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Occurrence of *Verticillium dahliae* defoliating pathotypes on olive trees in Tunisia

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Summary. Olive trees (cv. Chemlali) showing typical symptoms of wilt and dieback were collected from several areas in Tunisia. Visual diagnostic, isolation and microscopic observations identified the causal pathogen as *Verticillium dahliae*. The pathotype was determined using specific primers pairs for D (defoliating) and ND (non-defolitating) . Artificial inoculation of olive plants resulted in typical wilting with defoliation, proving that the isolate tested was pathogenic on olive, and this isolate belongs to the D pathotype. This is the first report of the defoliating pathotype causing wilting disease on olive in Tunisia.

Key words: Verticillium wilt, dieback.

Introduction

Olive groves are a very important cultivated crop in Tunisia with a total area of 2.3 million ha, and the annual production was 1183,000 tons of olive in 2008 (FAOStat, 2008). Olive trees are constantly at risk of attack by fungi which can cause severe damage in olive groves. Verticillium wilt is caused by the fungus *Verticillium dahliae,* which is one of the most important diseases occurring in olive growing Mediterranean countries. The disease on olive was first reported in the Mahres region of Sfax, Tunisia by Triki *et al*. (2006). Several other regions are also affected. Severity of attacks depends upon virulence of the pathogen isolates (Mercado Blanco *et al.*, 2001; Mercado Blanco *et al.*, 2002; Pérez Artés *et al.*, 2005). Isolates of *V. dahliae* infecting olive can be classified as defoliating (D) and nondefoliating (ND) pathotypes, according to their ability to completely defoliate plants (D) or to only cause wilt and partial or no defoliation (ND) (Schnathorst and Sibbett, 1971; Rodríguez Jurado *et al.*, 1993; Bejarano-Alcázar *et al.*, 1996). Infections caused by the D pathotype develop earlier and more rapidly, produce significantly greater yield loss and can be lethal to olive cultivars as compared to those caused by ND pathotype (Bejarano-Alcazar *et al.*, 1995, 1997). Therefore, the correct characterization of *V. dahliae* pathotypes would help in the decision making processes for disease management, such as the choice of host cultivar and avoidance of soil infested with *V. dahliae* D pathotypes (Tjamos, 1993).

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Materials and methods

Samples of branches, stems and shoots were collected in spring and summer from several dis-

eased olive trees located in the olive growing regions of Tunisia, including Monastir (Zarmdine), Sfax (Sidi Bouakkazine), Sidi Bouzid (Adhla and Hania), Kaïrouan (Chbika), Zaghouane (Nadhour), Kasserine (Oued eddarb), Mahdia (Bkalta), and Tataouine (Figure 1). They were brought separately to the laboratory for diagnosis. Samples showing symptoms were rinsed twice in sterilized distilled water, and then disinfected with ethanol 95%. Small fragments were cut aseptically and were placed on plates containing potato dextrose agar (PDA). Plates were incubated 25°C for 7 days. Identifications were based on culture morphology and microscopic characteristics of microsclerotia production (Hawksworth and Talboys, 1970; Triki *et al.*, 2006).

Mycelium of all isolates of *V. dahliae* (Table 1) was cultured on potato dextrose broth for 5 d with shaking at room temperature for DNA extraction. Each culture was centrifuged at 13,225× *g* for 15 min, and 50 to 100 mg of wet mycelium was used for DNA extraction. Primers ND1/ND2 and D1/D2 were used (Pérez Artés *et al.*, 2000). The PCR mix was performed in 25 µL containing 100 ng of the DNA of the isolates extracted with ZR fungal/bacterial DNA kit™ (ZYMO Research, Orange, CA, USA), 1× PCR buffer, 20 µM of each primer, 10 mM dNTP, and 1 U of Taq DNA polymerase. Amplification was performed with an initial denaturation at 94°C for 3 min, 35 cycles at 94°C for 1 min, 56°C for 1 min 72°C for 1 min 30 sec and a final extension at 72°C for 5 min. The PCR products (almost 1500 and 550 bp) were separated by electrophoresis on 1% agarose in 0.5× TAE buffer, stained with ethidium bromide and observed under UV light. Primers D1/D2 amplified a fragment of 0.55 kb in PCR reactions using DNA from D isolates of the pathogen, whereas Primers ND1/ND2 amplified a fragment of 1.5 kb in PCR reactions using DNA from ND isolates of

Figure 1. Localities of the olive tree orchards in Tunisian regions showing symptoms of Verticillium wilt.

Table 1. Details of *Verticillium dahliae* isolates from infected olive tree in Tunisian orchards, including regions, cultivars and plant parts from whence isolates were obtained, and their pathotype characterisations.

