A Comparison of Real-Time PCR Protocols for the Quantitative Monitoring of Asymptomatic Olive Infections by *Verticillium dahliae* Pathotypes

D. Gramaje, V. Pérez-Serrano, M. Montes-Borrego, J. A. Navas-Cortés, R. M. Jiménez-Díaz, and B. B. Landa

Department of Crop Protection, Institute for Sustainable Agriculture (IAS), Spanish National Research Council (CSIC), Campus de Excelencia Internacional Agroalimentario, ceiA3, Avda. Alameda del Obispo s/n, P.O. Box 4084, 14080, Cordoba, Spain.
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ABSTRACT

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Early, specific, and accurate in planta detection and quantification of *Verticillium dahliae* are essential to prevent the spread of Verticillium wilt in olive using certified pathogen-free planting material and development of resistance. We comparatively assessed the accuracy, specificity, and efficiency of eight real-time quantitative polymerase chain reaction protocols published since 2002 for the specific detection and quantification of *V. dahliae* in various host plant species and in soil, using a background of DNAs extracted from olive roots, stems, and leaves. Results showed that some of those protocols were not specific for *V. dahliae* or were inhibited when using backgrounds other than water. Ranking of protocols according to a weighted score system placed protocols TAQ (based on intergenic spacer ribosomal DNA target gene) and SYBR-4 (based on the β-tubulin 2 target gene) first in sensitivity and efficiency for the quantification of *V. dahliae* DNA in small amounts and different types of olive tissues (root and stem) tested. Use of TAQ and SYBR-4 protocols allowed accurate quantification of *V. dahliae* DNA regardless of the background DNA, with a detection limit being fixed at a cycle threshold of 36 (≈18 fg for SYBR-4 and 15 fg for TAQ) of *V. dahliae*. The amount of DNA from defoliating (D) and nondefoliating (ND) *V. dahliae* pathotypes was monitored in Verticillium wilt-resistant 'Frantoio' olive using the TAQ and SYBR-4 protocols. In the infection bioassay, higher amounts of D *V. dahliae* DNA were measured in olive stems, whereas the average amount of fungal DNA in roots was higher for ND-infected plants than D-infected ones. Overall, *V. dahliae* DNA amounts in all olive tissues tested tended to slightly decrease or remain stable by the end of the experiment (35 days after inoculation). The SYBR-4 and TAQ protocols further enabled detection of *V. dahliae* in tissues of symptomless plants, suggesting that both techniques can be useful for implementing certification schemes of pathogen-free planting material as well as helpful tools in breeding resistance to *V. dahliae* in olive.

Additional keyword: *Olea europaea*, olive resistance breeding, tolerance, vascular infection.

Verticillium wilt, caused by *Verticillium dahliae* Kleb., is currently considered the main soilborne disease threatening olive production worldwide (24). Severity of attack by this disease depends on the pathotype infecting the olive trees (41). Isolates of the defoliating (D) pathotype are highly virulent on olive compared with those of the nondefoliating (ND) pathotype. Infections by the D pathotype can be lethal to the plant, whereas ND-infected olive trees can eventually show symptom remission (12,24,34,37).

Effective management of Verticillium wilt of olive requires the implementation of an integrated disease management (IDM) strategy that combines preplanting and postplanting control measures to promote recovery from the disease and to reduce the risk of pathogen spread to new trees (24,41,51). Use of *V. dahliae*-free olive planting material for the establishment of new olive orchards in pathogen-free soils is a key component of that IDM strategy (24,34). Therefore, early and accurate in planta detection and quantification of *V. dahliae* pathotypes, particularly the D pathotype, that ensure healthy propagation of planting material are essential to prevent pathogen-infected olive trees from being planted and, thus, to facilitate the management of Verticillium wilt in olive (24,34,50). Accurate in planta quantification of the pathogen biomass would also facilitate the proper characterization of resistance or tolerance to *V. dahliae* in commercial olive varieties, olive germplasm (24,37), as well as in selection of wild olive genotypes potentially used as rootstocks, which has shown promise as an IDM strategy for Verticillium wilt in olive (9,24).

Over the last few years, several real-time quantitative polymerase chain reaction (qPCR) protocols have been developed for the specific detection and quantification of *V. dahliae* in different host plant species and in soil (Table 1). These protocols were each evaluated and optimized for individual pathosystems in compatible interactions; however, no comparisons of the protocols on effectiveness for the quantification of *V. dahliae* in asymptomatic plant tissues or resistant or tolerant reactions have yet been made. Furthermore, some of the protocols were developed specifically for soil samples, and their efficacy has not been evaluated on plant tissues. The accuracy and reliability of the detection and quantification protocols are essential for their use in the certification of *V. dahliae*-free planting material. Consequently, in this study, we present a comparative evaluation of the protocols referred to above together with a new SYBR-Green-based protocol (named SYBR-1) developed in this study using primers previously designed by Nigro (42) but without the "scorpion" chemistry. The main objective of this research was to compare the

Corresponding author: B. B. Landa; E-mail address: blanca.landa@csic.es

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weaknesses and strengths of each of the protocols and to assess their usefulness for monitoring the amount of pathogen DNA in time-course infection bioassays.

MATERIALS AND METHODS

Comparison of real-time protocols for the quantification of *V. dahliae*. *Fungal isolates.* In total, 77 fungal isolates representative of nine *Verticillium* spp., including *V. albo-atrum* (2 isolates), *V. alfalfae* (2 isolates), *V. dahliae* (49 isolates), *V. isacii* (1 isolate), *V. longisporum* (4 isolates), *V. nonalfalfae* (7 isolates), *V. nubilum* (1 isolate), *V. tricorpus* (2 isolates), and *Gibellulopsis nigrescens* (syn. *V. nigrescens*) (9 isolates) were used in this study. The 49 *V. dahliae* isolates represented a wide diversity in geographic origin, vegetative compatibility grouping, and D and ND pathotypes on cotton and olive (Table 2). Those isolates were stored on plum lactose yeast extract agar (PLYA) (49) covered with liquid paraffin at 4°C in the dark (5). Single-spore cultures of all *V. dahliae* isolates are deposited in the culture collection of the Department of Crop Protection, Institute for Sustainable Agriculture, Spanish National Research Council, Córdoba, Spain. Active cultures of the isolates were obtained on water agar amended with chlorotetracycline (0.3 g/liter) (CWA) and subsequent subculturing on potato dextrose agar (PDA) (250 g of unpeeled potato, 20 g of agar, and 20 g of glucose per liter of distilled water) or, alternatively, PLYA.

For fungal DNA extraction, actively growing cultures were placed onto a film of sterile cellophane layered over a plate of PDA and incubated for 5 to 7 days at 25°C in the dark. Then, mycelia growing over the cellophane surface were scraped directly with a sterile scalpel, lyophilized, and stored at –20°C until used.

Plant tissue samples. To compare the different real-time qPCR assays, samples of roots, stems, and leaves were obtained from certified olive rooted cuttings of 'Frantoio' grown in sterile sand in pots inside a walk-in growth chamber (Euroclima, Córdoba, Spain) adjusted to 23 ± 1°C in light and darkness, 40 to 70% relative humidity, and a 14-h photoperiod of fluorescent light of 360 μE m^{-2} s^{-1}. After 40 days, root, stem, and leaf samples were removed from plants, washed under running tap water, and surface-disinfested in 0.5% NaClO for 1.5 min, lyophilized, ground and stored at –20°C until use.

DNA extraction and quantification. Fungal and plant DNAs were extracted from 50 mg of lyophilized plant tissue or fungal mycelium using the G-Spin IIp Plant Genomic DNA extraction kit (Intron Biotechnology, Korea) and the Fast Prep System Bio 101 (Qbiogene, Illkirch, France) according to Landa et al. (30).

