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Dysfunctionality of the Xylem in *Olea europaea* L. Plants Associated with the Infection Process by *Verticillium dahliae* Kleb. Role of Phenolic Compounds in Plant Defense Mechanism

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Xylem ultrastructural modification and the possible participation of phenolic compounds in the natural defense or resistance mechanisms of olive plants infected with *Verticillium dahliae* Kleb. were studied. Microscopic study showed that the mycelium propagated and passed from one element to another through the pit. The formation of tyloses and aggregates contributed to obstruction of the xylem lumen. *In vivo* changes in the levels of these phenolic compounds in infected olive plants and their antifungal activity against *Verticillium dahliae* Kleb., as revealed by *in vitro* study, strongly suggest that they are involved in natural defense or resistance mechanims in this plant material, the most active being quercetin and luteolin aglycons, followed by rutin, oleuropein, luteolin-7-glucoside, tyrosol, *p*-coumaric acid, and catechin.

KEYWORDS: ***Verticillium dahliae* Kleb.; *Olea europaea* L.; plant defense mechanism; tyloses; antifungal acitvity; phenolic compounds**

INTRODUCTION

Verticillium dahliae Kleb. causes verticilosis, the most widespread disease to attack olive trees wordwide. It has several consequences, ranging from the limited loss of production on affected branches to the total destruction of the tree (*1*). Unfortunately, the variety Picual, which is the most commonly grown variety in the whole world due to its magnificent agronomic characteristics and the quality of its oil, is very susceptible to *V. dahliae* Kleb. (*2*). This fungus, which can persist in the soil as microsclerotia for more than 20 years, grows parasitically on the tree, while fungicides, besides having potentially toxic consequences and representing an environmental risk, have little effect on it (*3*, *4*).

The phenolic secondary metabolites, which are considered responsible for the special organoleptic properties of the oil and to play a role in the resistance to oil autoxidation, of some *Olea europaea* L. varieties were examined in previous studies (*5*, *6*). In addition, some reports have shown that some phenolic substances of olive trees may inhibit the growth of bacteria, such as *Lactobacillus plantarum* and *Leuconostoc mesenteroides* for example (*7*−*11*), and fungi belonging to the genus *Phytophthora* (*12*, *13*). It is possible, therefore, that increasing the endogenous levels of these secondary metabolites may act as a natural alternative for preventing such diseases.

The objective of this study was to analyze the ultrastructural alterations that occur in plants of *Olea europaea* L. infected by *V. dahliae* Kleb. and to characterize the growth mechanisms of the plant to establish possible physical and/or chemical barriers to prevent the advance of the fungus.

MATERIALS AND METHODS

Plant Material. The study was carried out with 8 year-old infected and noninfected olive trees (*Olea europaea* L. var. Picual) grown in a commercial plantation located in Jaén, Spain.

Chemicals. The standard phenolic compounds, luteolin-7-glucoside, oleuropein, and luteolin, were purchased from Extrasynthèse (Genay, France). Quercetin was purchased from Merck (Darmstadt, Germany). Tyrosol was from Aldrich (Madrid, Spain). Rutin, *p*-coumaric acid, and catechin were from Sigma (St. Louis, MO). Primers used were obtained from Biotools (B & M Labs, Madrid, Spain).

Extraction and Measurement of Phenolic Compounds. The plant materials (cortex plus phloem, and xylem plus pith), collected from the different trees, were mixed and divided, in each case, into three lots. These were ground and extracted with dimethyl sulfoxide (DMSO) (*14*) for 2 h at a ratio of 150 mg of fresh weight/ mL.

The resulting extracts were filtered through a 0.45-μm nylon membrane before analysis by (1) spectrophotometry, using a UNICAM UV−vis spectrometer UV2 (Unicam Limited, UK), to estimate total phenols, expresssed as caffeic acid/100 g FW, by the Folin Ciocalteu Method (*15*); (2) HPLC-MS with an Agilent liquid chromatograph (model 1050) (Agilent Technologies, Palo Alto, CA) coupled to a quaternary pump and automatic injector with a diode array detector (range scanned: 220−500 nm). In the HPLC analysis, the stationary phase was a Sherisorb ODS-2 analytical column with an average particle size of 5 μm (250 mm × 4 mm i.d.), thermostated at 30 °C. The solvent was a mixture of acetonitrile (A) and acetic acid/water (2.5:97.5) (B): 25−95% of A in 50 min.

