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First report of *Pseudocercospora cladosporioides*, the causal agent of Cercospora leaf spot of olive trees, in Tunisia

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Summary. Olive trees (*Olea europaea*) cv. Meski having leaves with yellow spots on the upper surface and grey blotches on the lower surface were found in three orchards located in the regions of Takelsa, Testour and Enfidha, central and northern Tunisia. The shoots of the olive trees had also become defoliated indicating a severe attack of a pathogen. *Pseudocercospora cladosporioides* was isolated from symptomatic leaves and Koch's postulates were fulfilled. This is the first Tunisian report of *P. cladosporioides* causing Cercospora leaf spot of olive trees.

Key words: cv. Meski, *Olea europaea,* olive leaf spot, cercosporiosis.

Introduction

Leaf spot of olive (*Olea europaea* L.), caused by *Pseudocercospora cladosporioides* (Sacc.) U. Braun (syn. *Cercospora cladosporioïdes* Sacc.), is an olive disease that causes premature leaf fall and hence a general weakening of affected trees. It is common in most olive growing regions in the world such as California (Viennot-Bourgin, 1949), Portugal (Pintoganhoa, 1963), Italy (Pettinari, 1952), Argentine, Spain (Del Moral and Cabezas, 1985), Greece (Pappas, 1975) and South Australia (Spooner-Hart, 2005).

The first symptoms of the disease are grey blotches on the underside of the leaves (López-Villalta, 1999; Jardak *et al.*, 2008), while the upper side of the leaves become yellow. Defoliation can occur. Mature and immature fruits sometimes develop small, brown spots and fruit ripening is not uniform (Del Moral and Cabezas, 1985; Sergeeva, 2008).

The disease cycle is similar to that of peacock spot caused by *Spilocaea oleagina* (Spooner-Hart, 2005) and the diseases often occur together on the same plant or even on the same leaf. Outbreaks of leaf spot are sporadic, and it may take several years before the disease becomes serious enough to cause economic damage. Not all infected leaves fall, and the fungus survives in those leaves that remain on the tree. The lesions enlarge in the autumn and a new crop of spores develops on them. New infections are associated with rainfall and mostly occur during winter. In the summer, most diseased leaves fall from the trees, leaving the crown partially defoliated.

Recently, leaf symptoms resembling leaf spot caused by *P. cladosporioides* were recorded for the first time in Tunisia, on cv. Meski olive orchards located in the regions of Testour, Enfidha and Takelsa. The aim of this work was to identify the causal agent of this disease and to fulfill Koch's postulates.

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Materials and methods

Location of the olive orchards

The orchards inspected were located in three regions: Takelsa, Testour and Enfidha (Fig. 1). These regions are characterized by high humidity and by the widespread occurrence of peacock spot caused by *Spilocaea oleagina*. In these regions, the symptoms of the new disease were often found associated with peacock spot.

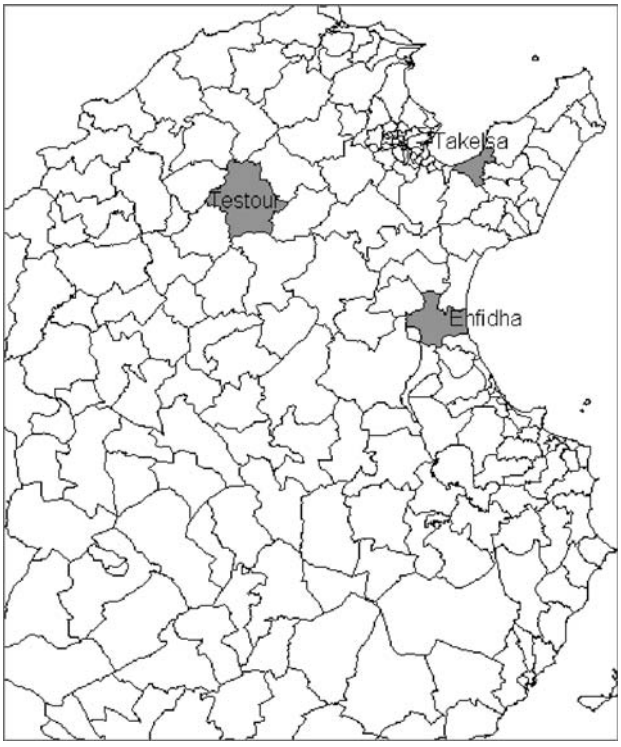


Fig. 1. Location of the olive tree orchards in Tunisian regions showing symptoms of Cercospora leaf spot.