DNA quality was assessed by gel electrophoresis and staining with ethidium bromide. DNA samples were quantified using the

TABLE 1. Real-time quantitative polymerase chain reaction protocols compared in this study for the efficacy in estimating *Verticillium dahliae* DNA in asymptomatic olive tissues, with detailed information on their properties

Protocol (denomination)	Gene target[y]	Cycling conditions	Amplicon size (bp)[z]	Primers	Primer/probe concentration	Reference
Scorpion (SCP)	IGS	94°C, 2 min 94°C, 45 s 55°C, 45 s 72°C, 1 min (40×) 72°C, 5 min	≈150	Vd10-scorpion, Vd7b	1.0 μM each primer	Nigro et al. (42)
SYBR Green						
SYBR-1	IGS	94°C, 2 min 94°C, 45 s 55°C, 45 s 72°C, 1 min (40×) 72°C, 5 min + melting curve	≈150	Vd10, Vd7b	1.0 μM each primer	This study; modified from Nigro et al. (42)
SYBR-2	Library	94°C, 4 min 94°C, 1 min 62°C, 1 min 72°C, 30 s (40×) 72°C, 6 min + melting curve	539 (D), 523 (ND)	DB19, DB22	0.1 μM each primer	Mercado-Blanco et al. (37)
SYBR-3	ITS	95°C, 10 min 95°C, 10 s 60°C, 5 s 72°C, 9 s (40×) 72°C, 6 min + melting curve	200	ITS1-F, ST-VE1	0.5 μM each primer	Lievens et al. (31)
SYBR-4	β-tubulin 2	95°C, 3 min 95°C, 30 s 63°C, 30 s 72°C, 30 s (40×) + melting curve	115	VertBt-F, VertBt-R	0.2 μM each primer	Atallah et al. (3)
SYBR-5	SCAR from RAPD	95°C, 2 min 95°C, 30 s 60°C, 30 s 72°C, 1 min (40×) 72°C, 5 min + melting curve	520	VDS1, VDS2	0.7 μM each primer	Gayoso et al. (17)
SYBR-6	ITS	94°C, 2 min 94°C, 1 min 60°C, 1 min 72°C, 30 s (40×) + melting curve	347	ITS1-F, ITS2-R	0.7 μM each primer	Markakis et al. (35)
Taq Man (TAQ)	IGS	95°C, 2 min 62°C, 30 s (40×) 72°C, 5 min	≈200	VD-F929-947 VD-R1076-1084	1.6 μM each primer, 0.4 μM TaqMan probe	Bilodeau et al. (6)

[y] IGS = intergenic spacer, Library = repetitive cloned sequence from *V. dahliae* library (Carder et al. [7]), ITS = internal transcribed spacer, SCAR = sequence characterized amplified region, and RAPD = random amplified polymorphic DNA.
[z] D and ND = defoliating and nondefoliating, respectively.

Quant-iT DNA Assay Kit Broad Range fluorometric assay (Molecular Probes Inc., Leiden, The Netherlands) and a Tecan Safire fluorospectrometer (Tecan Spain, Barcelona, Spain) (40). Special care was taken to get accurate concentrations of pathogen and host DNAs by quantifying each DNA sample in triplicate and in two independent microplates. DNA samples of known concentration were included in each quantification plate as an internal control of DNA quantification. DNA was diluted with sterile, ultrapure water, as appropriate.

DNA standard curves for real-time qPCR. DNA standard curves were obtained from 10-fold dilutions of *V. dahliae* isolates V781I (D pathotype) and V152I (ND pathotype) (Table 1). For this purpose, DNA (10 ng/µl) from the *V. dahliae* isolates was serially diluted (1:10, $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$, and $1:10^6$) in sterile ultrapure water as well as in a fixed background of host plant DNA (20 ng/µl) extracted from olive stem, leaf, or root tissue. Due to PCR inhibition when using DNA extracted from roots, additional DNA standard curves were obtained by spiking the dilutions with root DNA at 5 ng/µl. Plant DNA alone or no DNA served as negative controls for each standard curve. Two independent DNA standard curves were obtained using separate plant and pathogen DNA sources that were treated as independent experiments.

Real-time qPCR. All real-time PCR amplifications were performed using the iQ SYBR Green Supermix (Bio-Rad, Madrid) for SYBR green-based protocols, the iTaq DNA Polymerase and dNTP mix from Bio-Rad for the Scorpion assay, and the Real Master Mix without Rox (5 Prime; Fisher Scientific, Waltham, MA) for the Taqman assay. Scorpion primer and Taqman probe were synthesized by EUROGENTEC SA (Seraing, Belgium), and Sigma-Aldrich (Madrid, Spain), respectively. The remaining primers were synthesized by Sigma-Aldrich. Template DNA (1 µl) from each standard curve corresponding to either isolate V781I (D patho-

TABLE 2. *Verticillium* isolates used in this study with reference code, host source, geographic origin, vegetative compatibility grouping (VCG), and biological pathotyping on cotton and olive

Reference code and host source[w]	Geographic origin[x]	VCG[y]	Pathotype[z]
Verticillium albo-atrum			
Alfalfa			
DAOM 212676§	Canada	–	–
Potato soil			
CBS 130340	Canada	–	–
V. alfalfae			
Alfalfa	–	–	–
41§	Canada		
CBS 130603 (PD489)	United States	–	–
V. dahliae			
Vd52§	Germany	–	–
Artichoke			
V403II	Spain	1A	D
V485I , V534I	Spain	2A	ND
V613I	Spain	2B	ND
V574I	Spain	2Ba	–
V684I	Spain	4B	ND
Cotton			
V138I	Spain	1A	D
V610I	Spain	1A	–
V176I§, V185I, V249I	Spain	2A	ND
V4I	Spain	HSI	ND
V192I, V239I	Spain	4B	ND
V318I	California	2A	–
V356I (HB3)	China	1A	–
V357I (JY)	China	2B	–
V301I (cot 112)	Israel	2B	ND (DL)
V308I (cot 129), V293I (cot 87)	Israel	4B	ND
V646I	Greece	1A	–
V661I	Greece	1B HSI	–
V639I	Greece	2B	–
V669I	Turkey	1A	–
V656I	Turkey	2A	–
V668I	Turkey	2B	–
Olive			
V135I, V136I§, V150I, V781I	Spain	1A	D

(*continued on next page*)

[w] Reference code in parenthesis refers to that used by other authors in previous works. Fungal identification was given by providers or performed as described by Inderbitzin et al. (23), Karapapa et al. (26), Smith (47), and Zare et al. (54). Symbol: § indicates that isolates were assigned to the species level by sequencing the elongation factor (EF) gene using VEFf/VEFr primers and phylogenetic analysis of sequences with reference isolates as described by Inderbitzin et al. (23).

[x] Isolates from Cyprus, Greece, and Turkey provided by E. Paplomatas, Agricultural University of Athens, Athens, Greece; artichoke isolates provided by J. Armengol, Universidad Politécnica de Valencia, Valencia, Spain; isolates from China and Israel provided by T. Katan and N. Korolev, The Volcani Center, Bet Dagan, Israel; isolates from the United Kingdom and California provided by D. Barbara, Warwick HRI, England; isolates from Italy provided by F. Nigro, Università degli Studi di Bari, Bari, Italy; isolates from Syria provided by M. Al-Ahmad, Plant Protection Service, Damascus, Syria; isolates from Canada provided by G. Bilodeau, Canadian Food Inspection Agency (CFIA), Ottawa, Canada; isolates from Germany provided by A. von Tiedemann, Department of Crop Sciences, University of Göttingen, Göttingen, Germany; isolates from Slovenia provided by S. Radišek, Slovenian Institute for Hop Research and Brewing, Žalec, Slovenia. Reference isolates from CBS Fungal Biodiversity Centre were purchased or an aliquot of DNA was provided by G. Bilodeau, S. Radišek, and P. Inderbitzin, Department of Plant Pathology, University of California, Davis.

