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Morphological and molecular identification of *Fusarium oxysporum* f. sp. *lycopersici* associated with *Olea europaea* var. sylvestris decline phenomenon in Tunisia

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Abstract

Decline phenomena and mortality of *Olea europaea* var. *sylvestris* (oleaster) have been reported throughout the forest of Henchir Kort (northeastern of Tunisia). The affected plants show progressive dieback of shoots, twig blight symptoms and trunk canker. The fungi appear to have the most significant potential threat to the disease. However, it has been less well-studied in Tunisia. A survey on the causal agents of *O. europeae* decline attacked twigs with symptoms of wilting and vascular necrosis were collected. The causal agent of the syndrome was identified as *Fusarium oxysporum* f. sp. *lycopersici* based on morphological characteristics and molecular identification performed by sequencing the ITS region of the ribosomal DNA. *Fusarium* species are among the most aggressive telluric fungi, causing diebacks of many plant species, especially on *Olea europaea*. To the best of our knowledge, this is the first record on the occurrence of *Fusarium oxysporum* f. sp. *lycopersici* on *O. europaea* in the word and in Tunisia.

Keywords – Dieback – Fungi – Identification – ITS region

Introduction

The olive tree is present on six continents: Europe, North America, South America, Africa, Asia and Oceania. It's a very ancient Mediterranean species. It belongs to the family of *Oleaceae*, genus Olea, species *europaea*. Two subspecies distinguish it: *Olea europaea* var. *sylvestris* (oleaster) and *Olea europaea* var. *sativa* (cultivated olive tree) (Villa 2003). Its wild form *Olea europaea* var. *sylvestris* is mainly found in Mediterranean countries: Portugal, Spain, Italy, Turkey, Greece, Morocco, Syria and Tunisia (Villa 2003). Furthermore, *O. europaea* var. *sylvestris* seems to be the ancestor of the cultivated olive tree (Belaj et al. 2010). The economic and ecological interest of the oleaster is major. It is used as a firewall to maintain soils and limit erosion (Breton et al. 2006).

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However, this evergreen specie is withering in countries of the Mediterranean basin, including Tunisia (Lazzizera et al. 2008, Moral et al. 2009), due to the interaction of several abiotic factors (anthropic action, drought, water stress, and fires), and biotics (insect pests and pathogenic fungi) (Ben Jamâa & Hasnaoui 1996). Most fungal species causing dieback of oleaster are common saprophytes or secondary invaders usually penetrating through injuries made by biotic or abiotic factors (Lazzizera et al. 2008). Moreover, infections caused by fungi can cause very worrying diebacks, such as those caused by telluric fungi including *Fusarium* spp. which have been shown to cause symptoms of wilting and partial or total dieback of the olive (Jardak et al. 2007, Trabelsi et al. 2017) and caused extensive damage in several countries in the Mediterranean basin (Porras-Soriano et al. 2003). The *Fusarium* genus is one of the most complex and adaptive species in the Nectriaceae. The *Fusarium oxysporum* (Fo) species complex includes plant, animal and human pathogens and a diverse range of non-pathogens (Gordon 2017). This fungal pathogen is widely represented with a predominance of *Fusarium oxysporum* (Chliyeh et al. 2017), which are responsible for two distinct types of symptoms: wilting of the aerial part of the plant and root and/or collar rots (Gordon 1965). Members of *Fusarium* species are ubiquitous soil-borne pathogens of a wide range of horticultural and food crops which cause destructive vascular wilts, rots, and damping-off diseases (Bodah 2017). In particularly, *Fusarium oxysporum* f. sp. *lycopersici* first described in Europe at the end of the 19th century; it is present in dozens of countries on every continent (Blancard et al. 2009). Based on its economic importance and scientific interest, this species has been ranked among the "top 10" of plant pathogenic fungi (Dean et al. 2012).

In this context, this work consists of (i) the isolation of the pathogen *(Fusarium*) from symptomatic oleaster branches, (ii) its morphological identification (macroscopic and microscopic) and (iii) its molecular identification.

