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Changes in Phenolic Composition Induced by *Pseudomonas savastanoi* pv. Savastanoi Infection in Olive Tree: Presence of Large Amounts of Verbascoside in Nodules of Tuberculosis Disease

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A study was carried out to determine the changes in phenolic composition induced by tuberculosis infection in olive trees. Four ethanolic extracts were compared: olive leaf from shoots affected by *Pseudomonas savastanoi* pv. Savastanoi, nodules induced by this bacteria, leaf from healthy (asymptomatic) shoots, and shoots. Among the differences found, the presence of a phenolic compound in nodules was significant in much larger quantities than in leaf or shoots. Mass spectrometric analysis showed this compound to be verbascoside. The enhancement of its biosynthesis could be related to the defense mechanisms of the tree in the nodules induced by *P. savastanoi* and suggests the possibility of exploration of natural and biotechnological sources of this compound.

KEYWORDS: Verbascoside; *Pseudomonas* savastanoi pv. savastanoi; olive tree tuberculosis

INTRODUCTION

It is well known that simple biophenols, or esterified biophenols, are able to provide a natural defense against pathogen attack. Part of the defense mechanisms of the olive tree against pathogens is provided by *seco*-biophenols, responsible for resistance to infection through phytoalexin biosynthesis, controlled by β-glycosidase (*1*), as well as through their local accumulation in the infection sites, after inducible reactions such as cellular wall modification, enzymatic protection, and hypersensitivity reactions (*2*). *Olea europaea* shows high resistance to bacterial attack. In the leaf, two kinds of protection mechanisms could be significant: one is provided by bitter *seco*-iridoid glucosides, particularly oleuropein and ligustroside; the other is a physical barrier of oleanolic acid crystals, coating the leaf surface (*3*). Derivatives of oleuropein can perform multichemical defense bioactivity against microbe attack (*4*), not shown by the parent secoiridoid glucoside (*1*). An aglycone of oleuropein, either the hemiacetal or the corresponding aglycone cleaved enal−aldehyde structure, has been suggested as the antimicrobial active intermediate agent (*3*). More recently, a *seco*-biophenol defense mechanism against pathogenic elicitors has been described (*2*). Moreover, several authors have detected the antibacterial activity in vitro under different assay conditions of olive tree phenolics (*5*−*10*).

Interest in natural antioxidants as bioactive components of foods is increasing, and the antioxidant activity of phenolics extracted from *O. europaea* L. leaf has been proved (*11*−*12*). Indeed, there exists nutritional and medicinal interest in olive phenolic compounds (*13*). Verbascoside (**Figure 1**) has been used in traditional Chinese medicine as purified extract (*14*). Studies in vitro showed for verbascoside antiinflammatory (*15*), antitumor (*16*), antoixidative (*17, 22*), and reactive oxygen species (ROS) scavenging activities as well as being a muscle fatigue retardant (*18*−*21*). Sheng et al. (*22*) also reported that verbascoside may provide a useful therapeutic strategy for treatment of oxidative stress-induced neurodegenerative diseases, such as Parkinson's disease.

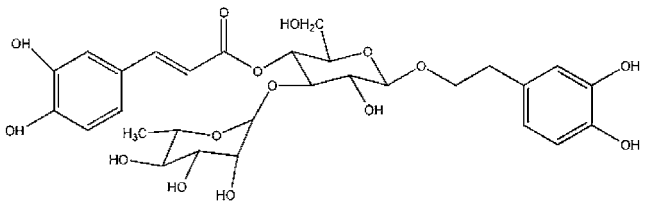

Figure 1. Verbascoside.

Tuberculosis of olive tree (*Pseudomonas savastanoi* pv. *savastanoi*) is a widespread disease, present in all olive growing areas. Affected trees show little vigor, growth reduction, and bitter taste of fruits, which also can be salty or rancid, with a decrease in oil quality. Highly affected shoots grow less, partially loose their leaves, and can die (*23*). The pathological cycle of *P. savastanoi* in olive trees consists of four fundamental phases. The bacteria survive in the nodules until the following season (summer phase). Exudates are produced in the presence