[y] VCG assessment of the isolates was previously reported (10,11,25). 2Ba = VCG2B isolates from artichoke in the Comunidad Valenciana Region that did not complement with VCG2B international reference testers but showed positive complementation with nitrate-nonutilizing (*nit*) mutants of VCG2B isolates from artichoke in that region (25); * = *nit* mutants were not produced.

[z] D = cotton defoliating, ND = cotton nondefoliating, DL = defoliating-like according to Korolev et al. (29), – = not determined. Pathotype assessment of the isolates was reported earlier in plant pathogenicity tests (5,25,28,29,44). D* = olive and cotton defoliating, as indicated by amplification of the 462-bp marker; ND* = olive and cotton nondefoliating, as indicated by amplification of 824-bp marker (38,39).

type) or isolate V152I (ND pathotype) was added to each reaction mixture, and the total volume was 20 µl in all assays. All real-time PCR assays were performed in an iCycler iQ3 (Bio-Rad) apparatus and results were analyzed with the manufacturer's software (Optical System Software v 3.0a). Assay details are outlined in Table 3 (cycling conditions, primer and probe concentrations, and so on).

DNA templates for comparisons were prepared in the same IQ 96-well microtiter plate (Bio-Rad). qPCR amplifications of each series of DNA standard curves included four replications per treatment and plate. All experiments were repeated twice independently (different PCR plates, operators, and DNA standard curves) and each included a common DNA standard curve to estimate the variability between and within experiments.

Olive–*V. dahliae* time-course of infection bioassay. *Fungal isolates and plant material. V. dahliae* isolates V781I (D pathotype) and V152I (ND pathotype) were used (Table 1). Plant material consisted of certified, 8-month-old rooted cuttings of Frantoio olive. Plants were kindly provided by Cotevisa (L'Alcudia, Valencia, Spain). Plants of this cultivar were obtained routinely by micropropagation techniques and exhibit resistance to Verticillium wilt when challenged with the D or the ND *V. dahliae* pathotypes (14,32,33,36).

Olive–V. dahliae *bioassay.* In total, 135 plants were used in the infection bioassay that included three treatments: (i) noninoculated control (45 plants), (ii) inoculation with isolate V781I (45 plants), and (iii) inoculation with isolate V152I (45 plants). From each treatment, 30 plants were used for isolation and in planta quantification of D and ND *V. dahliae* in a time-course sampling schedule after inoculation (3 plants each per 10 sampling times between 0 and 35 days after inoculation). The remaining 15 plants of each treatment were kept as a reference for the assessment of disease symptoms.

TABLE 2. (*continued from preceding page*)

Reference code and host source[w]	Geographic origin[x]	VCG[y]	Pathotype[z]
V149I, V152I	Spain	2A	ND
V789I	Spain	4B	–
V716I, V718I	Italy	2A	–
V361I	Syria	2A	–
V811I	Cyprus	2A	–
V674I	Greece	4B	–
V1383	Spain	1A	D*
V1288I, V1558I	Spain	2A	ND*
V1235I, V1374I	Spain	2B	ND*
V1242I, V1266I	Spain	4B	ND*
Pepper			
V561I (VdCa147a), V560I (VdCa83a)	California	6	–
Rape			
V-413[§]	Germany	–	–
V. isacii			
Vi-G[§]	Germany	–	–
V. longisporum			
V259	Germany	–	–
Cauliflower			
V559I (90-10)	California	–	–
Rape			
V558I (Vd-1)	Sweden	–	–
CBS 110229	Sweden	–	–
V. nonalfalfae			
V53I[§]	–	–	–
Vna-G[§]	Germany	–	–
Hops			
V48I (V-48 898, mild)[§]	United Kingdom	–	–
Rec[§]	Slovenia	–	–
Zup[§]	Slovenia	–	–
Petunia			
Surf[§]	Slovenia	–	–
Potato			
CBS 130339 (PD 592)	Japan	–	–
V. nubilum			
Potato			
CBS 456.51	United Kingdom	–	–
V. tricorpus			
54793[§]	–	–	–
54794[§]	–	–	–
Gibellulopsis nigrescens			
Cotton			
V-354I (Tester 115)[§]	Israel	–	–
Cotton soil			
PD 786 (Vn4)[§]	–	–	–
PD 787 (Vn5)[§]	–	–	–
Insulator wool			
CBS 123176	Finland	–	–
Potato			
V51I (V-51 1880)[§]	United Kingdom	–	–
Vn 8[§]	Israel	–	–
Weeds or potato soil			
Vn 7[§]	Israel	–	–
Vn 12[§]	Israel	–	–
Vn 14[§]	Israel	–	–

Plants were root-dip inoculated in a suspension of 10^7 conidia ml^{-1} of either *V. dahliae* V781I or V152I isolates for 20 min. Conidia were obtained from 7- to 10-day-old cultures in potato dextrose broth (PDB), as previously described (39). Plants were transplanted into sterilized soil (sand/loam, 2:1, vol/vol) in sterilized 3-liter clay pots (16.5 cm in diameter by 15.5 cm in height), and incubated in a walk-in growth chamber adjusted to 24 ± 1°C in light and darkness, 40 to 70% relative humidity, and a 14-h photoperiod of fluorescent light at –360 μE m^{-2} s^{-1}. Plants were watered every 1 to 2 days as needed and fertilized weekly with of Hoagland's nutrient solution at 110 ml/pot (21). The experiment was arranged as a completely randomized design. The severity of symptoms was assessed on a 0-to-4 scale (0 = no symptoms, 1 = 1 to 33% symptomatic leaves and twigs, 2 = 34 to 66% symptomatic, 3 = 67 to 100% symptomatic, and 4 = dead plant) at 35 and 122 days after inoculation (39).

Isolation of V. dahliae *from inoculated plants.* The roots and stems of inoculated olive plants were sampled separately in a time course after inoculation for isolation and real-time PCR quantification of *V. dahliae*. Three plants were destructively sampled at 0, 6, and 24 h and 2, 3, 7, 10, 15, 21, and 35 days after inoculation with the two *V. dahliae* pathotypes. Colonization of plant tissues by *V. dahliae* was determined in each sampled plant by isolating the fungus on CWA. For each plant, four 5-mm-long root and stem pieces were thoroughly washed under running tap water for 30 min, surface disinfested in 0.5% NaClO for 1.5 min (stems) or 2 min (roots), rinsed with sterile water, plated onto the medium, and incubated at 25°C in the dark for 9 to 21 days (39). For stem pieces, the bark was removed with a clean scalpel before disinfestation. The plated stem pieces were representative of the middle and lower parts of the total length of the sampled stems. Root pieces were arbitrarily chosen from the sampled plants. Bark was not removed from root pieces. The identity of *V. dahliae* was confirmed by microscopic observations of verticillate conidiophores and by the formation of microsclerotia.