Materials & Methods

Study area

The study was carried out in 2017, in the forest of Henchir Kort (36.30'.406" N; 10.38'.780"E) in Cap-Bon in the northeastern of Tunisia (Fig. 1). The vegetation is a mixture of pine trees with Mediterranean scrub composed mainly of *Olea europaea* var. *sylvestris.*

Fig. 1 – Localization of studied site (Henchira Kort forest is indicated with a red star).

Phytosanitary status

Damage degree was estimated based on visual appreciation of disease symptoms and a decline class of each *O. europaea* plant was assessed following the methodology described by

Franceschini et al. (2005), with: (C0) no attack /absence of symptoms; (C1) = 1 to 25%; (C2) = 26 to 50%; (C3) = 51 to 75% and (C4) = 76 to 100%.

Collection of samples and isolation

In December 2017, typical disease symptoms were collected from stems of *O. europaea* and transferred to the laboratory. First, all samples are superficially disinfected using the surface sterilization method described by Alves et al. (2013). Small pieces of necrotic tissue (3× 3 mm) were taken from the margin of infected tissues were placed in Petri dishes containing potato dextrose agar (PDA) added with streptomycin sulfate (0.05 g/l) antibiotic according to the technique used by Franceshini et al. (2005) and incubated in the dark at 25°C for 3 days. Pure cultures were obtained by plating a small piece of mycelium from the margin of each colony grown on PDA and incubating them under the same conditions described above.

Morphological and molecular identification

Fusarium oxysporum was identified based on its cultural traits, conidial morphological characteristics by referring to identification keys (Rayner 1970) and forest mycology guide (Lanier et al. 1976). Colony morphology, including color, shape, and growth rate, was determined after 7 days of incubation on PDA at 25°C in darkness. Microscopic characters were studied according to the technique explained by Arzanlou et al. (2007). Dimension of microscopic structures were calculated based on 30 measurements for conidia morphology (shape, color, and cell number), size (length and width). The percentage of isolation frequency (IF) was calculated using the formula of IF (%) Franceschini et al. (2005): IF = Ni/Nt x 100 with Ni (number of fragments colonized by the fungus) and Nt (total number of plated fragments).

Regarding the molecular identification, fungal DNA was directly extracted from mycelia growing on plates, using a commercial Kit Macherey-Nagel- 07/2014, Rev.09.PCR reactions were carried out with ITS1 and ITS4 primers (White et al. 1990) to amplify the ITS region of the ribosomal RNA as described by Alves et al. (2004). Products from PCR reactions were electrophoresed on a 1.5% agarose gel, then stained with GelRed, and visualized with UV transilluminator. The size of PCR products was estimated by comparison with a DNA ladder 100 bp plus, Transgen Biotech. All PCR amplifiers were sent for sequencing to the Interdepartmental Center for Chemical and Industrial Agricultural Biotechnology Services (Italy) laboratory. The representative sequence was deposed in GenBank.

Phylogenetic analysis

ITS sequences were used to conduct a phylogenetic analysis. Sequences of *Fusarium* species were retrieved from GenBank and aligned with sequence of the isolate (TN.24) obtained in this study. Sequences were aligned with ClustalX v. 1.83 (Thompson et al. 1997), using alignment parameters according to Linaldeddu et al. (2015). Phylogenetic tree was generated under Maximum Likelihood (ML) and analyzed to build the tree topology by the Neighbour-Joining method using MEGA 6.0 software (Tamura et al. 2013).

Results

Incidence

The Henchir kort forest investigation revealed that 40% of the examined oleaster plants showed dead twigs with necrotic lesions (Fig. 2). Subjects of decline class (C1) dominate with a rate of 50% followed by (C2) subjects with a rate of 34% and 16% for (C3).

