Phenolic Responses of Resistant and Susceptible Olive Cultivars Induced by Defoliating and Nondefoliating *Verticillium dahliae* Pathotypes

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ABSTRACT

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Verticillium wilt is the most serious olive disease worldwide. The olive-infecting *Verticillium dahliae* pathotypes have been classified as defoliating (D) and nondefoliating (ND), and the disease is mainly controlled in olive orchards by using resistant or tolerant cultivars. Limited information is available about the nature of resistance in most of the olive cultivars. In the present study, the phenolic responses of the susceptible to *V. dahliae* olive cv. Amfissis and the resistant cv. Koroneiki upon D and ND *V. dahliae* infection were monitored in relation to the fungal DNA levels in the vascular tissues with the purpose to explore the defense mechanisms of olive trees against *V. dahliae*. Quantitative polymerase chain reaction revealed that the decrease in symptom severity shown in Koroneiki trees was associated with significant reduction in the growth of both *V. dahliae* pathotypes in the vascular tissues compared with Amfissis. In Koroneiki trees, the levels of *o*-diphenols and verbascoside were positively associated with the DNA levels of the D and ND pathotypes. In addition, a positive association was observed between the levels of verbascoside and the fungal DNA level in Amfissis trees, whereas a negative association was revealed between the fungal DNA level and the total phenols and oleuropein content in both cultivars. The levels of verbascoside were clearly higher in Koroneiki trees compared with Amfissis trees, indicating for the first time in the literature the involvement of verbascoside in the defense mechanism of olive trees against *V. dahliae*.

Verticillium wilt, caused by the soilborne fungus *Verticillium dahliae* Kleb., is one of the most serious diseases affecting olive (*Olea europaea* L.) worldwide (20) and may cause severe losses and plant death (1,39). Microsclerotia, the resting structure of *V. dahliae*, constitute the main potential infective inoculum of the pathogen in the field and persist in the soil for more than 20 years. The use of fungicides, in addition to having potentially toxic consequences and representing an environmental threat, has little effect on them (14,45).

The olive-infecting *V. dahliae* pathotypes have been classified as defoliating (D) and nondefoliating (ND) according to their ability to defoliate the tree (38). The D pathotype is considered to be highly virulent and the ND one to be moderately severe, as has been documented in artificial inoculation studies (26,35,38). Although infections by the D pathotype can be lethal to the plant, olive plants infected by the ND pathotype can show complete remission from symptoms (20,35).

V. dahliae is generally controlled by a combination of preventive measures; among these is the use of resistant cultivars or root stocks (40,41). The use of host resistance is considered to be the most effective and ecologically sound method for managing *V. dahliae* in olive orchards (23). For this purpose, a large number of olive cultivars has been evaluated for resistance to Verticillium wilt (3,9,22,28). In these studies, 'Koroneiki' exhibited a high level of resistance against the D and ND *V. dahliae* pathotypes, showing less disease symptoms and vascular colonization by the pathogen compared with other olive cultivars; implying, in this way, the trigger of defense mechanisms against *V. dahliae* (10,25,27,33).

It is known that phenolic compounds accumulate in plant tissues and are challenged by fungal pathogens (14,35,43). Phenolics play an important role in the resistance of plants to pathogen attack because they belong to the antimicrobial defense arsenal (37,42,44). It has been shown that some phenolic substances of olive trees may inhibit the growth of fungi belonging to the genus *Phytophthora* (11,12). Furthermore, Cayuela et al. (7) reported enhanced levels of verbascoside in olive shoots infected by *Pseudomonas savastanoi* pv. *savastanoi* and Baidez et al. (4) reported the antifungal activity of oleuropein (an *o*-diphenol present in olive

trees) against *V. dahliae* in vitro. In the same study, a higher accumulation of total phenols was observed in the stems of *V. dahliae*-infected olive cv. Picual trees compared with the noninfected; however, these data were not correlated with symptom development and the actual time of pathogen invasion in the olive trees.

