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Screening of fungi implicated in the dieback of olive trees (*Olea europea*) in Chebika's area

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Several surveys were conducted during spring 2008 in Chebika's area in Tunisia. Samples were collected from infected plants showed different types of symptoms and they have been the subject of mycological analysis. The morphological identification of fungal colonies isolated from roots, crown and stems of two olive varieties Koroneiki and Chemlali Sfax, revealed the presence of a fungi complex including *Fusarium oxysporum*, *Fusarium solani*, *Rhizoctonia solani*, *Verticillium dahliae*, *Cladosporium fulvum*, *Alternaria solani*, *Alternaria tenuis*, *Bispora punctata*. and *Cylindrocarpon*.sp; Although, those fungi *Fusarium oxysporum*, *Fusarium solani*, *Rhizoctonia solani* and *Verticillium dahliae* are ubiquitous and the predominant one. Pathogenicity results revealed that the fungi isolated from olive trees exhibited typical symptoms on Koroneiki variety in controlled conditions.

Keywords: Surveys, *Olea europea*, fungi, pathogenicity

In Tunisia, where the olive is of great economic importance, fungi diseases are becoming an increasingly serious problem (Boulila and Mahjoub, 1994; Triki et *al*., 2006). Boulila and Mahjoud (1994) reported the infestation of olive by *Pseudomonas syringae* pv. *Savastanoi, Spilocaea oleagina* and *Glomerella cingulata*) that were identified also in other Mediterranean countries. Furthermore, *Rhizoctonia bataticola, Fusarium solani, F. oxysporum, Armillaria mellea et Corticium rolfsii* were identified from olve trees showing the decay symptom. In 2006, Triki et al. were reported dor the first time the attack of olive by *Verticillium dahlia* in Mahres area at the government of Sfax. This pathogen was also detected in Morrocco. *Verticillium dahliae* could be responsible for a stroke and a slow decline. The symptoms of decline appear on the olive trees in full production, which in some situations are completely decayed, while many others have a partial drying. It is obvious that these disorders also affect young olive trees after planting (Bellahcene et al., 2005).

The issue of the decayed olive tree is a fungal complex including several primary and secondary agents contributing to the onset of symptoms like yellowing, defoliation, stunting, wasting and withering of plants at any age.

The objective of this study is to (i) identify the fungi responsible of the dieback of olive trees in Chebika's area and to (ii) explore the pathogeny of the isolated species on young olive plants in controlled conditions.

Materials and Methods

Young olive plantations 4 years old were conducted in intensive drip irrigation and spaced at 6 × 6 m, located in chebika 's delegation of Kairouan governorate. Those plants have showed different symptoms of

yellowing leaf and decline during their first 2 years of growing with an infection rate of 33% in 2007. Six orchards, planted with Koroneiki and chemlali Sfax cultivars, were surveyed for detecting the disease incidence and for samples collecting for further identification of agents responsible for the observed symptoms (Table 1).

Table 1: Distribution of surveyed olive orchards

Orchards	Varieties	Total number of olive trees
S1	Arbequina	3000
S2	Chemlali Sfax	5146
S3	Chemlali Sfax	4000
S4	Chemlali Sfax	4200
S5	Koroneiki	2366
S6	Koroneiki	3525

Plants sampling

Roots and rootlets samples were randomly collected at two times of the year in 2008 (February and March) from symptomatic olive trees with typical symptoms of decline .

Isolation, Purification and Culture of fungal colonies

Samples of roots were removed carefully by washing them with running water, then cutting into small fragments of 5 to 10 mm and superficially disinfected by soaking for 5 minutes in sodium hypochlorite diluted to 10%. We cut the rootlets and roots at various levels to young shoots ascending to the vascular system. The dried fragments are placed in Petri dishes on PDA (Potato dextrose agar). The plates are then incubated at 25 ° C in the dark.

Purification of isolates was obtained by sub-culturing on PDA. For the mono-conidial culture, we added to the culture 5 mm of sterile distilled water, then we scrapped the mycelium to collect the inoculum. The spore suspension obtained was filtered. By the Malassez blade, we have adjusted the concentration to 10^6 spores / ml. After spreading this dilution in Petri dish containing 2% -Water Agar; the incubation was performed for 24 hours at 25 ° C in the dark. Each germinated spore was removed and grown separately on PDA to obtain a pure colony. Conservation and multiplication of these pure cultures were made from the tip of young developing colonies.

