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Full Length Research Paper

The role of phenolic compounds in the defense of sooty mold of olive leaves (*Olea europea* L.)

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The aim of this study is to demonstrate the role of phenolic compounds in the olive leaves infected by sooty mold. The results show that the presence of sooty mold induces a high production of polyphenols in infected leaves of olive compared to the uninfected ones. The high concentrations of flavonoids and alkaloids in the infected trees suggest that they make the olive tree resistant to this fungi disease. Analyses by high-performance liquid chromatography (HPLC) confirmed the presence of verbascoside acid, oleuropeinacid, caffeic acid and for the first time, tannic acid. These substances are good resistance markers and should help to make efficient strategies for the bio-control of this kind of fungal disease.

Key words: *Olea europea* L., fungi, phenolic compounds, defense, high-performance liquid chromatography (HPLC).

INTRODUCTION

The olive tree, *Olea europea* is in full expansion in many countries. Despite its importance, it faces several diseases that severely affect its tree production (Santos et al., 2013), one of which is sooty mold. It is accepted that sooty mold is a complex of dark-pigmented fungi of several genera, which have been described as non parasitic, saprophytic, and superficial on plants (Reynolds, 1999; Jouraeva et al., 2006). This fungal complex covers both leaf surfaces and small branches, giving a black aspect to the olive tree (Reynolds, 1999).

The black scale insect, *Saissetia oleae* (Olivier) (Hemiptera: Coccidae) excretes honeydew, facilitates the installation and growth of multiple fungi that cover the olive leaves and supports the proliferation of the sooty mold (Passos-Carvalho et al., 2003; Jouraeva et al., 2006). Heavy infestation reduces photosynthetic activity (Haniotakis, 2005) and a consequent alteration of the normal metabolism and physiology of the plant and ultimately its growth (Santos et al., 2013). For example, Passos-Carvalho et al. (2003) mentioned the negative effects of sooty mold on parameters such as photo-synthesis, chlorophyll, and respiration of the olive tree.

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Plants have complex mechanisms to protect themselves against pathogens. Phenolic secon-dary metabolites, which are involved in the special organoleptic properties of oil, have been shown to play a role in the resistance of some olive (*O. europea L.*) varieties to oil autoxidation (Botia et al., 2001). In addition, some reports (Marsilio and Lanza, 1998) have shown that some phenolic substances of olive trees may inhibit the growth of bacteria, such as *Lactobacillus plantarum*, *Leuconosto cmesenteroides* and fungi like *Phytophthora* (Del Rio et al., 2003). Similarly, the phenolic metabolism of the olive tree is considered as a plant-response to the infection caused by *Verticillium dahliae* (Daayf, 1993). Thus, increasing the endogenous levels of these secondary metabolites can improve the resistance properties of the plant and can be used as a natural alternative for preventing plant diseases. Methods for detecting and recognizing phenolic compounds rely mainly on chromatographic separation, using HPLC analyses (El Modafar and El Boustani, 2001) which allow their successful identification.

In Algeria, little is known about the resistance of *O. europea* L. The aim of this work is to isolate the pathogens involved in the sooty mold and detect the phenolic compounds potentially present in the defense of *O. europea* L. with the chemical nature of these compounds using HPLC method.

MATERIALS AND METHODS

Ten randomly selected olive trees of the Sigoise cultivar were sampled in an olive grove located in Tlemcen (in the northeast of Algeria). In each tree, 5 current season branch segments with visible sooty mold coverage and 5 healthy branch segments were detached from the south-facing canopy at about the same elevation (2.0 m). Branches were taken to a growth chamber at 22°C, placed in a container with water, and left overnight to avoid dehydration.

Isolation of the pathogen

To isolate the pathogens involved in the sooty mold disease, leaf samples were treated according to the method of Pinto (2007); they were placed on potato dextrose agar (PDA) medium amended with streptomycin (100 ppm) and incubated at 25°C in the dark, for 7 days.

