cropped with high susceptible crops, especially those belong to the solanaceous order such as potato and tomato, which are susceptibile to the same VCG groups [21]. By contrast, sunflower is not a usual plant in areas were olive is extensively cultivated which explain the lack of pathogenicity of olive isolates when inoculated to sunflower plants. Nevertheless, results of cross pathogenicity revealed that in some cases and when *V. dahliae* isolates sharing the same multilocus genotypes, they could have differential pathogenicity levels on different crops. This finding is supported by a recent comparative genomic study, involving 11 genome sequences of *V. dahliae* strains, recovered from different hosts and geographic origins. In that study, it was demonstrated that *V. dahliae* modulate its pathogenicity mechanisms through the establishment of highly dynamic lineage-specific (LS) genomic regions which lead to genetic variation and causes differential virulence [22]. In fact, under natural conditions, the significant implication of well-defined *V. dahliae* genotypes in the infection of specific host plants has been widely described in previous studies. For instance, *V. dahliae* assigned to the vegetative compatibility group VCG4A is usually associated with potato crops, while other VCGs are not well reported in this crop though they are present in the fields [23]. In fact, although the genotype VCG4A has demonstrated its ability to infect other crops by infection bioassays [23,24], isolates of this group are rarely encountered with crops other than potato [23]. By contrast, the group VCG2A is known by its ability to infect several hosts such as potato and olive. In this context, it was demonstrated that *V. dahliae* isolates infecting potato and olive presumably evolved from a common clonal lineage native to the studied area or introduced by plantation of infecting plant material. Indeed, during field prospections, it was noted that the majority of infected orchards are largely used for potato production, which suggest that *V. dahliae* may have adapted to the new introduced host (olive), but with no extensive genetic variation in the two *V. dahliae* populations [4].

Similar results were reported in previous studies aiming to define the genetic structure of *V. dahliae* populations infecting different plant hosts and collected from different geographic locations. For instance, study a relatively high degree of genetic diversity was revealed between *V. dahliae* isolates infecting artichoke, cotton and olive hosts [25]. Similar result was reported in Turkey where a relatively high level of genetic variation was observed among the isolates recovered from cotton plants in a small geographical area [26]. The genetic variation in *V. dahliae* collection infecting olive revealed that genetic distance ranged between 18% and 40%, although they are isolated from distant olive growing regions [27].

5. CONCLUSION

Overall, correlation of molecular traits, pathogenicity and host of origin revealed a significant relation between the genotype and host adaptation of isolates. This finding is in line with other genetic studies, which demonstrate that isolates recovered the same host are usually genetically similar to one another than isolates recovered from different host plants. This knowledge will foster our efforts in the development of a reliable disease management measures, and may lay the ground for the