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of free water, which can be washed off by rain drops, dispersing the pathogen. In addition, the bacteria are present on epiphyte phase, resident on the aerial parts of the plant, and able to live and grow without causing infection. Maxima of this epiphyte population in Andalusia (Spain) are in two seasons, around April and November (autumn and spring). During the winter phase, an epiphyte population with reduced infective activity is present on the shoots; although the temperature range which allows infection is 4−38 °C, optimum temperatures are 23−24 °C (*24*). Olive tree varieties immune to tuberculosis disease are not known. Although some are considered less susceptible, such as Spanish cvs. 'Picual' and 'Verdial de Huévar', the information available comes from field observations and there is very little data obtained from comparative inoculation experiments (*25*). Despite the wide spread distribution of this disease, there are no estimations of the harvest losses it can cause (*26*), and it is usually considered that, except in very heavy infection events, its economic importance is small. In this work, the phenolic composition of tuberculosis nodules has been studied to find out possible relations between these compounds and mechanisms of defense of the olive tree against the disease, identifying possible compounds of interest by their biological activity.

MATERIALS AND METHODS

Plant Material, Reagents, and Standards. The olive trees (*O. europaea* L.), cv. Picual, were grown in the experimental orchard of "Instituto de la Grasa", Seville. Samples were taken on November 26, 2004 (M1), April 26, 2005 (M2), and November 30, 2005 (M3). One additional sample, for which analytical results are not given, was taken on July 1, 2005. The autumnal rains to the date of the sampling were 97 and 114 mm, respectively, in 2004 and 2005. Nodules samples were always taken from fresh and active nodules only. Old dry and inactive knots, frequently present in the shoots of infected trees, were not taken. Healthy leaves were taken from asymptomatic shoots, being considered when no nodule was present in any branch from the shoot to the trunk of the tree. Affected leaves were taken from shoots in which active nodules were frequently present.

All reagents were of analytical grade. Acetonitrile (far-UV) and methanol were of HPLC grade (Romil Ltd., Cambridge, U.K). 3,4-Dihydroxycinnamic, betulinic, *o*-coumaric, and *p*-hydroxyphenylacetic acids standards were purchased from Sigma Aldrich (Sigma-Aldrich Química, Madrid, Spain). Folin-Ciacolteu phenol reagent was from Merck, KgaA (Darmstadt, Germany) and molasses alcohol from Alcoholes del Sur (Córdoba, Spain).

Preparation of Olive Leaf and Nodule Extracts. Olive tissues extracts (ALE, affected leaf extracts; HLE, healthy leaf extracts; NE, nodules extracts; ShE, shoot extract) were prepared as described previously (*27*) as soon as possible after sampling. Briefly, whole fresh olive leaves were washed with water, air-dried, macerated in the dark at room temperature with ethanol, with the proportion of leaf to solvent being 1/20 (g/mL), agitated for 30 s on alternating days during the extraction time for 4 weeks, and then passed through a filter sheet. The ethanolic leaves extracts were maintained at 25 °C until analysis. The same procedure was used for preparing ethanolic nodules extracts. The size of the nodules (the greatest dimension of it) used for extraction was 10−17 mm.

Internal Standards and Sample Preparation. Solutions of *p*-hydroxyphenylacetic acid (1 mg/mL), *o*-coumaric acid (0.5 mg/mL), and betulinic acid (1 mg/mL) in methanol were used as internal standards, according to methods previously described (*28*). Two milliliters of ethanolic extracts was concentrated to a syrup consistency (around 250 μL) in a rotary evaporator under vacuum at a temperature below than 40 °C. The syrup was treated with purified water (2 mL) and passed through a 0.45 μm membrane filter (Millipore Co., Bedford, USA), separating a solid fraction containing such compounds as chlorophylls, terpenic alcohol and terpenic acids from the extract. A 500 μL amount of the filtered aqueous solution, containing phenols and flavones, 250 μL of *p*-hydroxyphenylacetic acid, and 250 μL of *o*-coumaric acid standard solutions were added, and an aliquot was analyzed by liquid chromatography.