Isolation and culture of the causal fungus

Pieces of spotted leaves were removed from diseased olive trees cv. Meski with a sterile scalpel, plated on potato dextrose agar (PDA) (Tsopelas, 1999) and incubated at 25°C. Isolation was also done by transferring conidia directly from the underside of infected leaves to PDA using a sterile scalpel. After purification, the fungus was identified to species level using morphological characteristics and it was routinely cultivated and maintained on PDA without glucose or on Czapek-Dox medium (Herrero *et al*., 1996). Abundant conidia of *P. cladosporioides* were produced after incubating at 25°C, for 15 days.

A second isolation technique was also used in the study. A spore solution was prepared by dipping 2 or 3 infected leaves with a dusty grey underside (a typical symptom) in a tube containing sterile distilled water. After homogenisation, three dilutions of 10^{-2}, 10^{-3} and 10^{-4} were prepared. From each dilution an aliquot of 200 μl was placed with a sterile Pastor pipette on Petri dishes containing PDA without glucose or Czapek-Dox medium. After incubating for 4 days, several colonies had grown on the surface of the medium. Each colony (except for known saprophytes) was transferred to the same medium and the morphological identification was made in the same manner after incubating for 15 days at 25°C.

Pathogenicity tests

To verify the pathogenicity of isolates, ten detached leaves from an olive tree cv. Meski were inoculated by spraying their undersides with a conidial suspension of *P. cladosporioides* (10^7 conidia ml^{-1}). The inoculum was obtained by scratching a 15-day-old colony on PDA without glucose or Czapek-Dox culture at 25°C, with sterile distilled water. Conidia were harvested by filtration and the concentration was evaluated with the counting glass of Malassez. The desired concentration was obtained by dilutions in sterile distilled water. The inoculated leaves were placed in a humid plastic box and incubated at 25°C. After incubation, the typical symptoms of leaf spot were checked. Control leaves were sprayed with sterile distilled water and incubated separately under the same conditions.

The second technique consisted in spraying leaves of 2-year-old olive trees cv. Meski, with a conidial suspension of *P. cladosporioides* adjusted to 10^7 conidia ml^{-1}. Inoculated trees were covered for 48 hours with plastic film to maintain high relative humidity and were placed in a controlled-environment chamber at 25°C.

To re-isolate the fungus, 15 days after inoculation with *P. cladosporioides*, small pieces of diseased leaves were removed from all trees with a sterile scalpel, plated on PDA without glucose or Czapek-Dox medium, and incubated at 25°C.

Results and discussion

In all the 3 sites surveyed the presence of olive trees showing symptomatic leaves was recorded in some olive trees cv. Meski. The leaves observed during the survey were slightly chlorotic. The upper surface of infected leaves was yellow and some trees showed signs of defoliation. The undersides of the yellow leaves appeared discoloured because of the conidial stage of the fungus covering the leaf as a grey dust (Fig. 2).

Fig. 2. Symptoms of *Pseudocercospora cladosporioides* on the olive leaves.