Fungal identification

Morphological identification of isolates was performed with culture 8 days-old on PDA, using the criteria proposed by Booth (1971). The microscopic observation of the different structures of the fungus was performed, by the observation of the type of mycelium, sexual or asexual structure of multiplication.

Pathogenicity assay

Pathogenicity trials were conducted with four fungal species: *Fusarium oxysporum*, *Fusarium solani*, *Rhizoctonia solani* and *Verticillium dahliae* collected mainly from the surveyed olive trees. The plant material used is young olive plants of 3 varieties grown from softwood cuttings and one year-old: Arbequina, Chemlali Sfax and Koroneiki; They have been transplanted in 14 cm diameter plastic pots filled with peat. The plants are watered once a week during one month.

The preparation of inoculums was conducted by two techniques, the first technique is used for *Rhizoctonia solani* and *Verticillium dahliae*, which was consisted to grind in a blender the contents of the plate in 100 ml of sterile distilled water. The second technique was adopted for *Fusarium oxysporum* and *Fusarium solani*, by preparing a suspension of the inoculums in a flask containing 200 ml potato-dextrose broth (PDB). The flasks were incubated at 25 ° C in a rotative agitator for 3-4 days. The contents of each flask was filtered and the

concentration of spore suspension was adjusted to 10^6spores / ml through a Malassez blade (Boughalleb and El Mahjoub, 2005).

Pathogenicity essay was conducted by performing different combinations of inoculation:

- *Fusarium solani*
-*Fusarium oxysporum*
-*Rhizoctonia solani*
-*Verticillium dahliae*
- *Fusarium solani* +*Fusarium oxysporum* + *Rhizoctonia solani*+*Verticillium dahliae*

The experimental protocol adopted was a complete randomized experiment with 3 varieties, 6 treatments and 2 replicates for each treatment. Thus, two plants of each variety were sprayed with 50 ml of spore suspension of each pathogen and the combination of these four species, and a negative control of each variety. The experiment was repeated twice.

Results

Inventory of the fungal microflora

Eleven fungal species were identified from the roots, crown and stems of the two varieties of olive Koroneiki and Chemlali Sfax , namely *Alternaria solani*, *Alternaria tenuis*, *Bispora punctata*, *Cladosporium fulvum*, *Cycdrocarpon sp.* , *Fusarium oxysporum*, *Fusarium solani*, *Rhizoctonia bataticola*, *Rhizoctonia solani*, *Phoma sp* and *Verticillium dahliae*. Similar results were reported by Jardak et al. (2004) who reported that *Verticillium dahliae*, *Fusarium oxysorum*, *Fusarium solani* and *Rhizoctonia solani* could be responsible of olive trees dieback.

Morphological characterization

Fusarium oxysporum and *F. solani* mycelia colonies have invaded the Petri dishes after 6-7 days. Microscopic observation revealed the presence of chlamydospores in both species. *Fusarium solani* is characterized by long phialides with single macro-conidia or micro-conidia, whereas *Fusarium oxysporum* presented a short phialides.

Rhizoctonia solani and *R. bataticola* are completely overgrown after 2-3 days. Microscopic observation demonstrated that it is an anamorphic fungus characterized by the formation of a septate mycelium with an angle of 45° with the branch at which we note the presence of a slight constriction. This mycelium formed in defavorable growing conditions shaped articles including stromatic form plaques that will ensure the survival of the pathogen.

The mycelial colony of *Verticillium dahliae* developed on PDA medium appeared hyaline and produced only microsclerotia as resting structures and was identified as V. dahlia according to the description of Hawksworth and Talboys (1970). Thus we found that the decline of olive trees in Chebika's area is due to a fungal complex confirming the results of Boulila and Mahjoub (2008).

Pathogenicity of isolates

After 25 days of inoculation,no macroscopic symptoms were observed on seedlings of Arbequina and Chemlali Sfax varieties. However, by comparing the inoculated and the non-inoculated plants of Koroneiki, It appeared that the plants inoculated were dwarf and they lost their bluish-green to take one aspect of light green (Fig. 1).