On the other hand, in the few published studies (3,22,24,26,28) about the colonization pattern of the D and ND pathotypes in resistant and susceptible olive cultivars, the biochemical mechanisms involved in these interactions were not investigated. Nicholson and Hammerschmidt (29) have remarked that future research on defense responses should address the time, location, and concentration of phenols relative to the sequential development of stages of disease that lead ultimately to the resistance expression. Taken together, the aforementioned studies (3,4,7,22–24,26–28) conclude that, even if *V. dahliae* is a devastating pathogen for olive trees, limited information is available in the literature correlating the biochemical aspects of this plant–pathogen interaction with the symptom development and rate of colonization of the D and ND pathotypes in the susceptible and resistant olive cultivars.

In view of all the above, the main objective of this study was to investigate the involvement of phenolic compounds in the defense mechanisms underlying the observed resistance in Koroneiki compared with the susceptible 'Amfissis', in relation to the root colonization rate of the D and ND *V. dahliae* pathotypes and symptom development.

MATERIALS AND METHODS

Plant material. Plant material consisted of 3-month-old rooted cuttings of Amfissis and Koroneiki olive trees. Amfissis is susceptible to D and ND *V. dahliae* pathotypes while Koroneiki is resistant to both pathotypes (3,24,26,27,32).

***V. dahliae* isolates.** *V. dahliae* isolates PA and A6, representatives of the ND and D pathotypes, respectively, were used. These isolates have been characterized by biological and molecular pathotyping in a previous study (26).

Inoculum preparation. Microsclerotia of the PA (ND) *V. dahliae* strain were prepared in sucrose sodium nitrate (SSN) liquid culture according to Antoniou et al.

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(3). A6 (D) strain microsclerotia were prepared in minimal medium (MM) agar (33) according to Markakis et al. (26).

Olive-*V. dahliae* bioassay. Olive trees (3 months old) of each of the two cultivars were transplanted in plastic pots containing 7 liters of soil infested with D or ND *V. dahliae* isolate at 40 microsclerotia g⁻¹. Ten trees per cultivar and *V. dahliae* pathotype were destructively sampled at 15, 35, 55, 75, and 95 days after transplanting, for quantitative polymerase chain reaction (qPCR) quantification of the pathogen, determination of the total phenol and total *o*-diphenol content, and individual phenolic compounds in roots. In all, 10 trees per cultivar were retained for recording symptom development and 10 trees per cultivar were transplanted to plastic pots containing sterilized soil and kept as negative controls. The olive trees were placed at 23 ± 5°C with a 16-h light and dark cycle and neither herbicides nor pesticides were applied. The experiment was repeated three times in a greenhouse during spring.

Disease assessment. Verticillium wilt symptoms were recorded every week for 95 days after plant transplanting to infested soil. The disease severity index of each shoot was based on an arbitrary scale from 0 to 4, where 0 = healthy shoot, 1 = dull green leaves, 2 = internally rolled leaves, 3 = necrotic leaves, and 4 = defoliated and dead shoot (2).

The percentage of disease index was calculated from the disease rating by the formula disease severity index (%) = [Σ(rating score × no. of shoots in the rating class)/(total no. of shoots × highest rating)] × 100%.

Disease ratings were plotted over time to generate disease progress curves. Subsequently, the area under the disease progress curve (AUDPC) was calculated by the trapezoidal integration method (6). Disease was expressed as a percentage of the maximum possible area with reference to the maximum value potential reached over the whole period of the experiment and is referred to as relative AUDPC.

Pathogen quantification. Root tissues from the D and ND *V. dahliae* -inoculated trees were collected for DNA extraction. For each sampled plant, 20 randomly selected 8- to 15-mm-long root pieces (half of the root tissues) were collected. Bark was removed from the tissues and, subsequently, tissues were freeze dried and ground to a fine powder by using an autoclaved mortar and pestle in the presence of liquid nitrogen. Total DNA was isolated according to Dellaporta et al. (10), with the appropriate modifications (incubation time at 65°C was increased to 1 h) and was quantified by spectrophotometry and agarose gel electrophoresis (1% agarose in 1× Tris-acetate-EDTA buffer, stained with ethidium bromide).