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Eluent flow rate was 1 mL/min. The absorbance changes were recorded in the UV−vis diode array detector at 280 and 353 nm. The amounts of the principal phenolic compounds were determined from the area given by the integrator, using the response factor of the corresponding standards. The main phenolic compounds in these extracts were collected with a fraction collector (Gilson FC 203B) at the exit of the HPLC column for identification by mass spectrometry (Thermoquest TRACE/MS).

Fungal Cultures and Antifungal Activity of Phenolic Compounds. *Verticillium dahliae* Kleb. was isolated in the laboratory from infected olive trees and also was purchased from DSMZ (Germany). Fungi were cultured on potato dextrose agar (PDA) medium at 25 °C, to serve as inoculum.

The antifungal activity of the main isolated phenolic compounds against *V. dahliae* Kleb. were determined by *in vitro* assay. In each case, a 5 mm disk of culture medium, containing mycelium of the fungus, was placed in PDA culture medium (control) and in the PDA culture medium to which phenolic compounds had been added at different concentrations. In each case, mycelial radial growth was measured and mycelial growth inhibition (%) was calculated with respect to the corresponding control at 350 h.

The inhibition index (IC_{50}) was expressed as the concentration (mg/L) of phenolic compounds required to cause 50% inhibition of radial growth (centimeters). The IC_{50} was determined by linear regression.

DNA Extraction and Amplification. To identity the fungus isolated from infected olive trees, the PCR molecular technique was used. The method described by Milligan (*16*) was used to extract fungus genomic DNA from the stems and roots of 20 olive plants showing external signs of wilt. DNA amplification was carried out using a themocycler (Mastercycler, eppendorf) according to the method used by Mascarello et al. (*17*). The primers used were VMSP1 (5′-CAT AAA AGA CTG CCT ACG CCG-3′) and VMSP2 (5′-AAG GGT ACT CAA ACG GTC AG-3′), which amplify a specific fragment of 140 bp. The PCR products were separated by electrophoresis using a 1.5% agarose gel dyed with ethidium bromide (1.5 μg/mL gel) for 45 min at 120 V. The fragment size was compared with the marker size (Supperladder-low 100 pb Ladder) by visualizing the gel under UV light (TFX 35 M) coupled to a photographic camera to digitize the image (DP 001 FDC).

Scanning and Transmission Electron Microscopy. For microscopic studies, samples of mycelium grown in both PDA media (with and without the phenolic compounds) were fixed with glutaraldehyde (3%) for 4 h at room temperature. The samples were rinsed with buffer and then postfixed with OsO_4 (1%) at 0 °C for 2.5 h.

For scanning microscopy, the samples were dehydrated stepwise using a graded acetone series (10 min per step). The samples were critical point dried using a Balzers liquid CO_2 CPD 020 (Liechtenstein) for 2 h and then mounted onto aluminum stubs using double-sided adhesive tape. Finally, the samples were coated with 20 nm of gold for subsequent observation using a JEOL scanning electron microscope (JSM-6100, Japan) at an accelerating voltage of 15 kV.

In addition, for transmission electron microscopy, the samples were dehydrated stepwise using a graded ethanol series (50 min per step). They were gradually impregnated, starting with pure propylene oxide and finishing with 100% Spurr. The ultrathin sections were obtained with a diamond knife on a Reichert Jung ultramicrotome. The sections were placed on formwar-coated slot grids, stained with uranyl acetate and lead citrate, and examined in a Zeiss EM 109 electron microscope (Carl Zeiss, FRG) at an accelerating voltage of 60 kV.