implementation of genetic improvement programs aiming to develop tolerant cultivars to be cropped in infected regions. Thus, The screening of olive cultivars for their susceptibility to *V. dahliae* should be conducted using different genotypes that would reflect the genetic diversity reported in this study. Furthermore, the current research would be extended to include more isolates from other olive growing regions, which will provide valuable information for disease management in Tunisia. Given that strength of cultivars tolerance is largely linked to the evolutionary potential of the pathogen. Therefore, defining the factors that incite the evolutionary success of *V. dahliae* and its capacity of specialization is required to predict any resistance breakdown in the main olive cultivars.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Jiménez-Díaz RM, Cirulli M, Bubici G, Jiménez-Gasco MM, Antoniou PP, et al. Verticillium wilt, a major threat to olive production: Current status and future prospects for its management. Plant Dis. 2012;96:304-329.
2. Gharbi Y, Triki MA, Jolodara A, Trabelsi R, Gdoura R, Daayf F. Genetic diversity of *Verticillium dahliae* from olive trees in Tunisia based on RAMS and IGS-RFLP analyses. Can J Plant Pathol. 2014; 36:491–500.
3. Triki MA, Hassaïri A, Mahjoub M. Premières observations de *Verticillium dahliae* sur olivier en Tunisie. EPPO Bull. 2006;36:69–71.
4. Gharbi Y, Triki MA, Trabelsi R, Fendri I, Daayf F, Gdoura R. Genetic structure of *Verticillium dahliae* isolates infecting olive tree in Tunisia using AFLP, Pathogenicity and PCR Markers. Plant Pathol. 2015;64: 871-879.
5. Rodriguez-Jurado D, Blanco-Lopez MA, Rapoport HF, Jimenez-Diaz RM. Present status of verticillium wilt of olive in Andalusia (southern of Spain). EPPO Bull. 1993;23:513-516.
6. Bejarano-Alcázar J, Blanco-López MA, Melero-Vara JM, Jiménez-Díaz RM. Etiology, importance and distribution of verticillium wilt of cotton in southern Spain. Plant Dis. 1996;80:1233–1238.
7. Birem F, Alcántara E, Blanco-López MA, López-Escudero FJ. Physiological differences expressed by susceptible and resistant olive cultivars inoculated with *Verticillium dahliae*. 10th International Verticillium Symposium, Book of Abstracts, Corfu Island, Hellas. 2009;71.
8. Martos-Moreno C, López-Escudero FJ, Blanco-López MA. Resistance of olive cultivars to the defoliating pathotype of *Verticillium dahliae*. HortScience. 2006; 41:1313–1316.
9. Daayf F, Nicole M, Geiger J. Differentiation of *Verticillium dahliae* populations on the basis of vegetative compatibility and pathogenicity on cotton. Eur J Plant Pathol. 1995;101:69–79.
10. Korolev N, Pérez-Artés E, Bejarano-Alcázar J, RodríguezJurado D, Katan J, Katan T, Jiménez-Díaz RM. Comparative study of genetic diversity and pathogenicity among populations of *Verticillium dahliae* from cotton in Spain and Israel. Eur J Plant Pathol. 2001;107:443–456.
11. Jiménez-Díaz RM, Mercado-Blanco J, Olivares-García C, Collado-Romero M, Bejarano-Alcázar J, RodríguezJurado D, Giménez-Jaime A, García Jiménez J, Armengol J. Genetic and virulence diversity in *Verticillium dahliae* populations infecting artichoke in eastern-central Spain. Phytopathol. 2006;96:288–298.
12. Dobinson KF, Harrington MA, Omer M, Rowe RC. Molecular characterization of vegetative compatibility group 4A and 4B isolates of *Verticillium dahliae* associated with potato early dying. Plant Dis. 2000;84:1241–1245.
13. Pramateftaki PV, Antoniou PP, Typas MA. The complete DNA sequence of the nuclear ribosomal RNA gene complex of *Verticillium dahliae*: Intraspecific heterogeneity within the intergenic spacer region. Fungal Genet Biol. 2000;29:19–27.
14. Dobinson KF, Patterson NA, White G, Grant S. DNA fingerprinting and vegetative compatibility analysis indicate multiple origins for *Verticillium dahliae* race 2 tomato isolates from Ontario, Canada. Mycol Res. 1998;102:1089–1095.
15. Bellahcene M, Assigbetsi k, Fortas Z, Geiger JP, Nicole M, Fernandez M. Genetic diversity of *Verticillium dahliae* isolates from olive trees in Algeria. Phytopathol Mediterr. 2005;74:266–274.
16. Cherrab M, Bennani A, Charest PM, Serrhini MN. Pathogenicity and vegetative

compatibility of *Verticillium dahliae* Kleb. isolates from Olive in Morocco. J Phytopathol. 2002;150:703–709.
17. Berbegal M, Garzón CD, Ortega A, Armengol J, Jiménez-Díaz RM, Jiménez-Gasco MM. Development and application of new molecular markers for analysis of genetic diversity in *Verticillium dahliae* populations. Plant Pathol. 2011; 60:866–877.
18. Almany GR, De Arruda MP, Arthofer W, Atallah ZK, Beissinger SR. Permanent genetic resources added to molecular ecology resources database 1 May 2009–31 July 2009. Mol. Ecol. Resour. 2009;9: 1460–1466.
19. Nei M. Analysis of gene diversity in subdivided populations. Proceedings of the National Academy of Sciences, USA70. 1973;3321–3.
20. Excoffier L, Laval G, Schneider S. Arlequin ver. 3.0: An integrated software package for population genetics data analysis. Evol Bioinform Online. 2005;1:47-50.
21. López-Escudero FJ, Roca JM, Mercado-Blanco J, Valverde Corredor A, Blanco-López MA. Effect of agronomical factors in the importance of verticillium wilt of olive in the Guadalquivir Valley in Andalucía (Southern Spain). 10th Int Verticillium Symposium, Book of Abstracts, Corfu Island, Hellas. 2009;96.
22. Jonge R, Bolton MD, Kombrink A, Vanden Berg GCM, Yateda KA, et al. Extensive chromosomal reshuffling drives evolution in asexual pathogen. Genome Res. 2013; 23:1271-1282.
23. Rowe RC, Powelson ML. Potato early dying: Management challenges in a changing production environment. Plant Dis. 2002;86:1184–1193.
24. Dung JKS, Schroeder BK, Johnson DA. Evaluation of verticillium wilt resistance in Mentha arvensis and M. Longifolia genotypes. Plant Dis. 2010;94:1255–60.
25. Collado-Romero M, Mercado-Blanco J, Olivares-GarciaC, Valverde-Corredor A, Jimènez-Diaz RM. Molecular variability within and among *Verticillium dahliae* vegetative compatibility groups determined by fluorescent amplified fragment length polymorphism and polymerase chain reaction markers. Phytopathol. 2006;96: 485–95.
26. Erdogan O, Nemli S, Oncu T, Tanyolac B. Genetic variation among pathotypes of *Verticillium dahliae* Kleb from cotton in western Turkey revealed by AFLP. Can J Plant Pathol. 2013;3:354-362.
27. Dervis S, Bicici M. Vegetative compatibility groups in *Verticillium dahliae* isolates from cotton in Turkey. Phytoparasitica. 2005;33:157-168.

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