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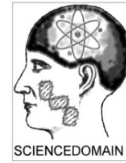

Phenotypic and Molecular Characterization of *Verticillium dahliae*, the Causal Agent of Verticillium Wilt of Olive in Tunisia

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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 were it causes severe yield losses and death of olive trees in southern and central regions of Tunisia. Therefore, identification of the causal agent as well as the study of its pathogenicity will be useful for design the appropriate management program.

Place and Duration of Study: This work was performed in the Laboratory of Phytopathology at the Olive Tree Institute (Sfax, Tunisia) between July 2014 and June 2015.

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Methodology: This study was conducted using phenotypic and molecular methods to identify the causal agent of the decline and death of olive trees. The pathogen was recovered from infested tissues using a potato dextrose agar medium. Identity of the isolates was confirmed by ITS-RFLP and sequencing. Pathogenicity of the isolates was evaluated by infection bioassay on young olive plants.

Results: All the fungal isolates were hyaline, flocculose and produced microsclerotia after 15 days of incubation at 25℃, which is in agreement with the identification key of *Verticillium* species. All the isolates were characterized by ITS-RFLP and sequencing of the internal transcribed spacer. All the isolates produced 470 bp using primers ITS1/ITS4. Digestion of the ITS product using *EcoRI* and *HaeIII* produced two fragment of 250 and 220 bp and three fragment of 300, 150, and 20 bp respectively. Pathogenicity was evaluated on two-year old olive plants using an artificial infection bioassay. After 15 days of inoculation, similar symptoms were produced as natural infection. Symptoms of wilt developed rapidly and caused the death of more than 50% of the inoculated plants.

Conclusion: *V. dahliae* is present in almost all growing olive regions of Tunisia. However, local *V. dahliae* populations in Tunisia is predominated by a highly pathogenic clone which is able to overcome the resistance of the main olive cultivar.

Keywords: Molecular identification; infection bioassay; ITS-RFLP; sequencing.

1. INTRODUCTION

Verticillium wilt of olive (VWO) caused by the soil-borne fungus *Verticillium dahliae* is one of the most diseases threatening olive orchards in the Mediterranean countries [1-4]. In Tunisia, this wilt was first reported in 2006 in south of the country and then has spread to other growing regions were it frequently causes decline and death of young olive plantations [5,6]. In Tunisia, where olive industry plays a central role in the national economy, Verticillium wilt is becoming an increasingly serious problem [7]. Unfortunately, once occurred, this wilt is difficult to manage and no efficient treatment was described until nowadays. Nevertheless, it has been reported that this wilt could be effectively controlled using an integrated management program, which involves many control measures such as, the use of tolerant cultivars, remediation of contaminated soils and the knowledge of genetic diversity of the pathogen. In fact, breeding programs aiming to develop performant cultivars usually requires a prior knowledge of the pathogenic and genetic structure of the pathogen. *V. dahliae* infecting olive trees were often characterized by morphological and pathogenicity assays. However, understanding the complex population structure of this fungus and its ability to infect different crops cannot be achieved using these techniques alone and usually require further molecular analysis [8]. Different molecular approaches have been employed to characterize *V. dahliae* isolates infecting several crops. For instance, genetic differentiation of *V. dahliae* isolates has been performed using several molecular techniques, including restriction fragment length polymorphism (RFLP) [9], phylogenetic analysis of rDNA sequences, including both the intergenic and the internal transcribed spacers and some conserved genes have been also used [10]. Random amplification polymorphism DNA (RAPD) has been widely used, but in general, it has yielded contradictory results [3]. Amplified fragment length polymorphism, (AFLP) which provide a higher resolution than RAPD is an alternative method to assess the genetic variation among *V. dahliae* isolates [11].