RESULTS AND DISCUSSION

Ultrastructural Changes of Xylem Vessels in Infected Olive Plants and Identification of the Mycelium by PCR. Scanning electron microscopy showed that the xylematic vessels of infected olive plants were obstructed, perhaps by aggregates or gums made of a polysaccharide-type material (**Figure 1A**). Obstructions were also formed by tyloses as a result of the invasion of the xylem lumen by parenchymatic cells adjacent to the xylem vessel. Tyloses of varying degrees of development were observed interspersed with fungal mycelium (**Figure 1B**). As time advanced, the tyloses increased in size until they completely blocked the xylematic vessels, findings which reflect those observed in vine plants infected by vascular pathogens (*18*).

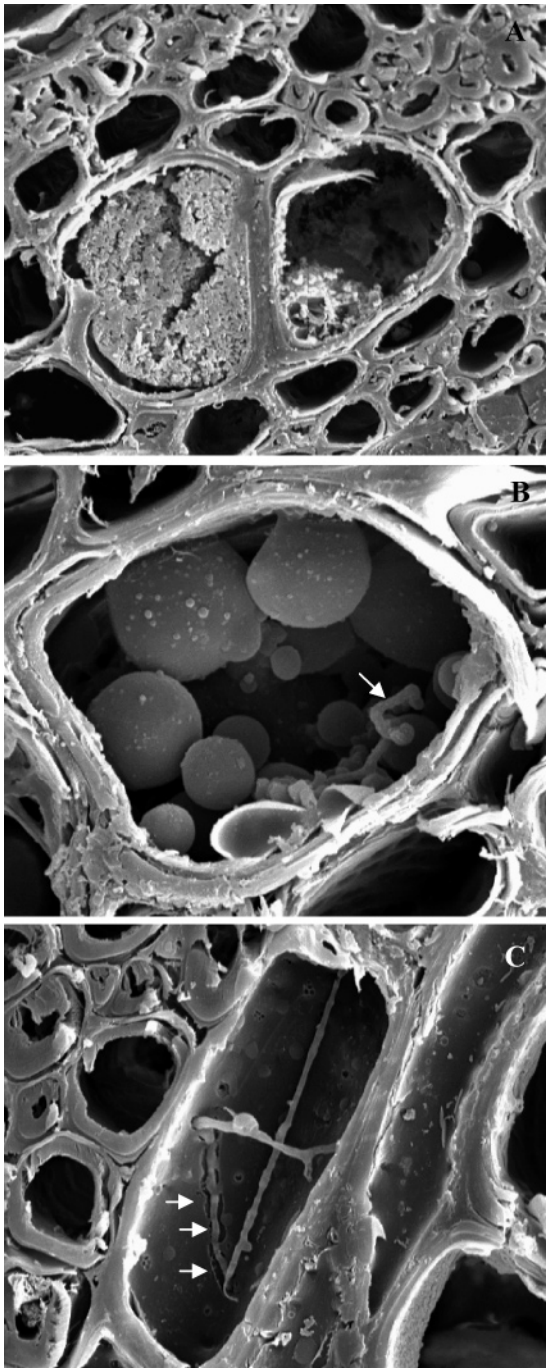

Figure 1. Scanning electron micrographs of cross-section of stem from infected olive var. Picual. (**A**) Completely obstructed xylem vessel next to another partially obstructed (×850). (**B**) Tyloses and hyphae in a xylem vessel (×1900). (**C**) Hypha adhering to wall (×1800).

The mycelium propagated and passed from one element to another through the pit. Occasionally, the adhesion of hyphae to the vessel walls resulted in degradation of the most external material of the vessel wall (**Figure 1C**), perhaps as the result of the secretion of extracellular enzymes on the part of the fungal enzymes since it is known that many phytopathogenic fungi release hydrolytic enzymes that degrade the cell components of the walls of xylematic vessels (*19*−*21*).