Isolate	Region	Pathotype	Cultivar	Plant organ	Date of isolation
V_{Za1}	Zarmdine (Monastir)	ND	Chemlali	Branch	Sept 2006
V_{16}	Sidi Bouakkazine -Sfax	ND	Chemlali	Branch	July 2005
V_{21}	Mahres (Sfax)	ND	Chemlali	Roots	July 2005
V_{d1}(Ref)	France	ND	-	Branch	-
V_{d2}(Ref)	Sousse	ND	-	Stem	July 2003
V_{FA}	Mahres (Sfax)	ND	Chemlali	Branch	Aug. 2006
V_{CH}	Sidi Bouakkazine -Sfax	D	Chemlali	Branch	June 2007
V_{M}	Monastir	ND	Sahli	Branch	June 2007
V_{BRm}	Mahres (Sfax)	ND	Chemlali	Roots	July 2007
V_{chbk}	Chbika (Kairouan)	ND	Chemlali	Collar	July 2007
V_{OKR}	Kairouan	ND	Chemlali	Branch	July 2010
V_{KS6}	Kasserin	ND	Chetoui	Collar	May 2006
V_{KS}	Kasserin	ND	Chemlali	Branch	June 2009
V_{RBS}	Mahres (Sfax)	ND	Chemlali	Branch	July 2008
V_{KS2}	Kasserin	ND	Chemlali	Root	May 2008
K_{S4}	Oued eddarb (Kassrin)	ND	Chemlali	Branch	Sept. 2008
Vj	Jammel (Monastir)	ND	Chemlali	Collar	July 2007
V_{Tt}	Tataouine	ND	Chemlali	Branch	June 2007
V_{RBS}	Mahres (Sfax)	ND	Chemlali	Branch	May 2008
Vo_2	Gremda (Sfax)	D	Chemlali	Collar	Oct 2007
V_{20}	Sidi Bouakkazine -Sfax	ND	Chemlali	Branch	Nov 2007
V_{26}	Gargour (Sfax)	ND	Chemlali	Root	June 2008
V_{Bak}	Sidi Bouakkazine -Sfax	ND	Chemlali	Branch	July 2009
V_{27}	Chott-Mariem (Sousse)	ND	Chemlali	Root	June 2008
$V_{P7.2}$	Mahres (Sfax)	ND	Chemlali	Branch	June 2007
V_{28}	Mahres (Sfax)	ND	Chemlali	Branch	Sept 2007
V_{30}	Gargour (Sfax)	ND	Chemlali	Collar	Oct 2009
V_{Hn}	Hania (Sidi Bouzid)	ND	Chemlali	Branch	June 2008
V_{OHF}	O.haffouz (Kairouan)	ND	Chemlali	Branch	Sept 2007
V_{Abd}	Nadhour (Zaghouane)	ND	Chetoui	Root	Oct 2009
V_{NADH1}	Nadhour (Zaghouane)	ND	Chetoui	Branch	May 2008
V_{NADH2}	Zaghouane	ND	Chetoui	Branch	June 2010
V_{19}	Gargour (Sfax)	ND	Chemlali	Collar	Sept 2009
V_{31}	Kaïrouan	ND	Chemlali	Branch	Nov 2009
V_{ZAF}	Zarmdine (Monastir)	ND	Chemlali	Branch	May 2009
V_{ZAC}	Zarmdine (Monastir)	ND	Chemlali	Branch	June 2010
V_{MAH1}	Mahdia	ND	Chemlali	Root	Nov. 2009
V_{MAH2}	Mahdia	ND	Chemlali	Branch	June 2010
V_{BCT}	Bkalta (Mahdia)	ND	Chemlali	Branch	June 2010
V138I	Spain	D	-	Stem	-
V4I	Spain	ND	-	Stem	-

V. dahliae. Amplicons of the D pathotype were purified with the ZymoClean™ DNA recovery Kit (ZYMO Research). The PCR products were directly sequenced in both directions using an automatic DNA sequencer (ABI 3100; Applied Biosystems, Foster City, CA, USA). Then the sequences were compared with those available in the Blastn program (http://www.ncbi.nlm.nih.gov/). The *V. dahl-*

iae isolates V4I and V138I, representatives of the ND and D pathotypes respectively, were supplied by Antonio Trapero Casas (Departamento de Proteccion de Cultivos, Instituto de Agricultura Sostenible, CSIC, Cordoba, Spain), and were used as a positive control in this study.

Pathogenicity of the isolates was tested on 2-year-old olive trees cv. Chemlali, the most commonly cultivated cultivar in Tunisia. Three plants were used for each isolate. Pure cultures of each isolate grow for 10 days on PDA were used to prepare a conidial suspension, by adding sterile distilled water to the plates and gently rubbing the surface of the colonies with a glass rod. Conidial suspensions of *V. dahliae* were adjusted to 10^5 conidia mL^{-1}. The roots of the olive trees were carefully washed under running tap water to remove soil and soaked for 1 h in spores or mycelia suspensions. Uninoculted control plants were soaked in water. The inoculated plants were transplanted into polyethylene pots of 3 L capacity, containing a sterile substrate (peat: sand, 1:1 v/v). The plants were incubated in a growth chamber at 24°C under high humidity and with a 16 h photoperiod. Plants were waterlogged twice per week. Pieces from roots of inoculated and control plants were plated on PDA to reisolate each inoculated isolate of *V. dahliae* present in the root tissues. Incubation conditions of plates and methods for identification of fungi isolated were as described above.