Data from the stem and root isolations of the pathogen were used to calculate an index of colonization (IC) determined as $IC = \Sigma(Ti \times Nj)/(T \times N) \times 100$, where Ti is the number of root or stem pieces from which the pathogen was isolated, Nj is the number of plants from which the Ti pieces were obtained, T is the total

TABLE 3. Regression equation parameters from standard curves of *Verticillium dahliae* DNA, sensitivity, specificity and weighted score index (WSI) of five real time quantitative polymerase chain reaction (qPCR) assays evaluated for quantification of *V. dahliae* in asymptomatic olive infections[u]

	Regression parameter					Specificity[w]								
Protocol[v]	Intercept	–Slope	R^2	AE (%)[x]	DL[y]	*Gn*	*Vaa*	*Va*	*Vi*	*Vna*	*Vn*	*Vl*	*Vt*	WSI[z]
SCP														
Water	22.75–22.90	4.04–3.78	0.968–0.992	76.8–99.0	1	…	…	…	…	…	…	…	…	19.0–19.0
Stem	22.18–26.25	3.84–3.44	0.973–0.772	82.1–95.4	1	…	…	…	…	…	…	…	…	18.0–19.0
Leaf	23.90–27.58	3.78–3.72	0.992–0.673	84.0–85.8	1	…	…	…	…	…	…	…	…	16.0–19.0
Root	–	…	–	–	–	…	…	…	…	…	…	…	…	0.00–0.00
Mean	24.33	3.76	0.876	84.79	1	No	Yes	No	No	No	Yes	No	No	13.75 bc
SYBR-1														
Water	12.18–13.40	3.60–3.67	0.996–0.997	87.2–89.6	0.1	…	…	…	…	…	…	…	…	21.0–21.0
Stem	12.57–13.42	3.28–3.55	0.996–0.999	91.2–101.7	0.1	…	…	…	…	…	…	…	…	21.0–23.0
Leaf	11.94–14.10	3.50–3.37	0.994–0.998	93.1–98.0	0.1	…	…	…	…	…	…	…	…	21.0–23.0
Root	17.59–18.40	3.51–3.61	0.944–0.957	89.2–92.6	0.1	…	…	…	…	…	…	…	…	20.0–21.0
Mean	13.60	3.45	0.985	92.83	0.1	Yes	Yes	No	No	No	Yes	No	No	21.38 b
SYBR-2														
Water	24.54–26.79	3.04–3.74	0.736–0.996	85.2–113.1	1	…	…	…	…	…	…	…	…	36.0–36.0
Stem	–	…	–	–	–	…	…	…	…	…	…	…	…	0.0–0.0
Leaf	23.85–24.40	5.35–3.91	0.823–0.955	53.9–80.3	1	…	…	…	…	…	…	…	…	35.0–36.0
Root	–	…	–	–	–	…	…	…	…	…	…	…	…	0.0–0.0
Mean	24.90	4.01	0.870	83.10	1	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	17.88 c
SYBR-4														
Water	21.43–21.39	3.33–3.34	0.973–0.986	99.3–99.8	0.1	…	…	…	…	…	…	…	…	43.0–43.0
Stem	21.41–21.48	3.34–3.27	0.993–0.992	99.4–102.4	0.1	…	…	…	…	…	…	…	…	43.0–43.0
Leaf	21.03–21.63	3.26–3.31	0.981–0.995	100.6–102.8	0.1	…	…	…	…	…	…	…	…	43.0–43.0
Root	21.21–21.34	3.17–3.17	0.995–0.989	100.7–106.9	0.1	…	…	…	…	…	…	…	…	41.0–43.0
Mean	21.36	3.27	0.990	100.80	0.1	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	42.75 ab
TAQ														
Water	15.06–15.27	3.39–3.60	0.971–0.996	89.7–97.0	0.1	…	…	…	…	…	…	…	…	46.0–48.0
Stem	14.69–14.76	3.61–3.62	0.990–0.996	88.7–89.2	0.1	…	…	…	…	…	…	…	…	46.0–48.0
Leaf	14.46–14.84	3.55–3.64	0.981–0.994	88.0–91.2	0.1	…	…	…	…	…	…	…	…	46.0–46.0
Root	15.09–15.61	3.15–3.60	0.822–0.990	89.6–107.9	0.1	…	…	…	…	…	…	…	…	46.0–48.0
Mean	14.97	3.52	0.968	92.68	0.1	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	46.75 a

[u] Standard curves were obtained by five-point 10-fold serial dilution of *V. dahliae* DNA of isolates V781I and V152I (10 ng/μl) to obtain 1 ng to 0.1 pg of DNA per PCR reaction diluted in sterile distilled water or in DNA extracted from olive leaves and stems (10 ng of background DNA) or roots (5 ng of background DNA) to investigate any possible influence of host DNA on sensitivity of PCR reactions.

[v] qPCR protocol and background DNA.

[w] No indicates that the protocol is not specific and cross-amplified *Gibellulopsis nigrescens* (*Gn*), *V. albo-atrum* (*Vaa*), *V. alfalfae* (*Va*), *V. isacii* (*Vi*), *V. longisporum* (*Vl*), *V. nonalfalfae* (*Vna*), *V. nubilum* (*Vn*), and *V. tricorpus* (*Vt*). Yes indicates that the protocol was specific for *V. dahliae*.

[x] Amplification efficiency (AE) was calculated from the slopes of the standard curves using the equation $AE = 10^{(-1/slope)} - 1$ (1,20). Range corresponds to results from two independent standard curves, each performed by two independent operators and four replications within each plate.

[y] Detection limit (pg/μl).

[z] Score system = points were given for each of the qPCR amplification traits that were consider important to choose a protocol as the most appropriate for our amplifications purposes: R^2 (score 0 = no amplification; score 1 = $R^2 < 0.85$; score 2 = $0.85 < R^2 < 0.95$; score 3 = $R^2 > 0.95$); AE (score 0, no amplification; score 1, AE < 85%; score 2, 85% < AE < 95%; score 3, AE > 95%), DL (score 0, no amplification; score 1, 1< DL ≤ 10 pg/μl; score 2, 0.1 < DL ≤ 1 pg/μl; score 3, DL ≤ 0.1 pg/μl). Additionally, scores of 0 were given if there was cross-amplification for the other eight *Verticillium* spp. different from *V. dahliae* that we tested, whereas a score of 1 was given if there was no cross-amplification for those species. Then, a WSI was calculated by using weights of 1, 2, 3, and 4 for R^2, AE, DL, and total specificity score, respectively, indicating the importance given to each parameter to appropriately quantify *V. dahliae* DNA in asymptomatic plant tissues under our experimental conditions. A total WSI of 54 points was possible. A Kendall-concordance analysis was performed using the WCI values obtained for each DNA background and experiment. Range corresponds to results from to two independent standard curves, each performed by two independent operators and four replications within each plate. Values followed by the same letter are not significantly different ($P = 0.05$).

number of pieces processed, and N is the total number of sampled plants. IC data were plotted over time in days to obtain vascular colonization progress curves.

All stem and root tissues of each sampled plant were immediately stored at –80°C for real-time PCR quantification assays.

In planta real-time PCR quantification of D and ND V. dahliae. Total DNA was extracted from 50 mg of lyophilized stems and roots of inoculated and noninoculated Frantoio plants. Extracted olive DNA was quantified using the Quant-iT DNA Assay Kit Broad Range fluorometric assay as described above and diluted to ≈20 ng/µl for stem samples or to 5 ng/µl for root samples. The amount of *V. dahliae* DNA in each sample was estimated using the SYBR-4 (3) and TAQ (6) qPCR protocols that were selected as the most efficient in the previous experiments for asymptomatic quantification of different *V. dahliae* isolates in the two types of olive tissues.

Amplification reactions were performed in 96-well microtiter plates. Three independent plant samples and two simultaneous, replicated amplifications were carried out for each DNA sample. Each plate contained all DNA samples corresponding to a single *V. dahliae* pathotype and tissue (stems or roots) assayed, and every reaction in the plate was made up using aliquots of the same master mix. Thus, all DNA samples (and their replicates) were subjected to the same experimental conditions. Additionally, each plate always contained the DNA standard curves obtained as described above, as well as DNA samples from noninoculated olive plants and a negative control reaction (no template DNA) that were used to test for interplate variability.

Additionally, to test for the absence of PCR inhibitors in the samples, the β-actin gene from olive was amplified using Act1-fw/Act1-rv primers (46). Results from quantification of the β-actin were referred to a DNA standard curve obtained as described above from six twofold dilutions of olive DNA (20 ng/µl) in sterile ultrapure water. These results were used to calibrate the quantification of *V. dahliae* DNA to the real amount of olive DNA template added to each qPCR assay (Supplemental Figure 1).