qPCR assays for quantification of D and ND *V. dahliae* DNA were conducted using

the primer pair internal transcribed spacer (ITS)1-F 5'-CCGCCGGTCCATCAGTCTCTCTGTTTATAC-3' and ITS2-R 5'-CGCCTGCGGGACTCCGATGCGAGCTGTAAC-3', designed on the ITS1 and ITS2 regions of the 5.8S ribosomal RNA gene (GenBank accession no. Z29511), generating a 347-bp amplicon (26). qPCR was performed in a Stratagene Mx3005P thermocycler and, for the amplification reactions, the TAQuarate GREEN Real-Time PCR MasterMix (Epicentre, Madison, WI) was used. The results were analyzed with the MxPro qPCR software. Levels of the *O. europea* L. actin gene (designed on GenBank accession no. AY788899), amplified using the primer pair OeACT-F 5'-ATCCTCAGAGCGTGG-3' and OeACT-R 5'-CGATCATTGAAGGCTGG-3', generating a 229-bp amplicon, were used as internal standards to normalize differences in DNA template amounts (26). PCR cycling started with an initial step of denaturation at 94°C for 2 min, followed by 40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 30 s. The primer specificity and the formation of primer-dimers were monitored by dissociation curve analysis. It was also verified that the ITS and actin primers do not amplify plant or fungal DNA, respectively. All qPCR reactions were performed as duplicates. To quantify the DNA relative amount of *V. dahliae* isolates, a PCR product of the primer pair ITS1-F/ITS2-R was cloned into the pGEM-Teasy (Promega Corp., Madison, WI) vector and eight 10-fold dilutions of the plasmid, ranging from 2.7 × 10⁹ to 2.7 × 10² molecules, were used to generate a standard curve ($r^2 \geq 0.99$; Fig. 1) (26,31).

Extraction of phenolic compounds.

Root tissues from the D and ND *V. dahliae*-inoculated trees were collected for phenolic compound extraction. For each sampled plant, 20 randomly selected 8- to 15-mm-long root pieces (half of the root tissues) were collected. The tissues were freeze dried and ground into powder by a mill (IKA, MF 10 basic), and the dry powder was stored at -80°C until the analysis. The extraction of phenolic compounds was accomplished by the use of 75% (vol/vol) ethanol in water. Approximately 100 mg of dried tissue was extracted with 2 ml of extraction solution in a water bath at 40°C for 30 min. The samples were then centrifuged at 4,000 × g for 6 min, the supernatant was retrieved, and the pellet was reextracted with another 2 ml of extraction solution, repeating the same procedure. The two supernatants were combined and used for the analysis of total phenol content, total *o*-diphenol content, and individual phenolic compounds by high-performance liquid chromatography (HPLC).

Colorimetric determination of total phenols. Colorimetric determination of total phenols was based on the procedure of Folin-Ciocalteu as described by Waterman and Mole (39) and results are expressed as milligrams of tannic acid equivalents per gram dry weight. There were two replications per assay of sample.

Determination of *o*-diphenols. Spectrophotometric determination of *o*-diphenols was based on the procedure of Gutfinger (17) and results were expressed as milligrams of caffeic acid equivalents per

Fig. 1. Standard curve obtained from plotting the log of known DNA concentrations (10 ng to 1 fg of DNA), generated from cloned (into the pGEM-Teasy vector) polymerase chain reaction (PCR) products of the primer pair *Verticillium dahliae* internal transcribed spacer (ITS)1-F/ITS2-R, against the cycle threshold (Ct) values obtained from quantitative PCR. This curve served to calculate the relative amount of *V. dahliae* DNA in total genomic DNA samples extracted from infected olive tissues. Vertical bars indicate standard deviation.

gram dry weight. There were two replications per assay of sample.

Individual phenolic compounds. The ethanolic supernatants were filtered through a nylon syringe filter (25 mm, 0.22- μ m pore size). Analysis was performed with a Hewlett-Packard 1050 HPLC instrument equipped with a UV-Vis detector on a Lichrospher RP-18 column (4.6 by 250 mm) at 28°C while phenolic compounds were detected at 280 nm. The mobile phase for the HPLC analysis consisted of 15% acetonitrile, 2% methanol, and 1% formic acid in water. The phenolic compounds were eluted from the column with a timetable flow rate as follows: from 0 to 5 min, flow rate of 0.2 ml min⁻¹, 5.5 to 15 min, flow rate of 0.5 ml min⁻¹; 15.5 to 22 min, flow rate of 1 ml min⁻¹; and 23 min and thereafter, flow rate of 1.8 ml min⁻¹. The two phenolic compounds with major participation in the HPLC phenolic profile of olive tissues were determined to be verbascoside and oleuropein, based on their retention times, co-elution with authentic standards, and absorbance spectra. Each sample was assayed twice.