Morphological characteristic

Morphological identification was based on an observation combining colony morphology and microscopic spore observation; it was taken place after 7 days of incubation. A collection of 20

isolates was obtained from infected samples collected from the oleaster plants at the Henchir kort forest. Preliminary identification of the genus was carried out by analyzing morphological traits based on the general appearance of colonies and the aspect of conidia. On PDA, colonies have a whitish, cottony and dense medium-growing mycelium (Fig. 3). Microscopic observation revealed the presence of very short conidiophore even invisible, septate macroconidia (3 to 7 septate) more or less curved fusiform measuring 2.9 to 4.9 µm by 23 to 53 µm. The apical cell is tapered and curved, and the basal cell is pedicelled. Microconidia are ellipsoidal and slightly curved, measuring 2 to 3.5 µm by 4.5 to 11 µm (Fig. 2).

In this study, *Fusarium* species were isolated from all sampled *O. europaea* plants showing disease symptoms and 45% of isolates were identical to *Fusarium oxysporum* f. sp. *lycopersici.*

Fig. 2 – Necrotic lesion on stem observed in naturally infected *Olea europeae* plant.

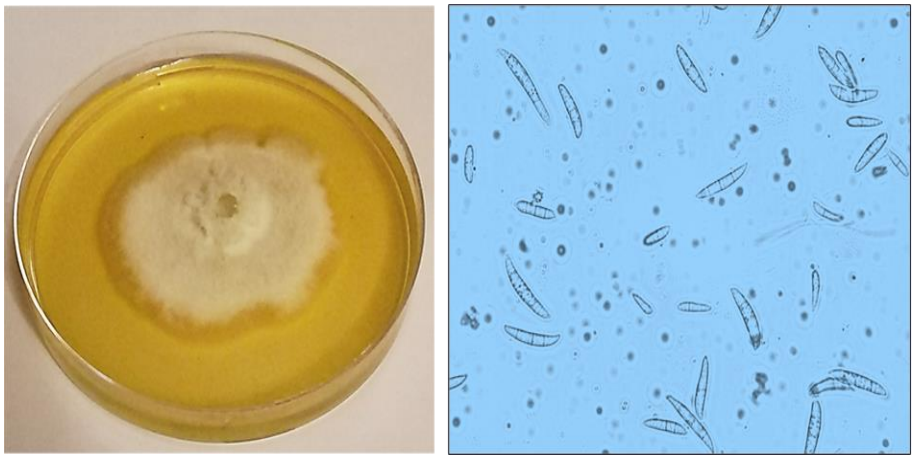

Fig. 3 – *Fusarium oxysporum* f. sp. *lycopersici*: Macroscopic appearance of the colony incubated on PDA at 25°C for 5 days (right) and microscopic appearance of conidia (40X) (left).

Phylogenetic analyses

A representative isolate was selected for molecular identification and the identity of the species was confirmed by DNA sequence analysis of the ITS regions. The BLAST research of the isolate TN.24 selected for molecular identification showed 98% homology with *Fusarium oxysporum* f. sp. *lycopersici* (FSOT) (KY100124) and the representative sequence was deposited in GenBank under the accession number (MN843963). The phylogenetic tree, resulting from the PCR amplification sequence of the ADNr nuclear operon using the ITS1 and ITS4 primers of the isolate TN.24 obtained in this study reveals that our isolate is grouped with other *F. oxysporum* f. sp. *lycopersici* (FOL) (Snyder & Hansen 1940) downloaded from the GenBank database and separate from the group of *F. equiseti* (Fig. 4).