Analysis and comparison of DNA sequences such as the ITS region of rDNA have been used to differentiate, and in some cases, detect and quantify, *Verticillium* isolates infecting diverse host plants [12,13]. The ITS region of nuclear rDNA repeat units evolve faster and may vary both among and within species [14]. The rDNA genes have been employed to analyze major evolutionary events because it is highly conserved, whereas the rDNA internal transcribed spacer (ITS1 and ITS2) was more variable so that it has been used for the investigation of the species-level relationship [15]. Thus, the ITS region has been used in classifying many other fungi species because of its systematic and taxonomic usefulness [16].

Therefore, studying the genetic diversity of *V. dahliae* populations infecting olive in Tunisia, along with their pathogenicity, will contribute to design the appropriate management program for this disease. The present study aimed to

(i) collect *V. dahliae* isolates from different olive growing regions and determine their pathogenicity on young olive trees (ii) confirm the identity of the isolates by amplification of ITS, restriction digestion and sequencing.

2. MATERIALS AND METHODS

2.1 Pathogen Isolation and Identification

Stems and roots of olive plants with typical wilt symptoms were collected from different olive growing regions in Tunisia (Sfax, Sidi Bouzid, Kairouan, Mahdia, Sousse, Monastir, Kasserine and Zaghouan). The diseased tissue plants were immediately packed in polythene bags and transported to the laboratory for initial diagnosis. The samples were immediately stored at temperature ranging between 4 and 8℃ until subsequent analysis. The diseased plant's stem and root was removed from the polyethylene bags, rinsed twice with sterile distilled water to remove all soil particles. The surface of the tissue was then sterilized using 75% ethanol solution and air dried at room temperature. The diseased part of the stem or the root was then cut into small pieces (0.5 cm) by a sterile scalpel. The obtained tissues pieces were transferred into Potato Dextrose Medium (PDA) and incubated for 7 days at 25℃. Colonies rising from plant tissues were transferred into new PDA medium for purification and fungal isolates were subjected to morphological and molecular identification as described by Gharbi et al. [7].

2.2 Plant Material

Two-years-old olive rooted cutting belong to the cultivar Chemlali were used in this study. This cultivar was previously assessed for resistance to VWO under controlled conditions using an artificial infection bioassay and was classified as extremely susceptible to this wilt. All cuttings were obtained from genetically authenticated mother plants from the Olive tree Institute (OTI). All plants were potted in plastic bags containing (50% peat; 50% sand) and placed in a greenhouse and irrigated weekly until subsequent use. Pathogen inoculation was performed as previously described by Gharbi et al. [7]. The experiment was conducted in a greenhouse under controlled conditions (23℃±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 1 h 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). The 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 Rodriguez-Juardo et al. [17]. Statistical analysis of variance was performed using SPSS software to determine the variability among the isolates.

2.3 Molecular Identification of *V. dahliae* Isolates

2.3.1 Genomic DNA extraction

Fungal DNA was recovered from mycelium growing in PDA medium by scraping the surface of the colony using a sterile scalpel. Indeed, 100 mg of mycelium were thoroughly ground in liquid nitrogen and resuspended in 200 µl sterile distilled water. The total volume was then extracted by ZR Fungal/Bacterial DNA mini prep D6006 Kit (Zymo Research, Irvine, CA, USA) as recommended by the manufacturer. The extracted DNA was re-suspended in 50 µl of elution buffer. DNA elution was quantified using a Nanodrop, ND-1000 spectrophotometer (Thermo scientific, Quebec, Canada) and analyzed in a 0.8% agarose gel to check its quality. Finally, DNA samples were kept at -20℃ until subsequent use.