Yields extraction

The leaves were washed and dried with paper towel; they were cut into approximately 1 cm squares, dried in an oven at 60°C for at least 24 h, crushed and degreased in a soxhlet, before use. All analyses were conducted in triplicate, and the results were based on dry weight per 100 g of sample.

Tannins extraction

Powdered material (100 g) was extracted at 4°C using 500 ml of a mixture of acetone-water (25/45, v/v) for 4 days (Bruneton, 1999). The extracts were filtered under vacuum through filter paper and the acetone was evaporated under reduced pressure. Subsequently, dichloromethane (2 × 25 ml) was used for the extraction of lipids and pigments from the aqueous extracts using a separating funnel. Afterward, the aqueous phase was extracted with 25 ml of ethyl acetate. This process was repeated four times. After filtration, the organic phases (ethyl acetate) containing tannins were recovered and concentrated to dryness under vacuum, using a rotary evaporator. The residue obtained after evaporation was kept at 4°C and used for further investigation.

Flavonoids extraction

A quantity of 10 g of dried material was extracted with 100 ml of methanol and 5 g of calcium carbonate by boiling for 1 h (Danguet and Foucher, 1982). After filtration, through Whatman filter paper, the methanol was evaporated under reduced pressure to eventually give an aqueous extract. Subsequently, the dry extract was recovered with 50 ml of boiling water. The aqueous extract was filtered and subjected to solvent fractionation; firstly with diethyl ether, then ethyl acetate and finally n-butanol, using separating funnel (pyrex). All fractions were concentrated, dried to constant weight in an oven at 45°C and kept at 4°C.

Extraction of alkaloids

An amount of 10 g of dried sample was mixed with 250 ml of HCl 2% and 110 ml of ethyl acetate. After cold soaking (4 °C) for 10 h, the mixture was filtered and basified with NH_4OH. The basic aqueous phase was extracted twice with ethyl acetate until no alkaloid was detected in the aqueous phase. The alkaloid residue was obtained by decantation and evaporation of the organic phase (Bruneton, 1999).

Plant extraction

The dried powder of olive leaves (10 g) was extracted in triplicate, using EtOH (96% v/v) at room temperature, under stirring. The aqueous suspension of the concentrated EtOH extract was evaporated to dryness and used for all investigations (Kukic et al., 2008).

Determination of total phenolic content

The amount of total phenolic content was determined by Folin-Ciocalteu procedure (Singleton and Rossi, 1965). Aliquot (0.1 ml) of each sample extract was transferred into the test tubes and their volumes were made up to 3 ml with distilled water. After addition of 0.5 ml Folin-Ciocalteu reagent and 2 ml of 20% aqueous sodium carbonate, tubes were vortexed and incubated at room temperature under dark condition. The absorbance was recorded after 1h at 650 nm JEN WAY 6405 UV/Vis spectrophotometer. The total phenolic content was calculated as a Pyrocatechol equivalent (mg PE/g DW).

High performance liquid chromatography (HPLC)

Total phenolics analyses on methanolic extract of infected olive leaves were carried out using Jasco HPLC. It consists (Jasco HPLC) of a pump (PU-2089 Plus) and UV detector model UV-2077 with ChromNAV on a XBridge analytical column (RP-C18 : 5 µm, 4.6 x 150 mm) (Waters Inc. USA), having gradient solvent system

Table 1. Gradient solvent composition in HPLC used in total phenolics analyses.