Table 1. Content of Phenolic Compounds (mg/g f.w.)[a]

	hydroxyt.	X	apigenin 7-glucoside	Y	Z	oleuropein	oleuropein aglycone	total phenols
				ALE				
M1	3.22	1.62	8.80	2.04	7.78	9.22	13.85	15.28
M2	1.61	2.85	1.93	0.49	2.16	2.12	2.83	6.56
M3	3.27	nd	6.09	3.36	7.71	15.50	9.44	17.60
				HLE				
M1	1.97	0.92	4.30	1.46	3.42	5.73	10.81	14.00
M2	2.13	0.42	3.94	0.92	3.69	2.88	76.16	11.49
M3	1.98	nd	7.43	2.89	10.59	13.92	11.86	18.40
				NE				
M1	1.81	79.93	nd	27.08	nd	16.69	nd	25.34
M2	0.69	46.76	nd	11.30	nd	5.18	nd	24.22
M3	nd	83.21	nd	5.68	9.04	14.17	nd	24.42
				ShE				
M3	1.08	nd	nd	1.75	2.34	13.65	nd	

[a] M1, 2004-11-26; M2, 2005-04-26; M3, 2005-11-30. Each reported value is the mean of two samples CV ≤ 5%.

The solid fraction, containing terpenic alcohols and acids, was dissolved in 2 mL of ethanol. A 200 μL amount of this solution was mixed with 200 μL of betulinic acid standard solution, and the resulting mixture was evaporated under vacuum to dryness at room temperature. The residue was treated with 200 μL of silylating reagent and kept at room temperature for at least 10 min. A volume (1 μL) of the final solution was injected into the gas chromatograph. The silylating reagent was prepared by adding 3 mL of hexamethyldisilazane and 1 mL of trimethylchlorosilane to 9 mL of anhydrous pyridine.

Phenols and Flavones Determination by HPLC. Quantification of phenols and flavones from extracts, purified as described previously (*27*), was performed in a Hewlett-Packard liquid chromatographic system (Hewlett-Packard, CA) equipped with a diode array UV detector and a Rheodyne injection valve (20-μL loop). A 250 × 4 mm i.d., 5 μm, Lichrospher 100RP-18 column (Merck, Darmstadt, Germany), maintained at 30 °C, was used. Elution was performed at a flow rate of 1.0 mL/min using as the mobile phase a mixture of water/phosphoric acid (99.5:0.5, v/v) (solvent A) and methanol/acetonitrile (50:50 v/v) (solvent B). The solvent gradient changed according to the following program: from 95% A to 70% in 25 min, 65% in 10 min, 60% in 5 min, 30% in 10 min, and 100% in 5 min, followed by 5 min maintained at 100% B. Detection was set at 280 and 335 nm. Quantification of phenols was carried out at 280 nm using *p*-hydroxyphenylacetic acid as internal standard. Quantification of flavones was done at 335 nm using *o*-coumaric acid as internal standard.

Gas−Liquid Chromatography Analyses of Terpenic Alcohols and Acids. Terpenic alcohols and acids, removed from extracts as described previously (*29*), were determined by GLC using a Hewlett-Packard 5890 Series II gas chromatograph (Hewlett-Packard, CA), equipped with split injection system, flame ionization detector (FID), and 30 m × 0.25 mm i.d., 0.1 mm film, Rtx-65TG Crossbond capillary column coated with 35% dimethyl−65% diphenyl polysiloxane as the stationary phase (Restek Co., Bellefonte, PA). Hydrogen was used as the carrier gas with a linear rate of 20 mL/min. The oven temperature was maintained at 260 °C and that of the injector and detector at 300 °C. Quantification of terpenic alcohols and acids was done using betulinic acid as internal standard.

Colorimetric Determination of Total Phenols. Total phenolic content was tested colorimetrically by the modified Folin−Ciocalteau assay (*30*). The calibration curve was calculated using pure caffeic acid concentrations ranging from 0.94 to 150.0 μg with a regression coefficient of 0.997. Briefly, aliquots (50 μL) of the aqueous solution, as described previously (*27*), were diluted in 7 mL of distilled water and mixed with 1 mL of sodium carbonate solution (20% aqueous Na_2CO_3) and 0.5 mL of Folin−Ciocalteau phenol reagent. The mixture was kept in the dark at room temperature (23−25 °C) for 1 h, and the absorbance was measured at 725 nm against deionized water as blank.