2.3.2 ITS amplification using primers ITS1/ITS4

The universal primers ITS1/ITS4 were used to amplify the ITS region from all the isolates. PCR reactions were carried out in 25 µl final volumes, containing 25 ng of fungal DNA, 2 mM $MgCl_2$, 200 µM of each dNTPs, 0.5 µM of each primer and 1 U of Taq DNA polymerase. Amplifications were carried out in a DNA Thermal Cycler (Biorad, Ontario, Canada) under the following conditions: initial denaturation cycle of 5 min at 95℃, followed by 34 cycles of denaturation at 95℃ for 1 min, annealing at 55℃ for 1 min and extension at 72℃ for 2 min, with a final extension step at 72℃ for 6 min. Tubes containing all reaction components except for

template DNA were included as controls. Amplification products were observed in ethidium bromide-stained 1.5% agarose gels, visualized under UV light, and photographed using a gel documentation system HP. The amplified fragments were purified and then subjected to cycle sequencing using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Lyon, France). The obtained sequences were compared with those available in GenBank by using the BLASTn (http://www.ncbi.nlm.nih.gov/BLAST).

2.3.3 Digestion of PCR products using HaeIII and EcoRI

PCR products were digested without prior purification using restriction endonucleases EcoRI and HaeIII following the protocol provided by the manufacturer (Invitrogen, Canada). Amplicons and their restriction fragments were visualized on 1.5% agarose gels containing 0.5 µg mL^{-1} of ethidium bromide at 60 volts for 45 min along a 1-kb DNA ladder (Invitrogen, Canada).

3. RESULTS

3.1 *V. dahliae* Isolation and Identification

Morphological and microscopic examination of the isolates revealed the presence of hyaline conidiophores with verticillate branches (Fig. 1A). All isolates produced conidia for which the size varies in an interval of 2.5–5 µm × 1.25–2.5 µm. Almost all isolates were able to produce microsclerotia after 15 days of incubation in the dark at 25℃. All isolates developed a milky white or white to grey mycelium after incubation for 7 days at 25℃. Based on the criteria described above, a total of 42 isolates were divided into two morphotypes: milky white and flocculose to slightly flocculose mycelium (36 isolates) (Fig. 1B) and greyish-white surface and slightly flocculose texture (six isolates) (Fig. 1C).

3.2 Pathogenicity Assay

The pathogenicity of 42 *V. dahliae* isolates on the cultivar Chemlali was assessed (Fig. 2). For any given isolate, disease scores were not significantly different between replicate inoculated seedlings ($P > 0.05$). Disease scores were used to rank isolates as highly, moderately or weakly aggressive towards olive. In fact, some of the olive isolates such as VD1 from Sousse, VD10 from Monastir, VD14 from Zaghouane, VD17 and VD22 from Sfax induced 90 to 100% of disease (including death) in the bioassay conducted at 25℃, whereas VD25, VD29 from Sfax and VD31 from Kairouan, were less aggressives. During the development of symptoms, vascular discoloration and root rot disease were evident in cross sections prepared from root and stems of diseased olive plants. The pathogen was recovered from nearly all the affected tissues, which fulfill the Koch's postulate.

3.3 ITS Amplification and Digestion

Amplification of the ITS region using the primers ITS1/ITS4 yielded a single PCR product of 470 bp from the 42 isolates without any amplification with the negative control (water, negative template control) (Fig. 3). In addition, digestion of ITS sequences with *EcoRI* and *HaeIII* generated 1–2 restriction fragments for each enzyme. The digestion profile obtained by *HaeIII* was common in almost all the isolates (300, 150, 20 bp) except for the isolate VD23, for which *HaeIII* had no restriction site (Fig. 4). Digestion of the ITS sequences with *EcoRI* produced similar patterns for the 42 isolates (Fig. 5). The representative 10 isolates are shown in Fig. 5. ITS sequences were analyzed using the BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were deposited in the GenBank database under the accession numbers (KM106203 to KM106212). The result of these alignments were in agreement with those of morphological and

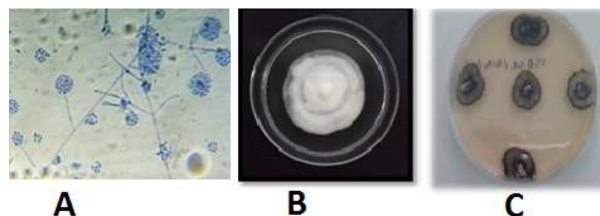

Fig. 1. Microscopic and morphological features of *V. dahliae* isolates recovered from diseased olive trees (A: hyaline conidiophores with verticillate branches; B: Milky white and flocculose morphotypes; C: grayish-white and slightly flocculose morphotypes)

species-specific PCR studies, with all, 42 sequences showing 100% homology to previously published sequences of the species *V. dahliae*.