Data analyses. In the real-time qPCR assays, the cycle threshold (C_T) values for each reaction were calculated first by determining the PCR cycle number at which the fluorescence signal exceeded the background using the default estimation criteria in the iCycler IQ software (version 3.0a; Bio-Rad). Then, to compare and establish relationships among the different DNA standard curves generated from different treatments, the threshold position was manually defined and fixed at the same position for all treatments and experiments (52). The amplification efficiency (AE) was calculated from the slopes of the standard curves using the equation $AE = 10^{(-1/slope)} - 1$ (1,20).

All data analyses were performed using Statistix v9.0 (NH Analytical Software, Roseville, MN). Linear regressions of the natural logarithm of known concentrations of the target DNA versus the C_T values were performed for each DNA dilution series. Standard regression lines from each plate chosen as reference curves were used for transforming the experimental C_T values into amounts of pathogen DNA (nanograms). All standard regression lines obtained for fungal genomic DNA in different backgrounds (water, stem, leaf, and root) were statistically compared for homogeneity ($P \geq 0.05$) of variance (Bartlett's test) and for equality of slopes and intercepts using an F test at $P < 0.05$.

Additionally, to objectively assess each protocol for selecting the most appropriate for our amplifications purposes, a score system was used whereby scores were assigned to each of the qPCR amplification traits that were considered important: R^2 (score 0, no amplification; score 1, $R^2 < 0.85$; score 2, $0.85 < R^2 < 0.95$; score 3, $R^2 > 0.95$), AE (score 0, no amplification; score 1, AE < 85%; score 2, 85% < AE < 95%; score 3, AE > 95%), and detection limit (DL) (score 0, no amplification; score 1, 1 < DL ≤ 10 pg/µl; score 2, 0.1 < DL ≤ 1 pg/µl; score 3, DL ≤ 0.1 pg/µl). Also, a score 0 was assigned if there was cross-amplification for any of the eight *Verticillium* spp. other than *V. dahliae* tested and a score of 1 if there was no cross-amplification for each of those species (a maximum score of 8 was possible for specificity; Supplemental Figure 2). Then, each score was weighted with 1, 2, 3, and 4 for R^2, AE, DL, and specificity, respectively, to indicate the weight (importance) given to each parameter for appropriately quantifying *V. dahliae* DNA in asymptomatic plant tissues. A total weighted score index (WSI) of 54 points was possible. Finally, a Kruskal-Wallis one-way nonparametric analysis of variance was performed to test for significance ($P < 0.05$) of the differences among the various protocols for the WSI.

In addition, the area under the IC curve (AUIC) plotted over time of sampling (estimated by positive pathogen isolation), as well as the area under the curve of the amount of *V. dahliae* DNA in olive tissue (AUAVDC) plotted over time of sampling (estimated by qPCR), were used to assess for significance of the differences among D and ND *V. dahliae* isolates by using Fisher's protected least significant difference at $P < 0.05$.

RESULTS

Comparison of real-time qPCR protocols for the quantification of *V. dahliae*. SYBR-3 (31), SYBR-5 (17), and SYBR-6 (35) protocols were initially screened but were rejected from further real-time evaluation because they showed cross-amplification with several *Verticillium* spp. or *G. nigrescens* (Supplemental Figure 2), as well as overall lack of reproducibility, or low sensitivity (data not shown). Similarly, under the conditions employed in this study, we found that primers Vd10 and Vd7b used in protocols SCP and SYBR-1 also cross-amplified DNA from some *Verticillium* spp. However, those two protocols were included in the analyses together with protocols SYBR-2 (37), SYBR-4 (3), and TAQ (6) because they performed well in the preliminary experiments (data not shown).

The performance characteristics of each assayed protocol and the weighted scores to select the most appropriate technique for the quantification of *V. dahliae* in asymptomatic olive tissues are listed in Table 3. The efficiency and reproducibility of some of real-time qPCR assays standard regression were significantly ($P < 0.05$) influenced by the presence of the host DNA (i.e., extracted from stem, leaf, and root tissues) (Table 3). PCR inhibition occurred for protocols SCP (42) and SYBR-2 (37) using root DNA, as well as for protocol SYBR-2 using stem DNA. Thus, a low reproducibility of amplifications occurred for those protocols and we failed obtaining a linear dynamic range of amplification after three orders of magnitude of DNA concentration (data not shown). Consequently, standard regression lines for those DNA backgrounds were not estimated (Table 3). Conversely, for the remaining SYBR-1 (this study), SYBR-4 (3), and TAQ (6) protocols, we achieved high reproducibility of amplifications ($0.88 > R^2 > 0.99$) with high efficiency ($87.2 > AE(\%) > 107.9$) over five orders of magnitude of DNA concentration for the different experiments and DNA backgrounds, which exhibited a linear dynamic range of amplification (Fig. 1; Table 3). *V. dahliae* DNA was accurately quantified at a concentration of 0.1 pg either if DNA was diluted in water or in DNA extracted from olive stem, leaf, or root tissues (Fig. 1; Table 3). *V. dahliae* DNA could also be quantified to 0.01 pg with the three protocols but the reproducibility of amplifications decreased at this lower concentration (data not shown); therefore, we fixed the limit of detection at the estimated C_Ts corresponding to this concentration (i.e., $C_T = 32$ or 36 for SYBR-1 and TAQ or SYBR-4 protocols, respectively) (Fig. 1).

Finally, the five q-PCR protocols were ranked according to a weighted score system (WSI) and analyzed together for both experiments and each of the DNA backgrounds: SYBR-4 (3) and TAQ (6) ranked first, followed by SYBR-1 (this study) (mean WSI of 46.8, 42.8, and 21.4, respectively), although there were no significant ($P \geq 0.05$) differences among SYBR-4 (3) and TAQ (6)

protocols (Table 3). Comparisons of the four standard regression lines within each of the protocols TAQ, SYBR-1, and SYBR-4 showed no significant differences among slopes or intercepts ($P > 0.3790$) for DNA template tested in the background of olive DNA from several tissues (stem, leaf, or root) (Table 3), suggesting that the performance of these three protocols was not influenced by the presence of host DNA (Table 3; Fig. 1). Finally, comparison of standard regression lines among protocols indicate that there were significant differences among intercepts ($P < 0.001$) but not among slopes ($P > 0.1931$). The lower value of intercept for protocol SYBR-1 and TAQ compared with that of SYBR-4 indicate a significantly higher ($P < 0.05$) sensitivity of the former two protocols.