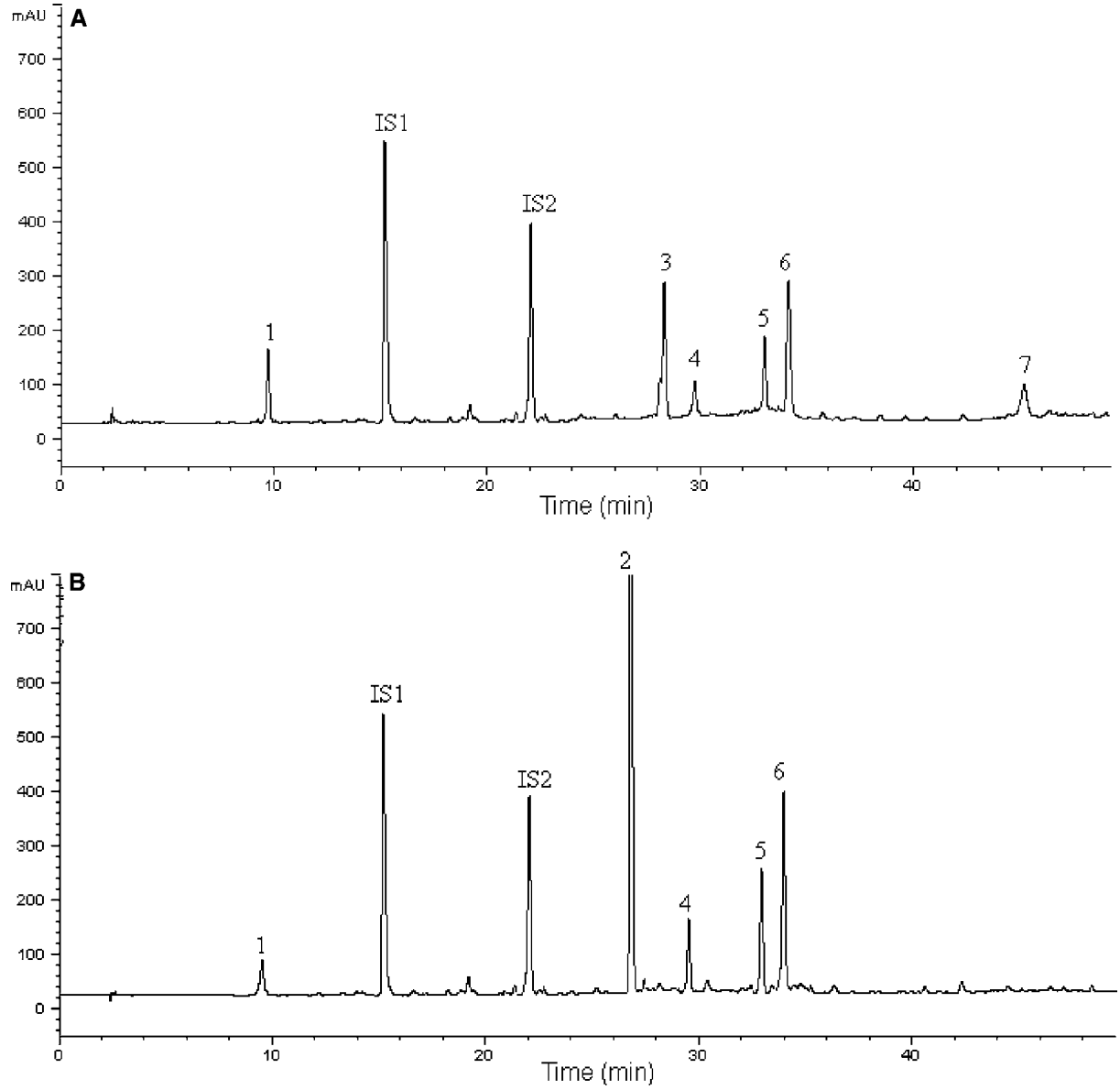

Figure 2. Example of a HPLC chromatogram (280 nm) of the phenolic compounds: (A) healthy leaf extract and (B) nodules extract. IS1, *p*-hydroxyphenylacetic (internal standard); IS2, *o*-coumaric (internal standard); 1, hydroxytyrosol; 2, verbascoside; 3, apigenin-7-glucoside; 4, Y (unidentified peak); 5, Z (unidentified peak); 6, oleuropein; 7, oleuropein aglycone.

Total phenolic content was expressed as micrograms of caffeic acid per milliliter of extract sample.