Isolation and identification of the fungi

The direct transfer of conidia and/or conidiophores from the underside of infected leaves to Petri dishes containing PDA without glucose or Czapek-Dox medium using a sterile scalpel proved to be the quickest, easiest and most effective way to isolate the fungus. However, the fungus grew very slowly on PDA and formed only a few conidia. Colonies obtained from the underside of infected leaves had limited growth, with an olive-brown colour on PDA without glucose after 15 days, indicating the production of conidia (Fig. 3). Microscope inspection revealed that conidia formed singly and were more or less cylindrical, septate (between 2 and 7 septa), with an obtuse apex and an obconically truncate or subtruncate base. Their size varied between 30–65×3–5 μm. By its morphological and cultural characteristics, this fungus was identified as *P. cladosporioides* (Viennot-Bourgin, 1949; Fvaloro, 1970; Avila *et al.*, 2005).

Pure colonies of *P. cladosporioides* were also obtained by the second technique based on the spore dilutions placed on PDA without glucose or Czapek-Dox medium. The 10^{-4} dilution proved to be the best because it gave a small number of pure fungal colonies that were easily purified and identified under the microscope.

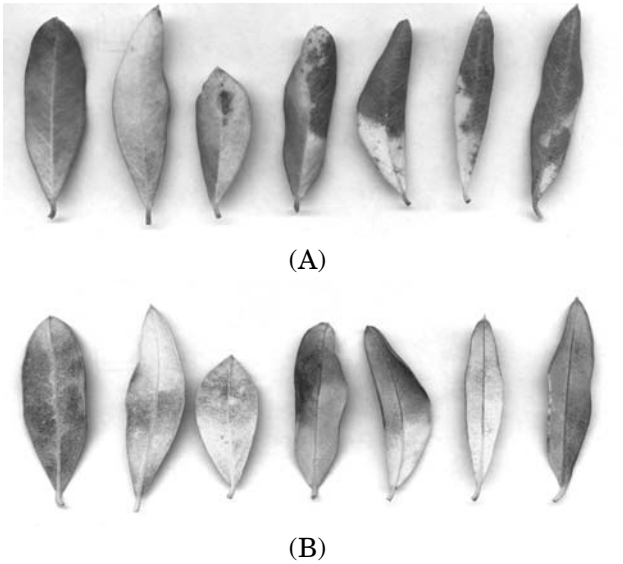
Fig. 3. Appearance of *Pseudocercospora cladosporioides* on PDA medium (without glucose) after incubation for 15 days at 25°C

Pathogenicity tests

After incubation for ten days, most inoculated leaves detached from the olive tree showed the typical symptoms of leaf spot caused by *P. cladosporioides*. Reisolation from these leaves on PDA without glucose or on Czapek-Dox medium produced mycelia and conidia having the same characteristics as the fungus inoculated (Fig. 4), confirming the its identification.

Also, the young olive tree inoculated by spraying a conidial suspension on the leaves showed the same leaf symptoms after 13 days. The underside of the leaves had grey blotches of conidia and the upper part of the leaves was yellow. Almost all the inoculated leaves were infected and some of them later fell.

The pathogen was successfully re-isolated from the



(A)

(B)

Fig. 4. Typical symptoms of Cercospora leaf spot recorded after inoculation for ten days on detached leaves of olive cv. Meski: (A) upper surface (B) lower surface.

infected leaves, and a pure culture of *P. cladosporioides* was obtained, confirming the disease aetiology.

Control of the disease

The occurrence of *P. cladosporioides* on the leaves of olive trees could be of economic importance because fruits can also be infected, causing a lower quality of olive oil due to higher levels of free acidity and peroxide number (Sergeeva, 2008). Nevertheless no fruit symptom was observed in the samples examined.

To control Cercospora leaf spot disease, fixed copper fungicide should be applied as early as possible after harvest and if at all possible before most of the autumn rains (Jardak, *et al*., 2008). A power sprayer with high pressure is necessary to cover the leaves completely on both sides, and also the inside of the crown. In cool, wet areas, preventive treatments should be applied to the olive trees after harvest but before the winter rains begin, and again in spring if wet, rainy weather persists. Copper fungicides were the most effective in controlling both leaf spot and peacock spot (Pappas, 1975, 1993; López-Villatta, 1999; Nigro, 2002).

Therefore, the occurrence of this disease not previously recorded in Tunisia needs to be closely monitored.

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