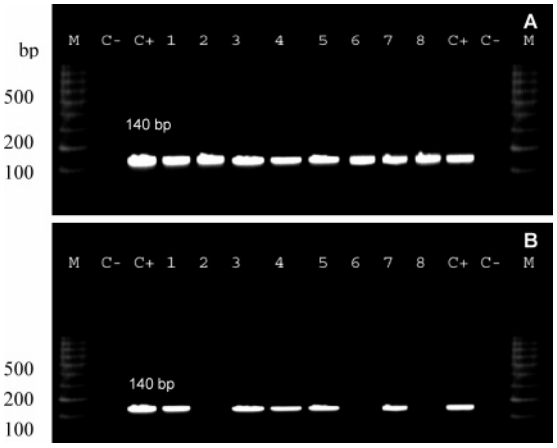

Figure 2. Detection of *Verticillium dahliae* Kleb. in olives var. Picual. (**A**) In stem samples. (**B**) In root samples. M: molecular-weight marker. C–: Negative control. C+: positive control of the fungus. Lanes 1–8: PCR products amplified by VMSP1/VMSP2 primers from fungal genomic DNA present in the plant.

Table 1. Concentration of Total Phenols (Caffeic Acid g/100 g FW) in Whole Stem and Its Tissues from Uninfected and *Verticillium dahliae* Kleb. Infected Olive Plants

	total phenols		
	whole stem	cortex + phloem	xylem + pith
uninfected	2.4 ± 0.2	3.4 ± 0.1	1.1 ± 0.2
infected	5.0 ± 0.3	7.1 ± 0.2	2.5 ± 0.3

[a] The data correspond to mean values ± SE ($n = 3$).

Figure 2 shows the agarose gels in which the DNA fragments amplified by VMSP1/VMSP2 primers, which generate a fragment of 140 bp, can be seen. Amplified fragments of fungus genomic DNA from stem and root samples can be seen in lanes 1 to 8 (**Figures 2A** and **2B**, respectively). The findings after analyzing several samples confirm that *V. dahliae* Kleb. occurs more frequently in the stems (100% of samples infected) than in the roots (60% of samples infected) (data not shown).

Phenolic Compounds Present in Olive Stems. Table 1 shows the total phenol content in whole stem and in both tissues (cortex plus phloem, and xylem plus pith) from uninfected and infected olive plants. The total phenol content of the whole stem of the infected plants was practically double that measured in the uninfected plants. The increase was evident in both tissues, although levels were three times higher in cortex plus phloem that in xylem plus pith.

HPLC studies point to the main phenolic compounds determined in olive stem extracts: oleuropein ($t_R = 23.8$ min, absorbance maximum at 279 nm), luteolin-7-glucoside ($t_R = 19.2$ min, absorbance maxima at 260 and 343 nm), rutin ($t_R = 18.2$ min, absorbance maxima at 260 and 358 nm), *p*-coumaric acid ($t_R = 17.0$ min, absorbance maximum at 322 nm), tyrosol ($t_R = 10.5$ min, absorbance maximum at 277 nm), and catechin ($t_R = 11.0$ min, absorbance maximum at 280 nm). The retention time, absorption spectrum (obtained by means of a UV/vis diode array detector), and mass spectra obtained for these compounds were identical to those obtained for the corresponding standards.

Table 2 shows the levels of these compounds in the different stem tissues (cortex plus phloem, and xylem plus pith) taken from uninfected olive plants. The major compound in both tissues was oleuropein, although the concentrations were higher in the cortex plus phloem (523 mg/100 g FW) than in the xylem plus pith (278 mg/100 g FW). However, the concentration of luteolin-7-glucoside in xylem plus pith (55.8 mg/100 g FW) is more than double that in the cortex plus phloem (20.1 mg/100 g FW), while rutin is only present in xylem plus pith (40 mg/100 g FW). Tyrosol levels are slightly higher in cortex plus phloem (4.2 mg/100 g FW) than in xylem plus pith (2.3 mg/100 g FW). The *p*-cumaric acid and catechin levels cannot be detected in xylem plus pith, but can be detected in the cortex plus phloem concentrations of 3.3 mg/100 g FW and 8.7 mg/100 g FW, respectively. A similar distribution was observed in infected olive plants, although the levels of oleuropein (836 mg/100 g FW in cortex plus phloem and 445 mg/100 g FW in xylem plus pith), rutin (44 mg/100 g FW), and luteolin-7-glucoside (22 mg/100 g FW in cortex plus phloem and 61 mg/100 g FW in xylem plus pith) were significantly higher than in the uninfected plants.