Identification and Analysis of Phenols and Flavones by HPLC-MS. The HPLC-MS analyses of phenolic extracts were performed in a Beckman Gold system using a model 126 pump with a model 168 diode array detector (Beckman Inc., Ramsey, USA) on-line with a AT95's magnetic sector mass spectrometer (Finnigan Mat, Bremen, Germany) equipped with an APcI ionization interface. A 250 × 4.6 mm i.d., 3 μm Lichrospher 100RP-18 column (Merck, Darmstadt, Germany), maintained at 30 °C, and a Rheodyne injection valve (200 μL loop) were used. Elution was performed at flow rate of 1.0 mL/min using a mixture of water/formic acid (99.9:0.1 v/v) (solvent A) and methanol/acetonitrile/formic acid (50:50:1 v/v) (solvent B) as the mobile phase. The solvent gradient was the same as that described above. Detection of phenols was carried out at 240 and 280 nm simultaneously. A split postcolumn of a 25% of the column flow was introduced in the APcI interfase.

The APcI mass spectra, in negative-ion mode, were obtained under the following conditions: capillary temperature of 220 °C; lens, skimmer, and octapole voltages were set to obtain optimal response for a pattern solution of gramicidine. Nitrogen at 150 Kpa was used as sheath gas. Afterward, partial defocusing of interface parameters was done in order to generate moderate collision-induced dissociation (CID) inside the ionic transport region. Under these conditions, the spectra showed enough ionic fragmentation to confirm or verify structural information from the deprotonated molecular ion.

Table 2. Terpenic Acids (mg/g f.w.)[a]

		erythrodiol	uvaol	oleanolic	ursolic	maslinic
ALE	M1	0.79	1.13	18.18	1.56	3.21
	M2	0.59	0.75	10.36	0.54	1.82
	M3	1.20	1.29	12.18	1.23	2.24
HLE	M1	0.93	1.08	16.29	1.20	2.81
	M2	1.30	1.80	17.04	1.17	3.17
	M3	2.26	3.06	15.12	1.53	2.96
NE	M1	nd	nd	nd	nd	nd
	M2	nd	nd	nd	nd	nd
	M3	nd	nd	nd	nd	nd
ShE	M3	0.39	nd	1.73	nd	0.54

[a] M1, 2004-11-26; M2, 2005-04-26; M3, 2005-11-30. Each reported value is the mean of two samples CV ≤ 5%.

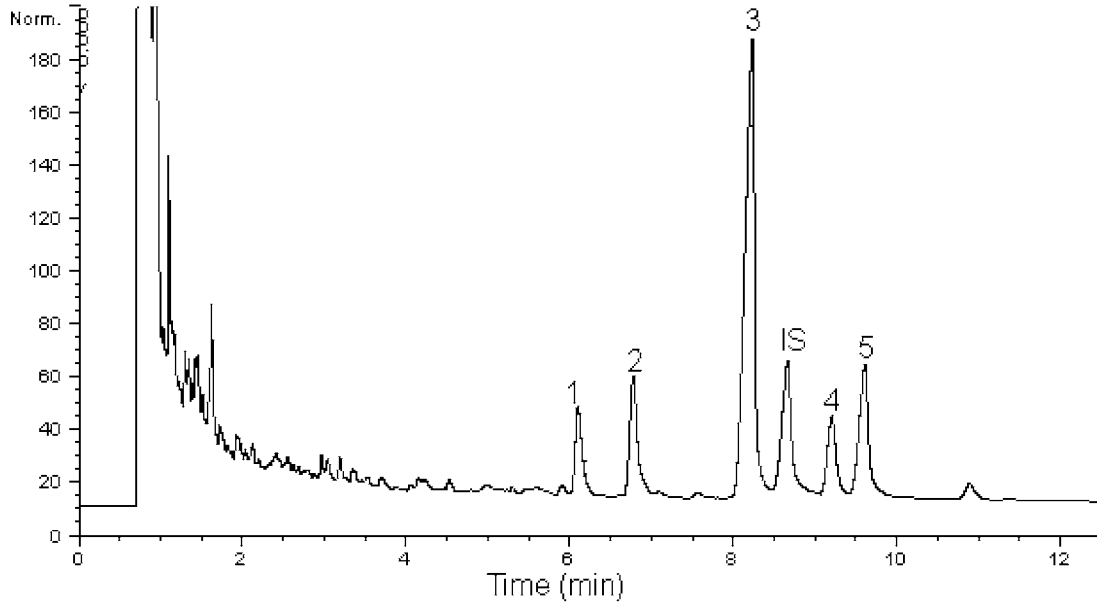

Figure 3. Example of a GC chromatogram of the terpenic compounds (TMS derivatives) from olive healthy leaf extract. Key: IS, betulinic acid (internal standard); 1, erythrodiol; 2, uvaol; 3, oleanolic acid; 4, ursolic acid; 5, maslinic acid.