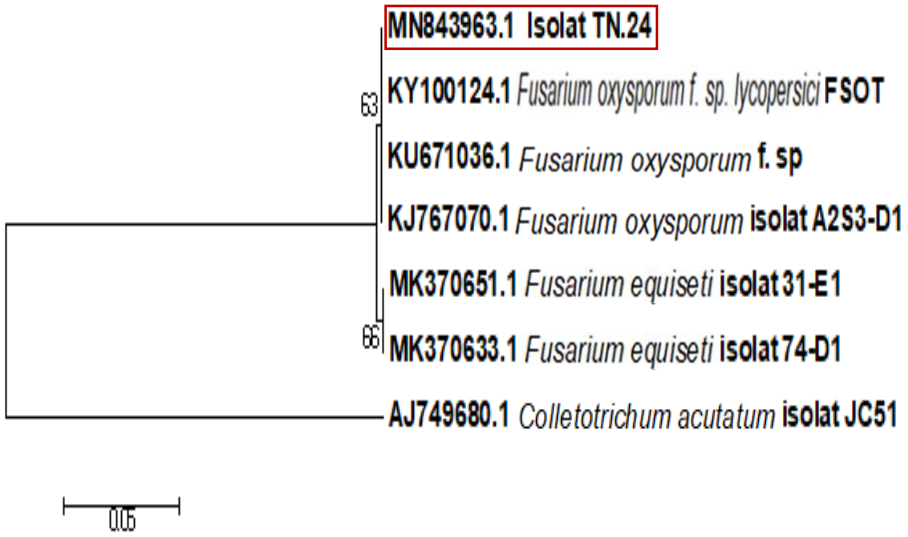

Fig. 4 – Phylogenetic tree obtained from ITS sequence data from the TN.24 isolate. Bootstrap support values (%) from 1000 replications are shown at the nodes. The tree is rooted in *Colletotrichum acutatum.* The scale bar shows 0.005 substitutions per site.

Discussion

Surveys in the Henchir Kort forest revealed the presence of dieback and necrosis on the twigs of olive shrubs resulting in defoliation and drying of new shoots. The pathogen was isolated from samples of the infected branches. Based on macroscopic and microscopic morphological criteria, the isolate TN.24 obtained in this study was identified as *Fusarium* sp. which is consistent with the results obtained by Isebaert et al. (2005). Moreover, the amplification of ribosomal DNA by the two universal primers (ITS1/ITS4) made it possible to distinguish the exact isolated species. According, Nasraoui & Lepoivre (2003) asserted that ITS regions are widely used for species identification. As a result, morphological and molecular phytopathological analyses have confirmed the presence of *Fusarium oxysprum* f. sp. lycopersici. Thus, *O. europaea* dieback is caused by this pathogen. The results obtained in this study are consistent with those of Cristobal-Alejo et al. (2016) in Mexico, which confirmed that *Fusarium oxysporum* f. sp. *lycopersici* (FSOT) caused significant damage to the stems of the *Saccharum officinarum.* Others researches, also such as those of Al-Ahmad (1984) in Syria and Sanchez et al. (1998) in Spain, have shown that telluric fungi causing threat to olive trees. In fact, the genus *Fusarium* includes many plant pathogenic species that can induce disease in many plants. Furthermore, symptoms of dieback caused by the genus *Fusarium* have been observed on young olive trees planted in Morocco (Chliyeh et al. 2014). As well, two species *F. oxysporum* and *F. solani* were isolated from crown and olive stems in Algeria (Merzoug et al. 2018). In Saudi Arabia, AL-Shebel et al. (2005) identified two olive dieback agents, *Fusarium* spp. et *Verticillium dahliae*. In the other hand, Messiaen & Cassini (1968) confirmed that *Fusarium oxysporum* f. sp. *radicis lycopersici* attacks the root parts and

Fusarium oxysporum f. sp. *lycopersici* (Sacc.) WC Snyder & HN Hansen attacks the aerial parts of the plant. In addition, Asha et al. 2011 reported that *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) WC Snyder & HN Hansen (FOL) caused vascular wilt of tomato disease and reduced the yield to the maximum extent.

Conclusion

This finding was the first record of *Fusarium oxysporum* f. sp. *lycopersici* as fungal pathogen associated with *Olea europea* var. sylvestris dieback in Tunisia. Despite the economic losses it causes, control of this pathogen is still limited to prophylactic measures, disinfection of the soil is never complete due to the difficulty of its production and to resistant strains (Benhamou et al. 1997). Therefore, and before the implementation of a strategy to control this phytopathogen, it is necessary to better understand the epidemiology and the mechanisms of *fusarium oxysporum* f. sp. *lycopersici* infection to assess its aggressiveness by artificial inoculations on the branches of oleaster. The assessment of the pathogenicity of this pathogen is being appraised.

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