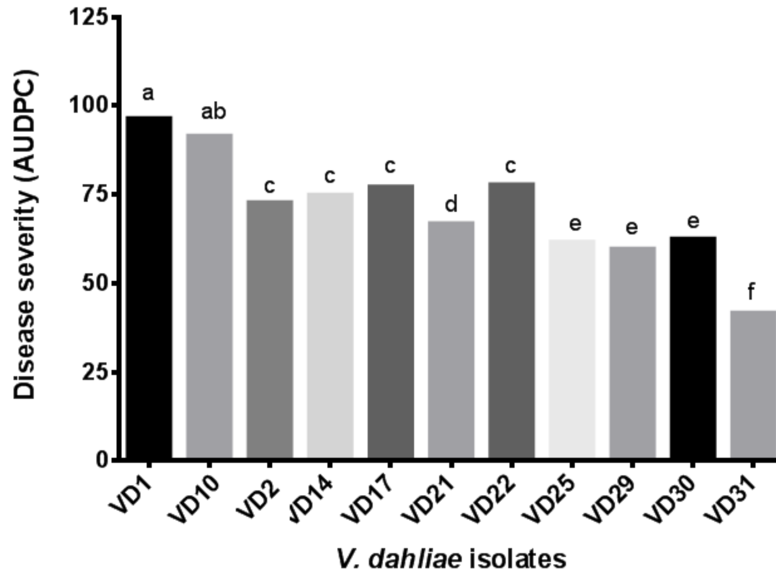

Fig. 2. Disease severity (DS) of *V. dahliae* isolates recovered from olive trees in Tunisia (histograms denoted by the same letter are not significantly different according to LSD test; α≤0.05)

Fig. 3. Gel electrophoresis of the amplified rDNA internal transcribed spacer (ITS) specific region of *Verticillium dahliae* isolates

(M: 1 kb DNA ladder, N: negative control)

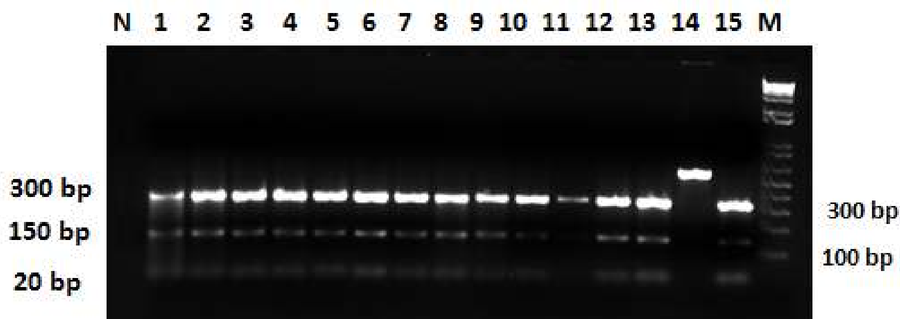

Fig. 4. Banding patterns of the amplified rDNA internal transcribed spacer (ITS) region after digestion with *HaeIII*

(1-15: V. dahliae isolates; M: 1 kb DNA ladder)

Fig. 5. Banding patterns of the amplified rDNA internal transcribed spacer (ITS) region after digestion with *EcoRI*

(1-10: V. dahliae isolates; M: 1 kb DNA ladder)