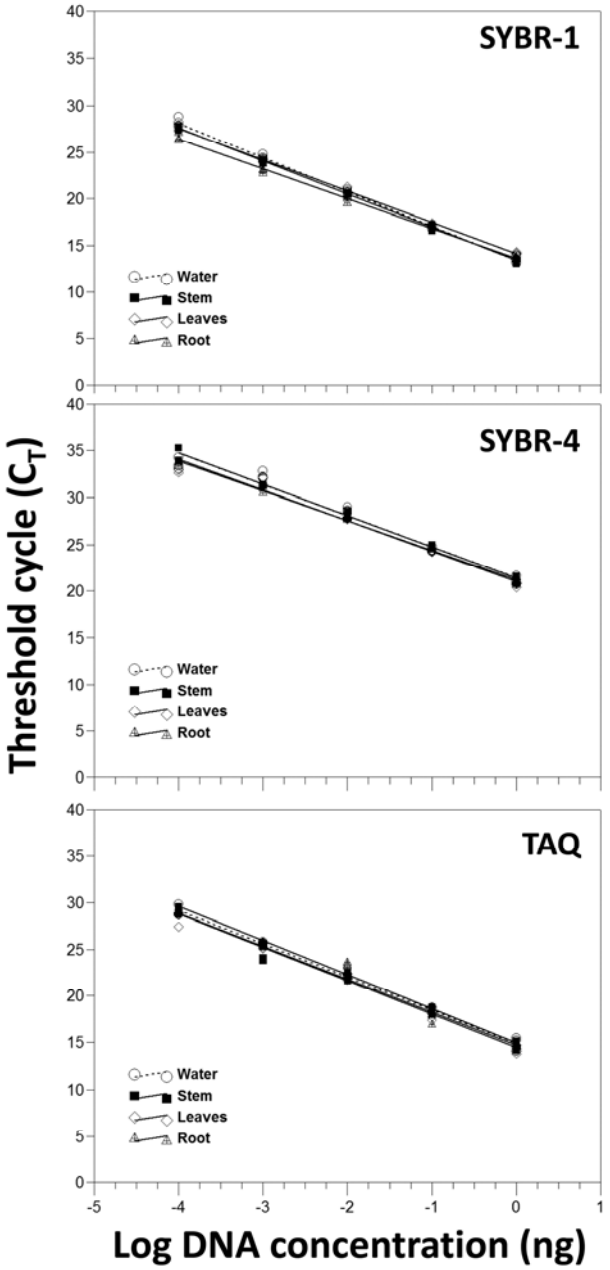

Fig. 1. Standard regression lines of a five-point 10-fold serial dilution of *Verticillium dahliae* (V781I) DNA (10 ng/µl) diluted in sterile, ultrapure water (Water series) or in DNA (20 ng) extracted from olive stems and leaves, or in DNA (5 ng) extracted from olive roots obtained with the SYBR-1 (this study) and SYBR-4 (3) and TAQ (6) quantitative polymerase chain reaction protocols. Cycle thresholds (C_T) were plotted against the log of genomic DNA standard curves of known concentrations. Data shown are from a representative experiment.

Finally, we selected the SYBR-4 protocol (based on the single-copy gene β-tubulin) and the TAQ protocol (based on the multi-copy region of ribosomal DNA [rDNA]) for subsequent analyses based on their increased specificity compared with SYBR-1 (Table 3). In silico analysis of the race 2 strain of *V. dahliae,* VdLs.17 genome has recently revealed that the VertBt primer pair occurs once (27).

Development of Verticillium wilt in Frantoio olive plants. Symptoms were absent in noninoculated plants after 35 and 122 days of incubation. Plants inoculated with *V. dahliae* were also asymptomatic at 35 days after inoculation. At 122 days after inoculation, two plants inoculated with D *V. dahliae* had a symptom severity score of 1 and 0.5, respectively, and one plant inoculated with ND *V. dahliae* had a symptom severity score of 1.5.

Isolations from inoculated plants indicated that infection of the root system by D and ND *V. dahliae* took place soon after inoculation. Thus, infections of root tissues by D and ND *V. dahliae* amounted to 15 and 50%, respectively, just after inoculation, and then increased to 55% for D *V. dahliae* at 2 and 10 days after inoculation and to 20% for ND *V. dahliae* at 2 and 15 days after inoculation. By 35 days after inoculation, percent root infections by D *V. dahliae* was 40%, whereas isolations failed to recover ND *V. dahliae* from root tissues at that same sampling time. The total amount of positive isolations estimated by the AUIC was significantly ($P < 0.001$) higher in roots inoculated with D *V. dahliae* than in those inoculated with ND *V. dahliae* (Fig. 2), with average infections per plant across the sampling period of 34.5 and 11.0% for D and ND *V. dahliae*, respectively.

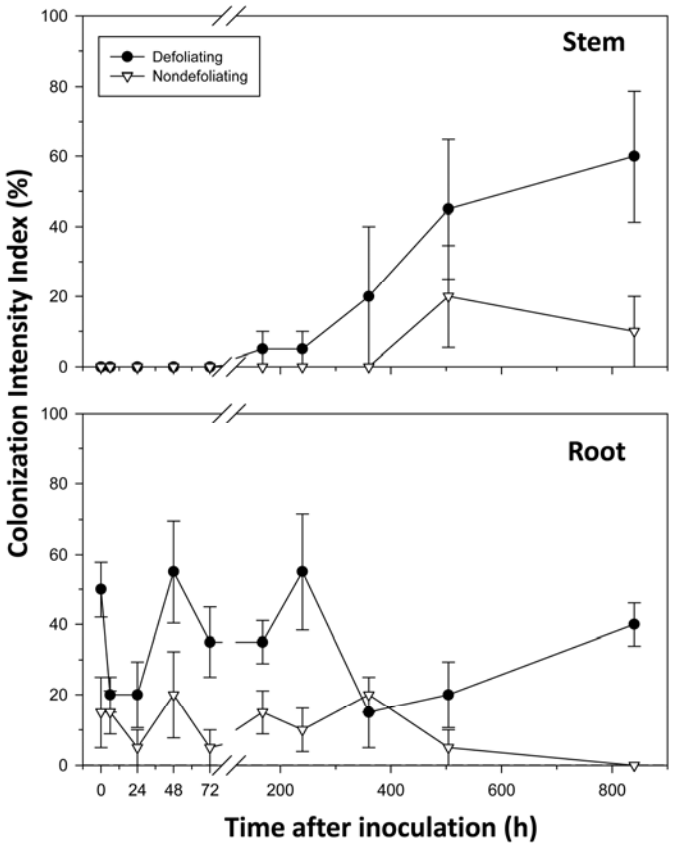

Fig. 2. Colonization of roots and stems of 'Frantoio' olive plants by *Verticillium dahliae* isolates V781I (defoliating pathotype) and V152I (nondefoliating pathotype). An index of colonization [IC = Σ(Ti × Nj)/(T × N) × 100] was calculated and plotted over time, where Ti is the number of plant pieces from which the pathogen was isolated, Nj is the number of plants from which the Ti stems or root pieces were obtained, T is the total number of plant pieces processed, and N is the total number of sampled plants. Each plotted point is the mean ± standard deviation value of five plants.

In contrast, the first *V. dahliae* isolation from stem was observed at 7 and 21 days after inoculation with D and ND *V. dahliae*, respectively. Later on, the IC in stems infected by the D *V. dahliae* pathotype steadily increased to 60% by the end of the bioassay, 35 days after inoculation. In contrast, the highest IC for ND *V. dahliae* was estimated at 20% by 21 days after inoculation. Globally, the AUIC was significantly ($P < 0.001$) higher in D *V. dahliae* infected stems compared with that in ND *V. dahliae* infected ones (Fig. 2).

Quantification of D and ND *V. dahliae* DNA in infected Frantoio olive plants by qPCR. All DNA samples extracted from olive root and stem tissues were suitable for qPCR experiments. Results of real-time qPCR assays using the SYBR-4 (3) and TAQ (6) qPCR protocols and samples of total genomic DNA extracted from stem and root tissues of D and ND *V. dahliae* inoculated plants are shown in Figure 3. Comparison of slopes and intercepts of regression lines generated for each protocol and DNA standard curves using a range of *V. dahliae* DNA (from 1 ng to 0.1 pg) in each of the assayed backgrounds showed no significant differences ($P > 0.05$) among all qPCR plates used, which allowed combining results from all experiments for analyses. For this assay, we fixed the detection limit at a C_T of 36, corresponding to ≈18 and 15 fg of *V. dahliae* DNA for SYBR-4 and TAQ protocols, respectively.