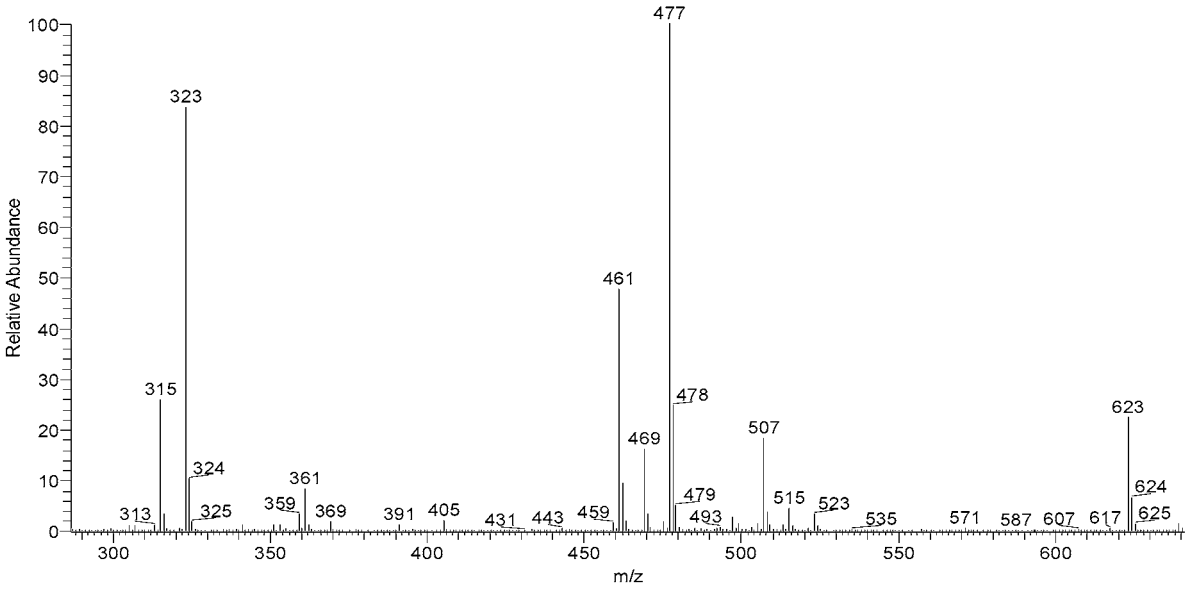

Figure 4. Mass spectrum of verbascoside.

RESULTS AND DISCUSSION

Nodule Extracts. The first and third samples (M1 and M3) (**Table 1**), taken at the end of November, coincident with the autumnal infection stage of the *P. savastanoi* cycle, showed the presence in NE, in higher quantities than in ALE and HLE, of three phenolic compounds X, Y, and oleuropein (**Figure 2**). It can be observed that apigenin-7-glucoside and oleuropein aglycone were not detected in NE. In summer a total inactivity and dryness of nodules was observed (analytical results not given), so the process of loss of activity and decay of nodules before summer is probably the cause of the lower quantities of phenolics detected in the spring samples with regard to the autumn stage sample (M2), when nodules seemed to reach maximum activity in the geographical area of the assay. No terpenic acids were detected in NE of any sample (**Table 2**). The sample of shoot extracts was analyzed to compare its composition with nodule extracts. It should be noticed, interestingly, that in ShE X compound was not present but a large amount of oleuropein was found, which has also been found in several parts throughout the tree in other studies (*31*).

Mass spectrometric analysis (**Figure 4**) revealed that the chromatographic X compound (**Figure 2**) is verbascoside. Probably, it's the same unidentified compound reported before (*32*). As can be seen, it showed the deprotonated molecular ion $[M - H]^-$ at *m*/*z* 623 and a dominant fragment ion at *m*/*z* 477, a result of the neutral loss of a deoxy-hexosyl residue [M − H − 146] from the rhamnose ring, which produced the ion at *m*/*z* 323 after subsequent hydroxytyrosol neutral elimination of 154 amu. The hydroxytyrosyl terminal was confirmed by the presence of an ion at *m*/*z* 469 as a consequence of direct neutral loss of hydroxytyrosol from the deprotonated molecular ion [M − H − 154]. In the same way, the ion at *m*/*z* 461 [M − H − 162] comes from neutral elimination of 162 amu from the caffeoyl residue after rearrangement of the caffeic terminal unit. Mass spectrometric results do not allow, at present, definite identification of Y and Z compounds (**Figure 2**), although these

results could make it reasonable to think that the Y compound could be a verbascoside isomer.