4. DISCUSSION

In this study, we examined the genetic diversity of *V. dahliae* isolates recovered from diseased olive trees in southern and central Tunisia. To achieve that, we employed of the ITS region for the detection and identification of *V. dahliae*. In addition, we compared this molecular technique with the morphological and microscopic identification of the pathogen. Morphological characterization of the pathogen isolated from infected olive trees revealed that *V. dahliae* population in Tunisia is dominated by two main morphotypes, with the predominant one having a milky white mycelium that gradually darkens with microsclerotia formation. In fact, the difference in color and texture of fungal colonies is probably due to the frequent mutations that could occur either by the composition of artificial medium or by application of fungicide in the field [18]. For instance, it has been reported that high temperatures lead to a higher frequency of morphological mutants [19]. Furthermore, correlation of the two morphotypes with the geographic origin of the isolates revealed that isolates are randomly grouped regardless of their morphological features, which suggests that there was admixture between the two observed morphotypes. The ITS region appeared to be little divergent both in length and restriction patterns among the examined *V. dahliae* isolates [20]. When the data obtained from ITS region is coupled with pathogenicity data, the ITS-RFLP failed to group the isolates according to their level of pathogenicity [1]. This result supported previous findings that ITS region is not a useful marker for intraspecific classification of *V. dahliae* isolates according to their pathogenicity levels.

Based on pathogenicity data, clear evidence was found that Tunisian *V. dahliae* isolates differed in their virulence towards olive, which suggests that *V. dahliae* populations, were relatively heterogeneous. Indeed, *V. dahliae* populations from center and coast regions in Tunisia were undifferentiated according to their virulence features, given that none of the virulence phenotypes was exclusively assigned to one of the two populations studied. Variation in virulence could be explained by the gain or loss of virulence as a result of changing the original host [21]. In fact, while the cultivar "Chemlali" is widely used in center of the country where the disease was first reported, other cultivars are used in the coastal region. The behaviour of these cultivars in response to infection by *V. dahliae* may have led to the emergence of highly aggressive pathotypes. This situation may have provided favorable conditions for pathogen selection, which is in agreement with previous reports supporting the hypothesis of host adaptation [22]. However, no association was found between the virulence phenotype and AFLP clustering of the isolates.

Like many asexual fungi, the sexual stage in *V. dahliae* is either unknown or has been observed only rarely in the laboratory and recent studies demonstrated that this fungus has a clonal population structure with little or no evidence of recombination. Despite the strong clonal structure and lack of evidence for sexual stage in *V. dahliae*, some population-genetic evidence suggests that recombination may occur or has occurred in the past. First, high diversity of multilocus genotypes for some pairs of microsatellite loci were interpreted as evidence of recombination in *V. dahliae*. A stronger argument for recombination derives from discrepancies in phylogenetic relationships among clonal lineages that could be explained more parsimoniously by recombination than mutation. The most obvious example is the polyphyletic nature of VCG2B, which comprises two distinct lineages, designated $2B^{334}$ and $2B^{824}$.

This study was carried out to determine the causal agent of the disease affecting olive trees in Tunisia and to study some of the phenotypic, pathogenic, and molecular properties of the fungus. The threat of this disease in Tunisia was increased by the fact that the extremely susceptible cultivar Chemlali is widely planted throughout the country. We concluded that cultural practice, the excessive watering, and plantation of susceptible cultivars act as risk factors for the development of the disease in the south and central Tunisia [23]. Controlling the spread of *V. dahliae* within and among production sites seems difficult because of the presence of inoculum sources and the broad

host range of the pathogen. In many production areas, plants do not show apparent symptoms before the infection is well established. Indeed, plants that have been treated with protective fungicides may appear healthy until the fungicides efficacy is lost and the pathogen population increases. Overall, removing undesirable weeds that could shelter high pathogen populations, use of drip irrigation instead of flood irrigation method and use of preventive fungicide are recommended for disease management.

5. CONCLUSION

In conclusion, *V. dahliae* infecting olive in Tunisia is characterized by genetic diversity with the predominance of one clone, which is probably the best adapted to infect olive. Analysis using ITS amplification did not possess any biological or ecological meaning but it can be applied as a technique for useful identification of *V. dahliae*. Therefore, more molecular approaches would be applied to define the genetic structure of this fungus in Tunisia.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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