Statistics. Analysis of variance (ANOVA) was used to determine the effects of replication (1, 2, or 3), *V. dahliae* pathotype (PA or A6), cultivar (Koroneiki or Amfissis), sampling interval (15, 35, 55, 75, or 95 days post inoculation [dpi]), and their interactions on disease severity, AUDPC, DNA level of the ND and D *V. dahliae* pathotypes, total phenols, *o*-diphenols, and oleuropein and verbascoside content (Table 1). ANOVA was performed using the software SPSS 10.0 for Windows (Microsoft Inc., Evanston, IL) and *F* values with *P* < 0.05 were considered significant. Prior to ANOVA, homogeneity of variance across treatments was evaluated and an arcsine transformation was needed to normalize variance. Data on relative AUDPC were subjected to means separation by Tukey's highly significant difference test.

RESULTS

Symptom development and quantification of the D and ND *V. dahliae* pathotypes. ANOVA revealed that the interaction of all the experimental factors (number of experimental repetitions, cultivar, *V. dahliae* pathotype, and harvest interval) affected disease severity, AUDPC, and the *V. dahliae* DNA level in the vascular tissues significantly (Table 1). The colonization pattern of the D and ND pathotypes in the roots of Amfissis and Koroneiki trees, along with symptom development, was monitored by real-time qPCR at 15, 35, 55, 75, and 95 days after transplanting into the *V. dahliae* microsclerotia-infested soil. Disease ratings and fungal biomass were plotted over time to investigate the relationship between the fungal colonization and the symptom ex-

pression in each cultivar–pathotype combination. Overall qPCR analysis showed a higher D and ND DNA relative amount in the tissues of Amfissis compared with Koroneiki; also, a higher DNA level of pathotype D than ND was observed in Amfissis trees (Fig. 2).

The D pathotype was first detected at 35 dpi in Amfissis, when the first symptoms were also observed (Fig. 2). The disease severity index and the DNA relative amount of the D pathotype in the root tissues of Amfissis trees were increased steadily until day 75 and then, at day 95, the relative fungal DNA amount was rapidly reduced, whereas the disease severity index was further increased. On the other hand, the ND pathotype was first detected in Amfissis at 55 dpi, when disease symptoms had already appeared. The maximum

Fig. 2. Verticillium wilt disease severity index on ‘Amfissis’ and ‘Koroneiki’ in relation to the relative DNA amount of the defoliating (D) or nondefoliating (ND) *Verticillium dahliae* pathotypes in the roots of both cultivars (molecules $\times 10^5$ per 100 ng total DNA), sampled at 15, 35, 55, 75, and 95 days after transplanting (d.p.i.) to the microsclerotia-infested soil. Fungal DNA levels were estimated by quantitative polymerase chain reaction using total plant DNA isolated from 10 plants per treatment. The experiment was repeated three times (Rep 1, 2, and 3). Vertical bars indicate standard errors.

Table 1. Analysis of variance for disease severity, area under the disease progress curve (AUDPC), DNA level of the nondefoliating (ND) and defoliating (D) *Verticillium dahliae* pathotypes, total phenols, *o*-diphenols, oleuropein, and verbascoside for Amfissis and Koroneiki cultivars (C) inoculated by the ND and D *V. dahliae* pathotypes (P), five harvest intervals (T), and three repetitions (R)