Both protocols yielded a similar trend in the amount of DNA of *V. dahliae* pathotypes quantified in olive tissues, with just some small differences that, overall, were not significant ($P > 0.05$) (Fig. 3). The amount of *V. dahliae* DNA differed significantly ($P < 0.05$) among roots and stems sampled from plants inoculated with D or ND *V. dahliae* at the different sampling times, being highest for root tissues. In roots and stems, the amount of DNA of D and ND *V. dahliae* was highest at 10 days after inoculation. Globally, for both protocols, the total amount of *V. dahliae* DNA in root tissues estimated by the AUAVDC was significantly ($P < 0.0001$) higher for the ND pathotype than the D one (Fig. 3), with mean values of 196 (SYBR-4) and 202 (TAQ) or of 40 (SYBR-4) and 35 (TAQ) ng of *V. dahliae* DNA per nanogram of olive root DNA, for the ND and D pathotypes, respectively. In contrast, for stems tissues, significantly ($P < 0.005$) higher AUAVDC values were estimated for D *V. dahliae* compared with that of ND *V. dahliae* (Fig. 3), with mean values of 48 (SYBR-4) and 46 (TAQ) or 5 (SYBR-4) and 8 (TAQ) ng of *V. dahliae* DNA per nanogram of olive stem DNA for the D and ND pathotypes, respectively.

In general, the amount of D and ND *V. dahliae* DNA in stems remained stable throughout the duration of the experiment, with the highest value occurring at 10 days after inoculation. Overall, in all olive tissues tested, *V. dahliae* DNA amount tended to slightly decrease or was stable by the end of the experiment, 35 days after inoculation.

DISCUSSION

Results of this comparative study demonstrated that some of the real-time qPCR protocols published in the last 10 years are not specific for *V. dahliae* or are inhibited when used in a background of plant tissue extracts. The study also selected two robust protocols (3,6) that were efficient in monitoring colonization of symptomless Frantoio olive plants by D and ND *V. dahliae* in a time course after inoculation.

Under our experimental conditions, protocols SYBR-1 (this study), SYBR-4 (3), and TAQ (6) performed best according to a WSI established for their objective evaluation (Table 3). The protocols were evaluated using R^2, AE, DL, and specificity.

When testing specific real-time qPCR protocols, it is important to determine that AE, sensitivity and reproducibility are not influenced by the presence of nontarget DNA from different sources (either plant or soil DNA). We statistically compared the slope and intercept parameters of the standard regression lines obtained for *V. dahliae* genomic DNA dilution series in a background of olive stem, leaf, or root DNA. A similar approach of validation procedure was used recently by other authors (3,13,40). Thus, statistical analysis of the obtained DNA standard regression lines

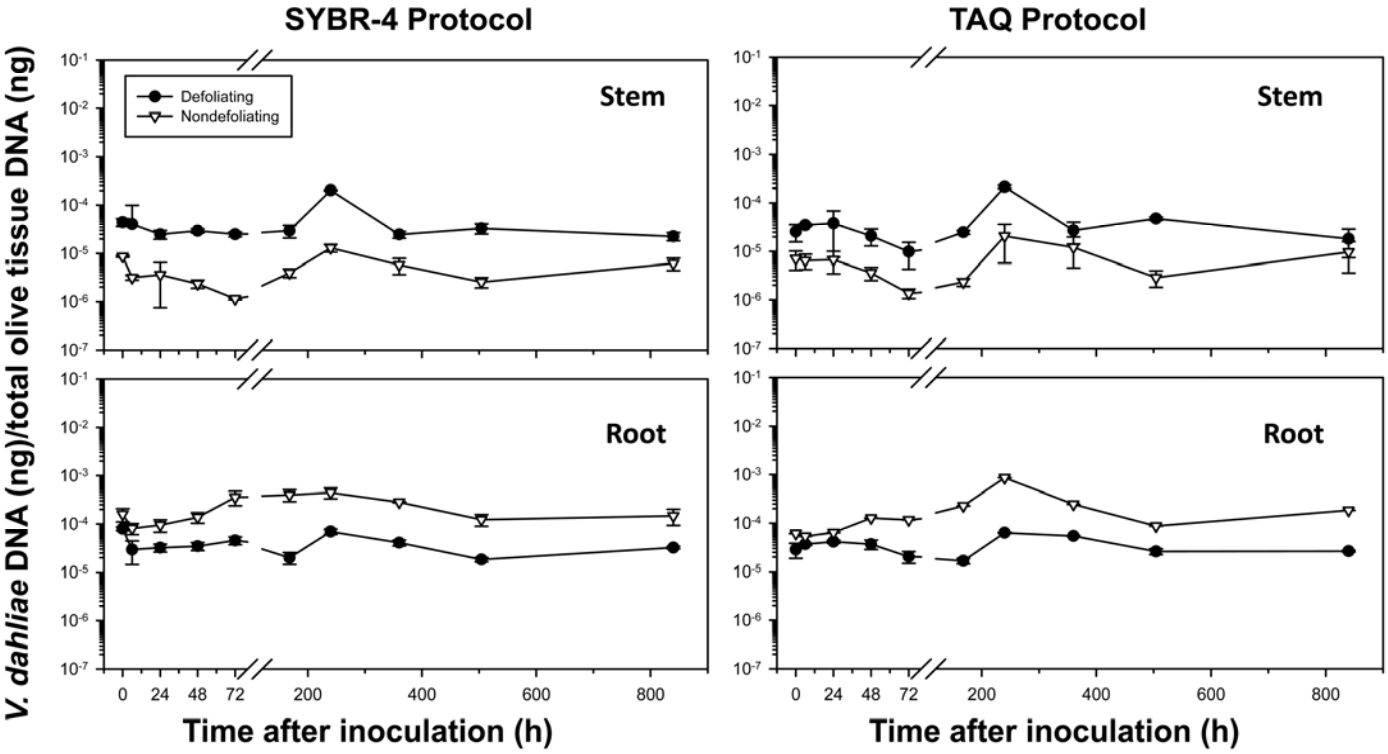

Fig. 3. Quantification of defoliating and nondefoliating *Verticillium dahliae* from roots, stems, and leaves of 'Frantoio' in a time course of infection bioassay. Each plotted point is the mean relative amount (nanograms of *V. dahliae* per nanograms of total genomic DNA extracted from olive tissue) ± standard deviation obtained from three independent DNA samples and two replications for each DNA sample.

indicated that neither background olive stem, leaf, or root DNA nor the operator performing assays significantly influenced performance of SYBR-4 (3), SYBR-1 (42), and TAQ (6) protocols demonstrating their robustness. Also, we demonstrated that sensitivity of the protocols was not affected by the plant tissue assayed because we could detect the fungus and quantify ≤100 fg of *V. dahliae* DNA in olive stem and root for SYBR-4 and TAQ protocols without loss of accuracy. A detection limit similar to that of the original protocol SYBR-4 was reported by Atallah et al. (3), who detected ≤148 fg of *V. dahliae* DNA in a background of potato tissue, which correspond to five nuclei based upon one genome in each nucleus ≈28 fg (48). The VertBt primers used in protocol SYBR-4 were recently applied with the use of an analytical grinder and an external control of spinach actin target DNA to quantify *V. dahliae* in spinach seed (13), showing a sensitivity limit of 1 infected seed per 100 seeds (i.e., ≈29 nuclei or 812 fg of DNA).