Verbascoside (caffeic acid-glucorhamnose-β-3,4-dihydroxytyrosol, a heterosidic ester of caffeic acid and hydroxytyrosol) was found in both olive fruit and leaf (*33*). It is the main hydroxycinnamic acid derivative of olive fruit (*34*), almost ubiquitous in the Oleaceae family (*31*, *33*−*34*), and distributed in many medicinal plants (*35*). Levels of verbascoside found in olive fruits average around 0.5% of dry weight. Amiot et al. (*38*) found in a study of phenolic contents in 11 olive varieties verbascoside contents from 0.2 to 9.5 mg/g dry weight; Romani et al. (*39*) reported the polyphenolic content in five Tuscany cultivars of olive and found in those verbascoside from 161.4 to 3202.1 mg/kg of olives (fresh weight). Vinha et al. (*34*) found the same compound in 29 different olive cultivars, from 0.7 to 209 mg/kg of fruit dry weight. However, different isomers of verbascoside have been suggested (*40*).

Extracts from Affected and Healthy Leaf. ALE obtained in M1 and M3 showed hydroxytyrosol in higher levels than HLE (**Table 1**). ALE in M2 has a larger amount (approximately seven times) of X compound than HLE; also in M1, X content was almost double that in ALE. Compounds Y and Z did not show a clear trend of difference in ALE and HLE. All samples showed, in both classes of leaf extracts, clearly lower concentrations of Y compound than in nodules, where the quantities found were high. Oleuropein aglycone was present in similar quantities in ALE and HLE. No significant differences were found in the quantities of terpenic acids (**Figure 3**), similar in ALE and HLE in all samples analyzed (**Table 2**); since terpenic acids are present in leaves but not found in nodules, they are probably not involved in important biological roles related to tuberculosis disease.

Olive leaf contents of verbascoside found in this work in healthy leaf (HLE) was 0.92 mg/g, 0.42 mg/g, and none detected (fresh weight) in M1, M2, and M3, respectively. Benavente et al. (*11*) reported the contents in olive leaf of this compound of a little more than 1% of the extracts (absolute content on dry basis).

Total Phenols. Total phenols content (**Table 1**) in NE of all the samples was higher than in ALE and HLE. These results agree with those previously reported by Roussos et al. (*32*), who found that nodules of *P. savastanoi* were rich in phenolic compounds and *o*-diphenols; they also contained higher amounts of oleuropein and another unidentified compound, in contrast to healthy shoots.

An inverse correlation between oleuropein and verbascoside contents in fruits of different olive cultivars has been observed (*34*, *38*, *41*); the higher the oleuropein content, the lower the verbascoside level. It has also been proposed as a possible basis for biochemical characterization of varieties of olive according to their polyphenolic profile, small fruit varieties being characterized by high oleuropein and low verbascoside content and large fruit varieties with a low oleuropein and high verbascoside contents (*38*). In the olive fruits, a rapid reduction in oleuropein and a peak accumulation of verbascoside and flavonols, closely associated with the "green maturation phase", characterized by a reduction in clorophylls and extensive lipid synthesis, has been described; this successive evolution of oleuropein and verbascoside and their biochemical relationship suggested a metabolic relationship between both compounds in the olive (*38*).

Quantities of verbascoside found in tuberculosis nodules in this work were considerably larger than that previously described in olive fruits, exceeding 8% of fresh weight and more than 15% of dry weight. The question of why verbascoside is the most abundant component of the phenolic composition of tuberculosis nodules and why it is present in this tissue at levels of 1 or even 2 orders of magnitude higher than in fruits and leaves, respectively, is interesting. The results obtained, considering the biological properties of the phenolics (*2*), suggest that those detected in larger quantities in the nodules, especially verbascoside, probably are implicated in the defense mechanisms of olive trees against tuberculosis disease. Verbascoside biosynthesis in tuberculosis nodules suggests the possibility of exploration of natural and biotechnological sources of this compound.

ABBREVIATIONS USED

ALE, affected leaf extracts; APcI, atmospheric pressure chemical ionization; HLE, healthy leaf extracts; LDL, low-density lipoprotein; NE, nodule extracts; ROS, reactive oxygen species; ShE, shoot extracts.

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