Source	df	<i>F</i> values ^a						
		Disease severity	AUDPC	DNA level	Total phenols	<i>o</i> -Diphenols	Oleuropein	Verbascoide
R	2	0.084	0.046	0.258	0.630	0.510	0.009	0.414
C	1	99.510***	77.891***	98.349***	41.753***	0.389	171.680***	94.793***
P	1	25.643***	30.696***	36.020***	2.050	2.665	0.233	12.185**
T	4	15.610***	21.220***	34.938***	111.455***	9.417***	94.740***	58.995***
R $\times$ C	5	19.907***	15.566***	19.836***	8.680***	1.930	34.133***	19.177***
R $\times$ P	5	5.149***	6.145***	7.421***	0.680	0.851	0.119	2.645*
R $\times$ T	14	4.453***	6.033***	9.932***	32.166***	3.211***	26.803***	16.922***
C $\times$ P	3	51.865***	49.212***	69.541***	20.890***	2.184	63.064***	52.138***
C $\times$ T	9	25.722***	30.511***	63.920***	100.223***	19.455***	147.373***	55.664***
P $\times$ T	9	11.075***	16.214***	30.187***	52.388***	4.775***	42.255***	29.366***
R $\times$ C $\times$ P	11	14.088***	13.359***	19.092***	5.818***	1.570	17.044***	14.346***
R $\times$ C $\times$ T	29	7.884***	9.335***	19.465***	31.090***	7.873***	44.872***	17.167***
R $\times$ P $\times$ T	29	3.402***	4.962***	9.226***	16.272***	1.881**	12.846***	9.015***
C $\times$ P $\times$ T	19	16.885***	23.062***	97.309***	64.957***	10.179***	83.223***	39.145***
R $\times$ C $\times$ P $\times$ T	59	5.283***	7.195***	30.572***	20.551***	4.309***	25.854***	12.290***

^a Symbols: *, **, and *** indicate significance at the *P* < 0.05, 0.01, and 0.001 levels, respectively, according to the *F* test.

ND DNA level was detected at 75 dpi and then, at day 95, it was reduced, despite further increase in the disease severity index (Fig. 2). In Koroneiki, the D and ND pathotypes were first detected on day 55. The relative DNA amount of the pathotype ND was further increased on day 75 and then was almost diminished, while the relative DNA amount of the pathotype D was almost zero since day 75. The first Verticillium wilt symptoms on the D- and ND-infested Koroneiki trees appeared on day 95.

In addition, correlation analysis revealed a strong positive correlation ($df = 28$, $r = 0.71$ and 0.92 , $P \leq 0.05$, for the D and ND pathotype, respectively) between disease severity and the levels of the D and ND pathotypes in Amfissis. On the other hand, a significant correlation was not observed between the DNA level of the two pathotypes and the disease severity in Koroneiki.

Furthermore, the relative AUDPC analysis demonstrated that symptom development in Koroneiki was significantly lower than in Amfissis, proving its resistance against the ND and D pathotype. The D isolate proved to be more severe than the ND one in Amfissis. On the other hand, Koroneiki showed the same level of resistance for both pathotypes.

Phenolic compounds quantification. ANOVA revealed that the interaction of all the experimental factors (number of experimental repetitions, cultivar, *V. dahliae* pathotype, and harvest interval) affected total phenols, *o*-diphenols, and oleuropein and verbascoside content significantly (Table 1). The concentration of total phenols in the roots of the D- and ND-infested Amfissis trees steadily decreased from 15 to 75 dpi and sharply increased on day 95 (Fig. 3). In the same time period, the DNA level of the D and ND pathotypes demonstrated a trend line pattern opposite to the concentration of total phenols. Indeed, correlation analysis revealed a strongly negative correlation ($df = 28$, $r = -0.87$ and -0.79 , $P \leq 0.05$ for the D and ND pathotype, respectively) between total phenol content and the DNA level of the D and ND pathotypes in Amfissis. On the other hand, a significant correlation was not observed between the DNA level of the two pathotypes and the total phenol content in Koroneiki, although a decrease in the content of total phenols was observed during the colonization process of the roots by both pathotypes.

The concentration of *o*-diphenols in the roots of the D- and ND-infested Amfissis trees steadily decreased from 15 to 75 dpi and sharply increased on day 95, as was also observed for total phenols (Fig. 4). The amount of these compounds in the tolerant cultivar was clearly higher than in the susceptible one for most of the sampling times. In the case of the D *V. dahliae* pathotype treatment, the concentration of

o-diphenols in tissues of Koroneiki was 17, 36, and 48% higher than those of Amfissis at 35, 55, and 75 dpi, respectively (Fig. 4). In the case of the ND *V. dahliae* treatment, the concentration of *o*-diphenols in tissues of Koroneiki was 2, 7, and 38% higher than those of Amfissis at 35, 55, and 75 dpi, respectively (Fig. 4). The correlation analysis between the *o*-diphenol content and the relative *V. dahliae* DNA quantity of the D and ND pathotypes showed a strong negative correlation in Amfissis ($df = 28$, r

$= -0.81$ and -0.59 , $P \leq 0.05$ for the D and ND pathotype, respectively). On the other hand, a significantly positive correlation was observed between the relative DNA quantity of both pathotypes and the *o*-diphenols content in Koroneiki ($df = 28$, $r = 0.54$ and 0.74 , $P \leq 0.05$ for the D and ND pathotype, respectively).