Figure 1: Olive leaf from the variety Koroneiki decolorized after 25 days of inoculation with *Fusarium oxysporum*

Plants inoculated with *Fusarium solani* and *Fusarium oxysporum* showed symptoms of leaf curling (Fig. 2) and presented a rotten root (Fig. 3). In the case of plants inoculated with *Fusarium oxysporum*, a unilateral yellowing (hemiplegic) was noted, indicated the presence of vascular attack. However, the leaves of plants inoculated with *Rhizoctonia solani* are not wrapped, but have yellowing from the tip and a yellowing from the base for *Verticillium dahliae* inoculation.

Figure 2: Appearance of decolorized olive plants of the variety Koroneiki, after 25 days of inoculation with *Fusarium solani*

Figure 3: Root rot of the plant of Koroneiki after 25 days of inoculation with *Fusarium solani*

For the olive plants inoculated with the four identified species, the results showed the presence of a slight winding up and a lighter yellow leaves. *Fusarium oxysporum* was re-isolated from the plants with a height of 75 cm and *Verticillium dahliae* was re-isolated from 65 cm of height far above the ground plants, representing 86, 6% of the total height of the plant.

It appears that the fungi isolated from olive trees were pathogenic on Koroneiki variety showing typical symptoms. Inoculation of plants with *Rhizoctonia solani*, *Fusarium solani*, *Fusarium oxysporum* and *Verticillium dahliae* together showed high aggressiveness indicating the presence of synergy between *Fusarium sp.* and *Verticillium dahliae*. The re-isolation of the fungi was positive for all inoculated plants, except *Rhizoctonia solani*, which showed a weak competitive as the development of *R. solani* mycelium is inhibited in the presence of other isolates (Table 2).

Discussion

Decay symptoms, observed in the surveyed olive trees orchards in the region of Chebika, appeared to be caused by several fungi, including *Verticillium dahliae*, *Fusarium oxysporum*, *Fusarium solani* and *Rhizoctonia bataticola*. Our results concord with those obtained by Boulila et al. (1993) in other regions of Tunisia: Southwest (Gafsa, Ben Aoun), Centre (Ennadour, Kairouan, Sbikha) and northern part (Bousalem).

The symptoms were usually localized on a branch. Leaves from the infected area were curled and were tinged with a light brown (Bellahcene et al., 2005). The young shoots are completely defoliated at the base so they can keep a few brown leaves at the end (Sanchez-Hermandez et al., 1998). El Bsir (2007) estimated the infestation rate at 10 to 40%.

Verticillium wilt of olive trees has also been reported in Tunisia by Triki et al. (2006) during surveys conducted in the olive trees of the delegation of Mahres (Sfax). Indeed, some decayed Chemlali old trees have been observed in two neighboring orchards and the symptoms were located per area. Field investigations and laboratory showed the presence of *Verticillium dahliae*. According to Bellahcene et al. (2005), *Verticillium dahliae*

could be responsible alone for a stroke and a slow decline. Similar results were reported by Jardak et al. (2004) who reported that *Verticillium dahliae*, *Fusarium oxysorum*, *Fusarium solani*, *Rhizoctonia solani* and *Phoma sp*. could be the principal agents of dieback of olive trees. *Verticillium dahliae* was isolated from olive trees in some regions in Spain (Sanchez-Hermandez et al., 1998) and Algeria (Bellahcene et al., 2005). More recently, Boulila and Mahjoub (2008) also mentioned the pathogenenicity of *F. solani* and *F. oxysporum* in Tunisian olive trees. The decline symptoms appear on the olive trees in full production, which in some situations are completely decayed, while many others have a partial drying.

Table 2: Pathogenic species of *Fusarium sp.*, *Verticillium dahliae* and *Rhizoctonia solani* alone and their combination

Species	Number of symptomatic plants/ 4	Diseased plants after 25 days (%)	Re-isolation	Frequency of re-isolation for Koroneiki (%)
Fusarium solani	4	0	+	47,25
F.oxysporum	4	0	+	71,66
Verticillium dahliae	4	0	+	29
Rhizoctonia solani	4	0	+	75
R.solani + *F.oxysporum*	4	0	-	0
			+	49,77
F.solani + *Verticillium dahliae*			+	15
			+	22,7
Témoin	0	0	-	0

\+ Positive Re- isolation - Negative Re- isolation

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