Results

The majority of colonies obtained in culture developed a dense mycelium all around the periphery of each colony after 8–16 days. Microscopic observations revealed that vegetative mycelium was hyaline, septate, and multinucleate. Conidia were ovoid or ellipsoid and usually single-celled. They were borne in masses on verticillate phialides. Some isolates produced abundant irregular microsclerotia after 4–19 days on medium. These characters confirm that the isolates were *V. dahliae*.

The two isolates (V_{CH} and V_{O2}) and the reference strain V138I, used as positive control, yielded an amplicon of about 550 bp. However, with the other isolates as the reference strain V4I a fragment of 1500 bp was amplified using ND isolates of *V. dahliae* (Figure 2). A BLAST search of the NCBI GenBank database with the two sequences generated in this work gave 98 % similarity to the RAPD genomic sequence of *V. dahliae*. The closest sequences were affiliated to accession number AJ302674.

Inoculated plants were evaluated for symptoms after 1 month. Moreover, pieces from inoculated and control roots were plated on PDA to reisolate the fungus. Incubation conditions of plates and methods for identification of reisolated fungi were as described above. Five weeks

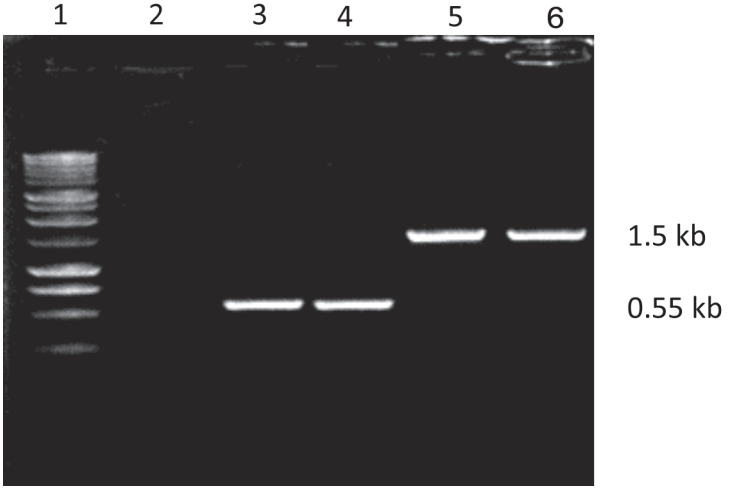

Figure 2. Specific PCR of total DNA from D and ND *Verticillium dahliae* isolates using the D and ND-specific primers. Lane 1, 1 kb DNA ladder (Promega); 2, negative control; 3, V138I; 4, D. *V. dahliae* isolate V_{CH}; 5, V4I and 6, ND *V. dahliae* isolate VZa1..

after inoculation, all of the inoculated plants developed the same symptoms of the disease as observed in the fields from which diseased samples were collected. Only plants inoculated with isolates V_{CH} and V_{O2} were shown to be highly aggressive on young olive trees cv. Chemlali, causing severe defoliation, wilting and dieback. However, the ND isolates showed only partial wilting and dieback typically developed on one main branch (Figure 3).

Discussion

The defoliating pathotype of *V. dahliae* was detected by PCR in 5% of the isolates analysed. This pathotype was found in samples from Sidi Bouakkazine and Gremda olive growing areas (Sfax region). The defoliating pathotype of *V. dahliae,* which is common in some countries of the Mediterranean basin such as Spain (Péréz Artez *et al.*, 2000; Mercado Blanco *et al.*, 2003), Italy (Colella *et al.*, 2008) and Turkey (Dervis *et al.*, 2010), is reported in this study for the first time in Tunisia. However, in Algeria, only the ND pathotype of *V. dahliae* was detected from olive (Bellahcene *et al.*, 2005).

The occurrence of this pathotype in Tunisia led to speculation about its origin. The D pathotype may have been introduced from outside the country. Some factors might contribute in the spread of the D pathotype from the Mediterranean basin to distant countries. These include previous field planting with susceptible crops (before establishment of olive tree plantations), changes from dryland farming to irrigation, very susceptible cultivars, and the use of infected planting material. Intercropping of olive trees with sensitive vegetables may increase pathogen inoculum potential in soils (Jiménez Diaz *et al.*, 1998). The use of molecular techniques such as RAPD and the comparison of profiles obtained could be helpful in determining the origin of infection by this pathotype.

The use of primers ND1/ND2 and D1/D2 is of particular interest for implementing certification schemes for the production of *V. dahliae*-free olive planting stock, but also for epidemiological studies on Verticillium wilt of olive. Information of this nature would be of much interest in the design and application of potential disease management strategies for Verticillium wilt.

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Figure 3. Symptoms induced by inoculation of olive plants with *Verticillium dahliae*: a, plant inoculated with isolate Z_{a1} showing wilting on the branch; b, plant inoculated with isolate V_{Ch} showing wilting and defoliating symptoms.

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