HPLC analysis showed the presence of two main phenolic compounds in the olive root extracts of both cultivars during the root infection process by both *V. dahl-*

Fig. 3. Time course formation of total phenols (milligrams of tannic acid equivalents per gram dry weight of plant tissue) in relation to the relative DNA amount of the defoliating (D) or nondefoliating (ND) *Verticillium dahliae* pathotypes in the roots of 'Amfissis' and 'Koroneiki' (molecules $\times 10^5$ per 100 ng total DNA), sampled at 15, 35, 55, 75, and 95 days after transplanting (d.p.i.) to the microsclerotia-infested soil. Fungal DNA levels were estimated by quantitative polymerase chain reaction using total plant DNA isolated from 10 plants per treatment. The experiment was repeated three times (Rep 1, 2, and 3). Vertical bars indicate standard errors.

Fig. 4. Time course formation of *o*-diphenol content (milligrams of caffeic acid equivalents per gram dry weight of plant tissue) in relation to the relative DNA amount of the defoliating (D) or nondefoliating (ND) *Verticillium dahliae* pathotypes in the roots of 'Amfissis' and 'Koroneiki' (molecules $\times 10^5$ per 100 ng total DNA), sampled at 15, 35, 55, 75, and 95 days after transplanting (d.p.i.) to the microsclerotia-infested soil. Fungal DNA levels were estimated by quantitative polymerase chain reaction using total plant DNA isolated from 10 plants per treatment. The experiment was repeated three times (Rep 1, 2, and 3). Vertical bars indicate standard errors.

iae pathotypes (Fig. 5). One of the two compounds was identified as oleuropein ($t_R = 28.10$ min, absorbance maximum at 279 to 280 nm) and the other as verbascoside ($t_R = 21.10$ min, absorbance maximum at 329 nm). The retention time and absorption spectrum were identical to those obtained for the corresponding standards.

The variation pattern of oleuropein content in the roots of both cultivars showed high similarity with that of total phenols content over time in each cultivar-pathotype combination (Figs. 3 and 6), and a strong positive association was revealed after correlation analysis ($df = 28$, $r = 0.97$, $P \leq 0.05$, for both pathotypes in Amfissis; $df = 28$, $r = 0.98$ and 0.70 , $P \leq 0.05$, for the D and ND pathotype, respectively, in Koroneiki). HPLC analyses revealed a rapid reduction of the oleuropein content in roots of the D- and ND-infected Amfissis trees from 15 to 75 dpi, followed by an increase on day 95. On the other hand, the oleuropein content in the tissues of Koroneiki gradually decreased from 15 to 55 dpi and then increased from 55 to 95 dpi (Fig. 6). Overall, a negative correlation was observed between the relative DNA quantity of both pathotypes and the oleuropein content in Koroneiki ($df = 28$, $r = -0.43$ and -0.73 , $P \leq 0.05$, for the D and ND pathotype, respectively) and Amfissis ($df = 28$, $r = -0.93$ and -0.79 , $P \leq 0.05$, for the D and ND pathotype, respectively).

Interestingly, a remarkable increase in the content of verbascoside in the roots of Koroneiki was revealed by HPLC analyses (Fig. 7). The amount of that compound in Koroneiki was clearly higher than in Amfissis in most of the sampling times. In the case of the D *V. dahliae* pathotype treatment, the concentration of verbascoside in

the tissues of Koroneiki was 58, 83, 158, 73, and 78% higher than in those of Amfissis at 15, 35, 55, 75, and 95 dpi, respectively, whereas, in the ND *V. dahliae* treatment, the concentration of verbascoside in the tissues of Koroneiki was 60, 7, and 25% higher than in those of Amfissis at 35, 55, and 75 dpi, respectively (Fig. 7). In addition, the verbascoside amount in the ND-infected Amfissis trees was 82, 32, 84, 41, and 61% higher than in the D-infected Amfissis trees at 15, 35, 55, 75, and 95 dpi, respectively.