Time (min)	Composition (%)	
	Solvent A (ACN)	Solvent B (H_2O, pH 2.5)
Initial	2.0	98.0
5.00	2.0	98.0
15.00	5.0	95.0
17.00	100.0	0.0
35.00	100.0	0.0
Flow rate (ml/min)	0.7	
Method time	35 min	

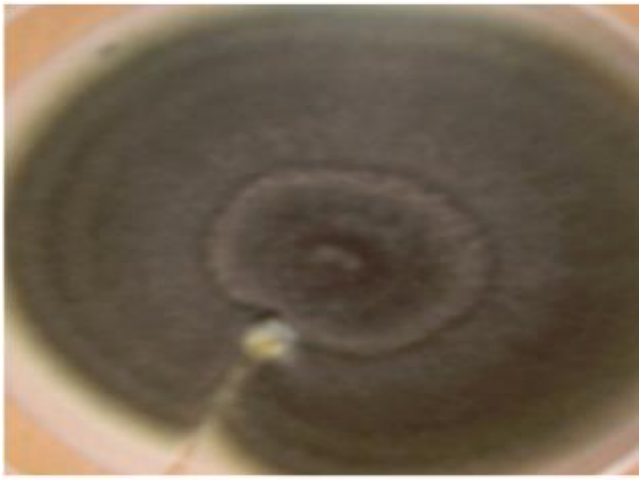

Figure 1. Colony type of *Alternaria* spp.

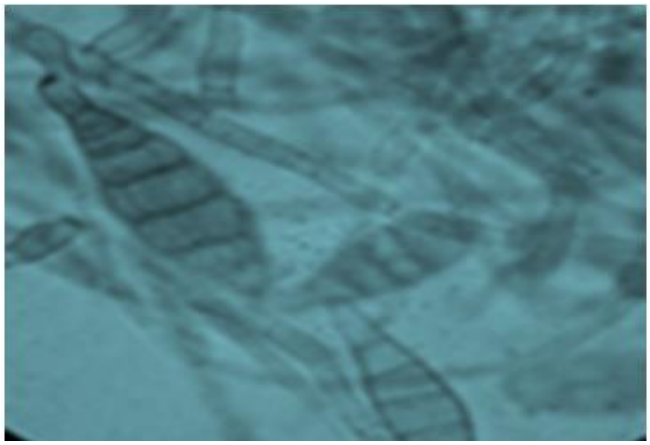

Figure 2. Mycelium and conidiophores of *Alternaria* spp.

and parameter condition as shown in Table 1. The chromatograms were observed at wavelengths of 254, 270, 280 and 329 nm. All the analyses were carried out at sample concentration of 1 mg/ml and injection volume of 20 µl.

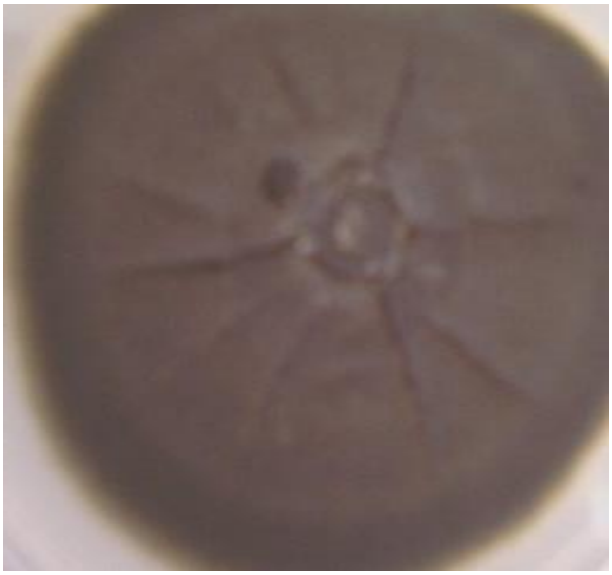

Figure 3. Colony type of *Ulocladium* spp.

RESULTS

Identification of fungi

Leaves with sooty mold showed a dark color covering large areas of both surfaces. The symptoms from infected leaves were very similar to those described for sooty mold of olive tree. Fungal colonies present in our leaves as sooty mold are: *Alternaria* spp. (Figure 1 and 2), *Ulocladium* spp. (Figures 3 and 4) and *Penicillium* spp. (Figures 5 and 6). Mycelia settle on the surface of leaves to form a black film which causes premature aging by suffocation, blocking of photosynthesis and decreasing of gas exchange. It slows growth and leaves a black layer on leaves; it makes xylem to become brown and leaves roll to their inner face, with color changing from yellow to brown. These funguses are responsible for sooty mold in Algeria with the high dispersion of their spores.