Other real-time qPCR protocols (i.e., SYBR-1, SYBR-2, SYBR-6, and SCP) assayed in this present study under our experimental conditions had shown lesser specificity or sensitivity levels for detection of *V. dahliae* in olive compared with that of SYBR-4 and TAQ. Thus, the high specificity and sensitivity of TAQ and SYBR-4 protocols make them suitable for accurately detecting and quantifying *V. dahliae* in asymptomatic reactions or incompatible combinations, for which the amount of pathogen DNA is expected to be rather low. Protocols SYBR-1 and TAQ were more sensitive than SYBR-4 because the intercepts of the standard DNA regression lines of those assays were significantly lower than that of SYBR-4. The higher sensitivity of SYBR-1 and TAQ protocols may result from the primers developed for them targeting the intergenic spacer (IGS) region of the rDNA occurring in multicopies. The fact that such targets within the rDNA occur in high copy numbers makes PCR assays using primers derived from rDNA regions easy, sensitive, and reliable in the detection of fungal pathogens. However, it should be taken into account that the number of rDNA copies in a microorganism may fluctuate with its age and stage of growth (43), as has been reported for different *Verticillium* spp. (45,47) as well as for different isolates of *V. dahliae* (6,45). For instance, Bilodeau et al. (6) compared the amplification of the rDNA and of several single-copy genes among isolates of *V. dahliae* and concluded that the number of rDNA copies was ≈24 to 73 per haploid genome (average of ≈46), depending upon the isolate. This variation in the copy number among *V. dahliae* isolates, together with findings in this present study of risk with the SYBR-1 protocol of cross-amplification with *V. albo-atrum, V. tricorpus*, and *V. longisporum*, led us to discard this latter protocol for further assays and to select SYBR-4 (3) and TAQ (6) as the protocols most appropriate for our purposes. One advantage of the SYBR-4 protocol over the TAQ protocol is that the SYBR-4 protocol is based on the nuclear gene β-tubulin which, in *V. dahliae*, occurs in single copy (27), thus allowing for potential comparisons among samples and consistent pathogen quantification regardless of age, growth stage, and nature of the *V. dahliae* isolate. In fact, Atallah et al. (3) observed that sequences of β-tubulin 2 provide polymorphisms that separate *V. dahliae* from closely related species such as *V. albo-atrum* and *V. tricorpus*. One possible shortcoming when using the SYBR-4 protocol could be the cross-amplification with *V. longisporum* (13), as we also found in our study. However, natural infections of olive trees by *V. longisporum*, a crucifer-specific pathogen, are yet to be demonstrated (15,18,24,34,48).

The selected SYBR-4 and TAQ protocols were further used for quantitatively monitoring the amount of pathogen DNA in the colonization of reportedly resistant 'Frantoio' by the D and ND *V. dahliae* pathotypes in a time-course infection bioassay. Although previous reports have highlighted the resistance of Frantoio olive to D (14,32,36) and ND (33) *V. dahliae* pathotypes under artificial and natural infections, in our study, we showed the ability of those two pathotypes to extensively infect and colonize systemically symptomless resistant Frantoio plants that had not been previously documented. This symptomless infection was consistently demonstrated in this present work both by means of direct isolation of the pathogen from stems and roots of inoculated plants on culture and by real-time qPCR assays using the SYBR-4 protocol and total DNA extracted from stems, roots, and leaves of those plants. Mercado-Blanco et al. (37) observed that slight wilt symptom expression of the highly resistant Acebuche-L olive genotype correlated with resistance to infection by the pathogen, as indicated by failure to quantify *V. dahliae* DNA in the stem. In this regard, it is worth mentioning the high inhibition of the primers and SYBR-2 protocol employed by Mercado-Blanco et al. (37) found in our study when spiking the standard curves with stem tissue DNA.

Based on our results, 'Frantoio' should be considered tolerant rather than resistant to *V. dahliae* pathotypes according to the concept of tolerance as a plant's ability to sustain extensive systemic colonization but express few, if any, symptoms and provide a reasonable crop yield (4,16). Nevertheless, the term "tolerance" might still be somehow controversial in Verticillium wilt of olive. Thus, whereas histopathological observations and frequency of *V. dahliae* isolation from olive root and stem convincingly indicated that colonization by the pathogen is hindered in resistant plants, corresponding to true resistance (2,34), Jiménez-Díaz et al. (24) indicated that the resistant reaction of certain olive cultivars does not exclude the pathogen from reaching the upper plant parts and being isolated from symptomatic or symptomless leaves. In our study, lesser amounts of pathogen DNA were quantified in Frantoio olive stem tissues compared with those in root tissues, which agrees with results reported by Mercado-Blanco et al. (37).

Differences in the degree of systemic colonization of resistant and tolerant plants by *V. dahliae* appear to be clearer in herbaceous hosts. For example, assessment of stem colonization in tomato plants by qPCR assay (8), and in *Arabidopsis thaliana* plants by tissue maceration and plating (53) indicated that the average amount of *V. dahliae* in tolerant plants showing few or no symptoms was very similar to that in susceptible plants but much higher than in the resistant ones. Further studies including more tolerant and resistant olive genotypes are needed to better understand differences in olive–*V. dahliae* interactions leading to true tolerant and resistant reactions.

Monitoring of colonization of symptomless Frantoio plants by *V. dahliae* indicated that differences in virulence of D and ND pathotypes on susceptible olive cultivars correlated with differences in the amount of *V. dahliae* DNA revealed by the SYBR-4 and TAQ protocols and percentage of positive pathogen isolations. However, the amount of each pathotype biomass varied with the olive tissue assayed. In general, the amount of *V. dahliae* DNA in stems was higher for the D than for the ND pathotype, whereas the opposite occurred for the amount of *V. dahliae* DNA in roots. Also, percentage of positive isolations from root and stem tissues was higher for the highly virulent D isolate than for the less virulent ND one. These findings are in agreement with those of Mercado-Blanco et al. (37), who observed higher average DNA amounts of ND pathotype in roots of 'Arbequina' and 'Picual' olive compared with that of the D pathotype but that the opposite occurred in the stem. Nevertheless, those authors and Markakis et al. (35) concluded that differences in the in planta amount of pathogen DNA was influenced to a larger extent by the olive genotype than by virulence of the infecting D or ND pathotype, suggesting that the extent of pathogen colonization does not solely determine the level of symptom expression or virulence phenotype (35,37).

In this present study, peaks of *V. dahliae* DNA occurred in all sampled tissues at different times after inoculation followed by intermittent periods of decreasing fungal biomass. However, increased pathogen biomass that occurred early in root tissues did

not exactly correspond to subsequent high *V. dahliae* biomass in stem tissues. The fluctuations in the fungal biomass in Frantoio olive plants observed in this study suggest that the pattern of colonization by *V. dahliae* was cyclical in nature, and similar to that reported for colonization of alfalfa and tomato by *V. albo-atrum* (19,22). Nevertheless, significant differences might occur between that pattern in resistant, tolerant, and susceptible reactions. For instance, Heinz et al. (19) observed that substantial amounts of *V. albo-atrum* were present in the upper tissues of resistant tomato plants within 2 to 4 days after root-dip inoculation, and concluded that this initial burst of colonization resulted from the earliest rounds of sporulation by the fungus. Thereafter, a decrease in the amounts of detected pathogen DNA may correspond with partial lysis of *Verticillium* sp. propagules as a consequence of defense mechanisms in the infected plant that are not sufficient to interfere with subsequent events of fungal conidiogenesis and cyclic colonization during host growth (19, 35,37). In our study, we observed a steady and progressive decrease in the amount of *V. dahliae* DNA over time, especially in root tissues, and a complete failure of positive isolation of the ND, which is similar to that reported by Mercado-Blanco et al. (37) and Markakis et al. (35). This decrease in fungal biomass could be attributed to the natural phenomenon of recovery from disease of tolerant genotypes associated with mechanisms that allow olive trees to overcome injury and decay, and can be activated after infections by *V. dahliae* (24).

In conclusion, this present work identified SYBR Green and TAQ protocols targeting the β-tubulin (3) and the IGS genes, respectively, as superior real-time PCR techniques for the specific detection and quantification of *V. dahliae* in different asymptomatic tissues of resistant or tolerant olive genotypes. The procedures used in this study revealed the presence of *V. dahliae* in upper tissues of symptomless plants, which makes implementation of these techniques suitable for certification schemes of pathogen-free planting material as well as breeding programs for development of new olive genotypes highly resistant to the D pathotype of *V. dahliae*, the current most important pathogen for olive production.

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