The correlation analysis showed that there is a significant positive association

between the relative DNA quantity of both pathotypes and the verbascoside content in each cultivar ($df = 28$, $r = 0.97$ and 0.68 , $P \leq 0.05$, for the D and ND pathotype, respectively, in Amfissis; $df = 28$, $r = 0.67$ and 0.83 , $P \leq 0.05$, for the D and ND pathotype, respectively, in Koroneiki). Furthermore, a strong negative correlation was observed between the verbascoside and the oleuropein content in each cultivar ($df = 28$, $r = -0.93$ and -0.88 , $P \leq 0.05$, for the D and ND pathotype, respectively, in Amfissis; $df = 28$, $r = -0.81$ and -0.77 , $P \leq 0.05$, for the D and ND pathotype, respectively, in Koroneiki).

Fig. 6. Time course formation of oleuropein content (milligrams per gram dry weight of plant tissue) in relation to the relative DNA amount of the defoliating (D) or nondefoliating (ND) *Verticillium dahliae* pathotypes in the roots of 'Amfissis' and 'Koroneiki' (molecules $\times 10^5$ per 100 ng total DNA), sampled at 15, 35, 55, 75, and 95 days after transplanting (d.p.i.) to the microsclerotia-infested soil. Fungal DNA levels were estimated by quantitative polymerase chain reaction using total plant DNA isolated from 10 plants per treatment. The experiment was repeated three times (Rep 1, 2, and 3). Vertical bars indicate standard errors.

Fig. 5. Example of a high-performance liquid chromatography phenolic profile of olive root extracts from a defoliating *Verticillium dahliae* pathotype inoculated tree of A, 'Amfissis' and B, 'Koroneiki', 55 days after transplanting into the microsclerotia-infested soil. Detection at 280 nm of two major compounds: verbascoside and oleuropein.

DISCUSSION

Verticillium wilt has often been reported as the main phytopathological problem of olive trees in orchards of Mediterranean countries and in California (5,39). Because Verticillium wilt cannot currently be controlled by chemicals, the use of resistant and tolerant olive cultivars is considered to be the most efficient method for disease management. Although a large number of olive cultivars has been evaluated for resistance to Verticillium wilt (3,9,22–24,26–28,32), limited information is available in the literature about the defense mechanisms underlying the observed resistance (4). Two olive cultivars with known level of resistance against the D and ND *V. dahliae* pathotypes were used in the present study (3,24,26,27,32). The phenolic responses of susceptible Amfissis and resistant Koroneiki against the D and the ND *V. dahliae* pathotype were monitored in relation to the fungal DNA levels in the root tissues with the purpose of exploring the defense mechanisms of olive trees against *V. dahliae*.

Furthermore, qPCR analysis along with the symptom assessment revealed a significant positive correlation between the DNA levels of the D and ND pathotypes and disease severity in Amfissis. This correlation is even stronger when day 95 is excluded from the correlation analysis. On day 95, the fungal DNA amount of both pathotypes and in each olive cultivar decreased, despite the observed increase of disease severity (Fig. 2). This failure to increase after 75 dpi probably reflects reduced sporulation in the roots and coincides with the cyclical periods of fungal elimination that characterize the lifestyle of *V. dahliae* in the vascular system of trees and plants (8,16,19,26,28). On the other hand, a correlation was not observed between the DNA levels of the D and ND pathotypes and disease severity in Koroneiki. The DNA levels were nearly zero in most of the sampling time points, indicating the operation of defense mechanisms aiming to reduce fungal proliferation in the vascular tissues. Lopez-Esudero and Blanco-Lopez (22) have also correlated the presence of *V. dahliae* in olive tissues with the level of resistance and the virulence of the pathogen. They observed that pathogen isolation was significantly higher from susceptible cultivars (85%) than from resistant ones (33%) and, studying the natural mechanism of recovery, they observed that the 53% of 464 trees showed recovery after being transferred to pathogen-free soil, indicating the activation of defense mechanisms in the infected trees.