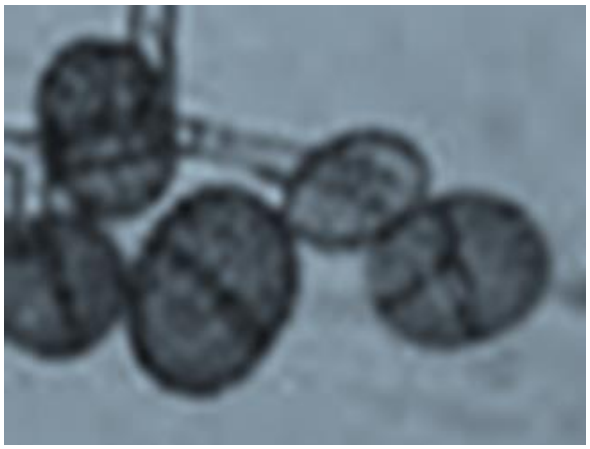

Figure 4. Mycelium and condiophores of *Ulocladium* spp.

Figure 5. Colony type of *Pinicillium* spp.

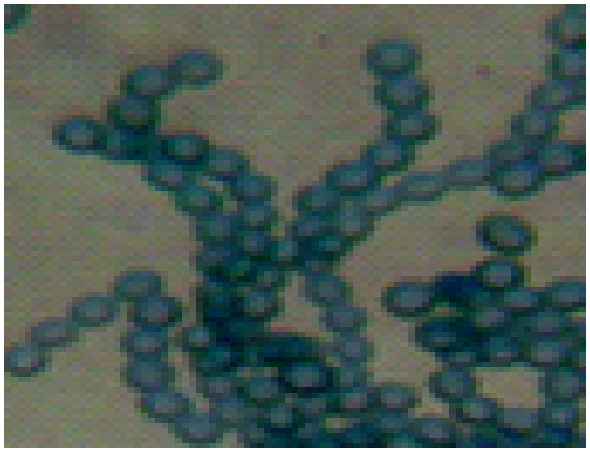

Figure 6. Mycelium and conidiophores of *Penicillium* spp.

Total phenol content

Figure 7 shows the total phenol content in a whole leaf from uninfected and infected olive plants. The total phenol contents in the infected plants (40.8 mg/g) were practically higher than those measured in the uninfected plants (21.7 mg/g).

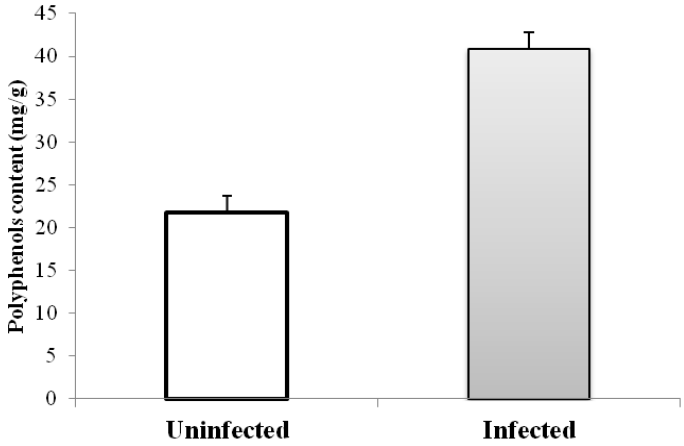

Figure 7. Polyphenols content of uninfected and infected leave of olive.

Yields extraction

The yields of tannins, flavonoids and alkaloids are presented in Figure 8. The yield of tannins in whole leaf from infected and uninfected olive plants was 1.18 and 2.3%, respectively. The yield of flavonoids and alkaloids was higher in infected plants: 4.05 and 2.1% for flavonoids and 2.56 and 1.1% for alkaloids content in uninfected and infected plants, respectively.