Table 2. Phenolic Compounds (mg/100 g FW) in the Different Stem Tissues of Uninfected Olive Var. Picual[a]

	stem tissues	
phenolic compounds	cortex + phloem	xylem + pith
rutin		40.0 ± 3.8
oleuropein	523 ± 41.0	278 ± 25.0
luteolin-7-glucoside	20.1 ± 0.7	55.8 ± 2.2
p-coumaric acid	3.3 ± 0.4	
tyrosol	4.2 ± 0.6	2.3 ± 0.4
catechin	8.7 ± 0.9	

[a] The data correspond to mean values ± SE ($n = 3$).

Table 3. IC_{50} Values in mg/L of the Different Phenolic Compounds for *Verticillium dahliae* Kleb.[a]

phenolic compounds	IC_{50} (mg/L)
rutin	80 ± 10
quercetin	6 ± 0.4
oleuropein	170 ± 20
luteolin-7-glucoside	260 ± 30
luteolin	7 ± 0.6
tyrosol	660 ± 50
p-coumaric acid	1470 ± 200
catechin	2100 ± 125

[a] The data correspond to mean values ± SE ($n = 3$).

These findings, then, suggest that the first step of the defense mechanism in olive plants responding to infection is a rapid accumulation of phenols at the infection site, which restricts or slows down pathogen growth, as has been suggested by several authors for several different vegetal materials (*18*, *19*, *22*, *23*).

Antifungal Action of Phenolic Compounds Present in Olive Stems against *Verticillium dahliae* Kleb. Table 3 shows that of the different phenolic compounds detected in olive stems, the most toxic for *V. dahliae* Kleb. is rutin, which has an IC_{50} of 80 mg/L, followed by oleuropein, luteolin-7-glucodide, and tyrosol with IC_{50}s of 170, 260, and 660 mg/L, respectively. The compounds showing the lowest antifungal activity were *p*-coumaric acid with an IC_{50} of 1470 mg/L and catechin with an IC_{50} of 2100 mg/L (**Table 3**). Moreover, in vitro experiments carried out after the incorporation of rutin and luteolin-7-glucoside in the corresponding PDA culture medium before inoculation with *V. dahliae* Kleb. revealed the hydrolyzing action of this fungus on these compounds, the appearance of the corresponding aglycons being detected: quercetin and luteolin, respectively (data not shown). When the potential antifungal activity of quercetin, the aglycon of rutin, was studied, the IC_{50} for *V. dahliae* Kleb. was 6 mg/L, meaning its antifungal capacity was on the order of 13 times that of rutin. Similarly,

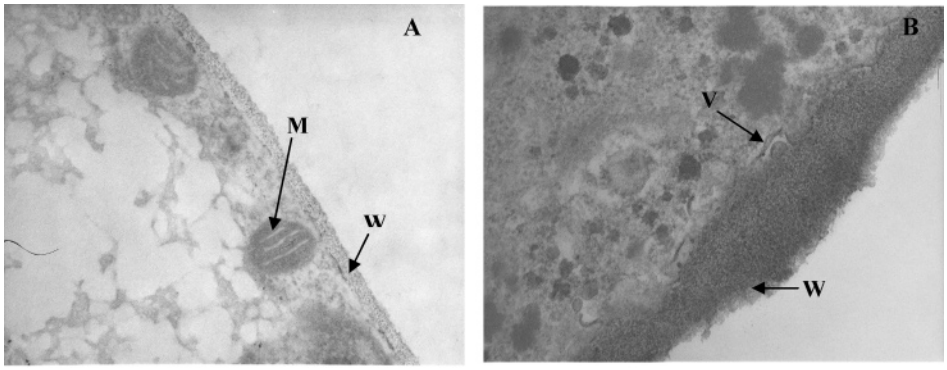

Figure 3. Transmission electron micrographs of the hyphae of *Verticillium dahliae* Kleb. in PDA culture medium (**A**) and in the same PDA culture medium to which luteolin (7 mg/L) had been added (**B**). W, wall; M, mitochondria; V, vesicles. ×40000.