In several plant–pathogen interactions, the role of phenols to restrict or slow down pathogen growth has been suggested by several authors (4,7,13,29,30). In olive trees, the accumulation of total phenols upon infection by *P. savastanoi* pv. *savas-*

tanoi and *V. dahliae* has been shown (4,7,36). Although, the results of Baidez et al. (4) demonstrate the important role of phenolic compounds in the defense mechanisms of olive trees against *V. dahliae*, only the phenolic response of naturally infected olive trees of the susceptible Picual compared with the noninfected was studied.

Several authors have reported the increased accumulation of phenolic compounds in plant tissues after pathogen infection (4,7,13,29,36); however, in the present study, the total phenol content was decreased in both cultivars upon infection by *V. dahliae* pathotypes (Fig. 3). Interestingly, this decrease was negatively associated with the fungal DNA level in Amfissis. A possible explanation is that many plant pathogens, among them *V. dahliae*, actively suppress the expression of plant defense reactions during successful infection (15,18,21). On the other hand, another explanation could be that total phenols may serve as a pool required for diversion to other products (29), presumably associated with cellular browning and tissue necrosis that may result from oxidation and polymerization of *o*-diphenols (34). The *o*-diphenols have been associated with increased resistance against plant pathogens and the onset of defense mechanisms such as the hypersensitive reaction (29). Indeed, in the present study, a role for *o*-diphenols in the observed resistance of Koroneiki against *V. dahliae* can be proposed, because the *o*-diphenols content in this olive cultivar was positively correlated with the relative DNA quantity of both *V. dahliae* pathotypes, in contrast to Amfissis, where a negative association was observed.

Furthermore, the *o*-diphenol concentration was higher in Koroneiki compared with Amfissis at the time points during which a boost in *V. dahliae* DNA level was detected (Figs. 2 and 4).

HPLC analyses of plant extracts during the *V. dahliae* colonization process offers the possibility of detecting and quantifying individual compounds which possibly could be implicated in fungal restriction and plant tolerance. In the present study, two major phenolic compounds were determined in olive root extracts: oleuropein and verbascoside (Fig. 5).

Oleuropein has been associated by Baidez et al. (4) with the defense mechanisms of olive trees against *V. dahliae*; however, in the present study, a negative association between oleuropein content and relative DNA quantity of each *V. dahliae* pathotype was observed for both cultivars. In contrast, verbascoside levels were positively associated with the relative DNA quantity of the D and ND pathotypes in both cultivars. Moreover, oleuropein and verbascoside amounts were negatively associated with each other in both cultivars and *V. dahliae* pathotypes. This phenomenon has been also reported by Amiot et al. (2), suggesting a metabolic relationship between these two compounds. Overall, verbascoside content was higher in Koroneiki compared with Amfissis, indicating a correlation between the enhanced levels of verbascoside and the observed resistance of Koroneiki to both *V. dahliae* pathotypes. Similarly, high levels of verbascoside in shoots of olive trees have been detected as a response to infection by the nodule-inducing plant-pathogenic bacterium *P. savastanoi* pv. *savastanoi* (7,36).

Fig. 7. Time course formation of verbascoside content (milligrams per gram dry weight of plant tissue) in relation to the relative DNA amount of the defoliating (D) or nondefoliating (ND) *Verticillium dahliae* pathotypes in the roots of 'Amfissis' and 'Koroneiki' (molecules × 10⁵ per 100 ng total DNA), sampled at 15, 35, 55, 75, and 95 days after transplanting (d.p.i.) to the microsclerotia-infested soil. Fungal DNA levels were estimated by quantitative polymerase chain reaction using total plant DNA isolated from 10 plants per treatment. The experiment was repeated three times (Rep 1, 2, and 3). Vertical bars indicate standard errors.

Therefore, the present data support further the involvement of verbascoside in the defense mechanism of olive trees against plant pathogens.

In view of all the above, the data of the present study suggest the important role of the phenolic and separated compounds in the defense mechanism of olive trees against *V. dahliae*, as has already been stated in other plant–pathogen interactions (4,7,29,36). It is evident that further study is needed to elucidate and explore the biochemical pathway leading to the observed *V. dahliae* resistance of Koroneiki and other resistant olive cultivars, because this would be a substantial breakthrough in the effort to control one of the most serious olive diseases.

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