Identification of phenolic compounds by HPLC

The data (retention time, λ max in the visible region, and tentative identification) obtained for the phenolic compound peak in the HPLC- analyses are presented in Table 2 and Figure 9. HPLC studies point to four phenolic compounds determined in olive leaves extracts: caffeic (*tR* = 9.850 min, maximum absorbance at 249 nm), Verbascoside (*tR* = 11.419 min, maximum absorbance at 240 nm), tannic (*tR*= 18.41 min, maximum absorbance at 253 nm) and oleuropein (*tR* = 20.06 min, maximum absorbance at 233 nm).

DISCUSSION

The highest numbers of fungal species causing fruit rot of olive are common saprophytes or secondary invaders that normally penetrate through injuries made by biotic or abiotic factors (Lazzizera et al., 2008). However, this is the first report of *Alternaria* spp*., Ulocladium* spp*.* and

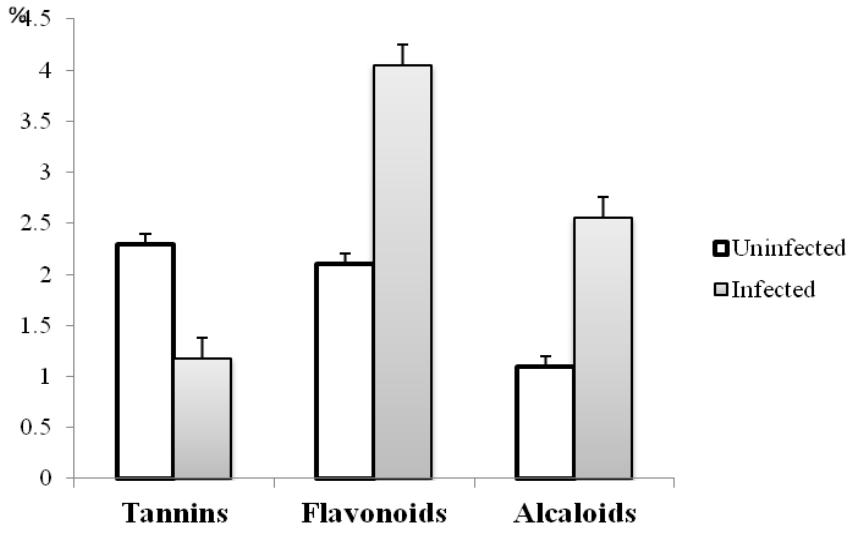

Figure 8. Tannins, flavonoids and alkaloids contents of uninfected and infected leave of olive.

Table 2. Retention time (Rt), wavelengths of maximum absorption in the visible region (λmax) and tentative identification of phenolic compounds in olive leaves.

Peak	Rt (min)	Λmax (nm)	Tentative identification
1	0.650	243	N.D
2	2.022	250	N.D
3	2.538	252	N.D
4	9.850	249	Caffeic
5	11.419	240	Verbascoside
6	18.41	253	Tannin
7	20.06	233	Oleuropein