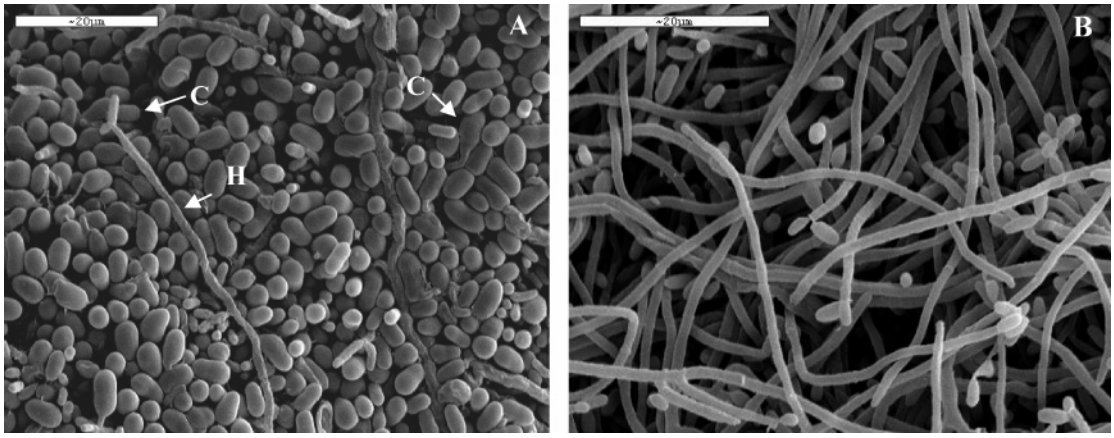

Figure 4. Scanning electron micrographs of the hyphae of *Verticillium dahliae* Kleb. PDA culture medium (**A**, ×1500) and in the same PDA culture medium to which luteolin (7 mg/L) had been added (**B**, ×2000). C, conidia; H, hyphae.

luteolin, the aglycon of luteolin-7-glucoside, showed an IC_{50} for *V. dahliae* Kleb. of 7 mg/L; that is, the aglycon is 37 times more toxic for the fungus than its corresponding glycosylated compound. These findings agree with those observed by several authors in different plant materials infected with a variety of pathogenic fungi, which point to the hydrolyzing action of some of these fungi on the some flavonoid glycosides and the greater fungicidal effect of the aglycons compared with the corresponding glycosides (*23*, *24*).

Besides inhibiting the radial growth of the fungus, these compounds produce ultrastructural and morphological alterations in the fungus, especially in the case of luteolin and quercetin (**Figures 3** and **4**). At the ultrastructural level, the most notable effect of these compounds is on the thickness of the hyphal wall, which increases by about 250%. This is accompanied by an increase in the number of vesicles that deposit their content in the wall (**Figure 3**). The same phenomenon has been observed in *Penicillium digitatum* exposed to different flavonoids (*25*). Another effect observed was the increase in the number and size of mitochondria, which double the dimensions of those present in the fungus hyphae grown in the control medium, implying greater energy generation in reply to the increased metabolic activity necessary for the greater synthesis of material which will form part of the wall. This increase in the number of mitochondria has also been seen by authors after submitting *Fusarium culmorum* to fungicidal treatment (*26*). On the basis of these observations, it can be concluded that in response to the stress produced by these phenolic compounds, the fungus activates the machinery necessary to synthesize the material necessary to form the wall, in order to thicken it and to protect itself from their toxic effect. Morphologically, the most noticeable effect is the much reduced sporulation. In addition, the conidia produced do not mature and therefore do not germinate to create new hyphae (**Figure 4**).

In conclusion, the results obtained in this study strongly suggest that some of the phenolic compounds present in olive act as phytoanticipins and/or phytoalexins in the plant's natural defense mechanisms, as has already been established for other phenolic compounds in different plant materials infected by pathogenic fungi (*18*, *23*, *25*, *27*−*30*).

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