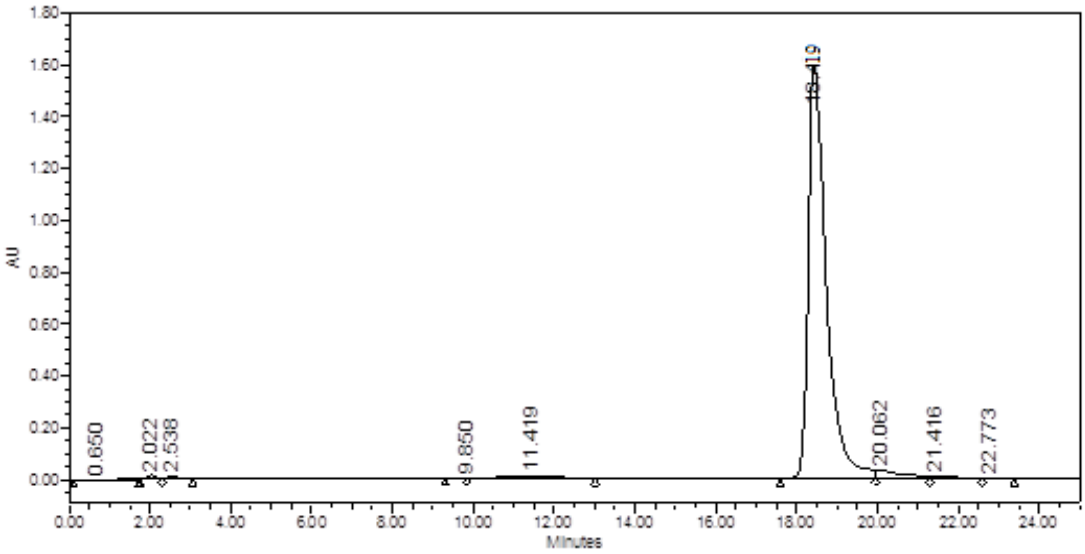

Figure 9. Chromatogram (zoom) recorded at 254 nm showing the phenolic compounds profiles identified and not identified of olive leaves (*Olea europea* var. Sigoise).

Penicillium spp. causing leaf chlorosis and olive fruit rot in Algeria.

The obtained results show that total polyphenols were present in infected olive trees at higher levels than in uninfected olive trees; and the difference was statistically significant ($p< 0.001$). However, the difference in tannin and flavonoid yields was significant ($p < 0.05$).

This is certainly due to the type of analysis which shows that the total polyphenols synthesis was better after infection by the fungi. The total polyphenols content obtained confirms this idea because the total polyphenols analysis gives a quantitative result, whereas the yield gives a qualitative one.

The inoculation of the olive twigs by a conidial suspension of *Verticillium dahlia* resulted in important modifications in flavone and phenol levels (El Boustani et al., 1998). These findings suggest that the first step of the response mechanism to infection in olive plants is a rapid accumulation of phenols at the infection site, thus reducing or slowing the pathogen growth, as reported for other vegetal materials (Del Rio et al., 2004). Therefore, in contrast to flavonoids and alkaloids, the tannin content of the uninfected sample was higher than that of the infected one. Our results are in agreement with those of Corbaz (1990), whose study results show that the young leaves at the cotton plant are often resistant to *V. dahlia* and become sensitive as they grow older. This phenomenon might be ascribed to the inhibition of mycelium growth in young tissues, which contain higher concentrations of substances such as tannins than those in the old leaves.

In selective extractions, those concentrations of alkaloids in infected olive plants were higher than in uninfected ones also suggest that alkaloids may have a role in the response mechanism of olive plants to sooty mold. These results are similar to that found by Bensalah et al. (2014) who found olive infected by *V. dahliae*.

Our main findings were that the HPLC analyses revealed the presence of some phenolic compounds in infected olive leaves, namely verbascoside and tannic acid. Bensalah et al. (2014) found verbascoside for the first time in olive leaves infected by *V. dahliae*. This result confirms that verbascoside compounds have a role in the resistance or defense of olive against fungi attacks. Tannic acid is reported to have a role in the resistance of plant to insects. In this study, we have found that this acid has a role in the defense mechanism to sooty mold. We suggest that this compound have a role or function in the resistance to fungi.

Conclusion

This study strongly suggests that some of phenolic compounds present in olive leaves variety Sigoise play a role in the natural defense mechanism, as it has been established for other phenolic secondary metabolites in different plant materials infected by pathogenic fungi. The HPLC analysis revealed the presence of new phenolic compounds, namely tannin.

Conflict of Interests

The authors have not declared any conflict of interests.

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