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Quantitative monitoring of colonization of olive genotypes by *Verticillium dahliae* pathotypes with real-time polymerase chain reaction

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Abstract

For Verticillium wilt diseases, quantitative measurement of the pathogen in infected tissues is necessary for characterizing the disease reaction of host genotypes to pathogen strains. The amount of pathogen DNA in the plant should be representative of the number of potential propagules. In this work, we quantified the DNA of highly virulent, defoliating (D) and mildly virulent, nondefoliating (ND) *Verticillium dahliae* in Verticillium wilt resistant (Acebuche-L), and -susceptible (Arbequina and Picual) olive genotypes by real-time polymerase chain reaction (PCR) assays. We show that the amount of pathogen DNA quantified in each olive genotype correlated with susceptibility to Verticillium wilt, but was influenced less by virulence of the infecting *V. dahliae* pathotype, suggesting that the extent of pathogen colonization does not clearly determine the virulence phenotype. Maximal pathogen DNA occurred in root and stem tissues before disease had fully developed in the susceptible olive genotypes, with pathogen DNA content in stem tissue being lower than that in root tissues. A boost in *V. dahliae* propagules in root tissues, as indicated by the amount of D and ND DNA, took place 1 week after inoculation, followed by a decrease over time. That decrease was sharp in Arbequina and Acebuche-L plants compared with a progressive reduction in Picual. The amount of *V. dahliae* DNA in roots of Picual plants remained at a high level for a long period of time following inoculation, but it eventually decreased too. Similar changes in pathogen DNA amounts over time occurred also in stem. Verticillium wilt hardly developed in Acebuche-L plants, but the amount of D and ND *V. dahliae* DNA in roots was comparable to that found in Arbequina plants. However, pathogen DNA could not be quantified in stems of Acebuche-L plants though it was detectable in them by nested-PCR. The results in our study show that real-time PCR is an excellent tool for quantitative diagnosis of olive infection by D and ND *V. dahliae*; as well as for monitoring pathogen colonization and assessing resistance and tolerance to Verticillium wilt in olive genotypes. This would help in olive breeding programs for resistance to the disease.

Keywords: Fungal propagules; In planta DNA quantification; *Olea europaea*; Real-time quantitative Polymerase chain reaction; Resistance; Tolerance; Verticillium wilt

1. Introduction

In Verticillium wilt diseases, the extent of vascular colonization may correlate with resistance or susceptibility to the pathogen. However, mild symptoms may occur in tolerant hosts showing resistance to disease without actual reduction in the pathogen growth within the plant [8,21]. Quantitative measurement of pathogen biomass in the plant would help in the characterization of host resistance or susceptibility to strains of *Verticillium* spp., as well as virulence of those strains to susceptible host genotypes. Biomass quantification of *Verticillium* pathogens in planta is intrinsically difficult as the fungus is characteristically confined to nonliving elements of the xylem throughout most of the period of pathogenesis [21]. In addition, quantitative assessment of *Verticillium* colonization have been hindered by lack of suitable methodology, which was based on culturing colonies from tissue sections or plating out fungus–host comminutes on nutritive media, e.g. [9].

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More recently, polymerase chain reaction (PCR)-based procedures were developed and used successfully to determine pathogen DNA in *Verticillium*-infected herbaceous hosts such as alfalfa (*Medicago sativa*) [12] and tomato (*Lycopersicon esculentum*) [10]. However, PCR-based methods have not been used for pathogen quantification in Verticillium wilt diseases of woody hosts such as olive (*Olea europaea*).

Verticillium wilt, caused by *Verticillium dahliae*, severely affects olive worldwide [13]. In Spain, severe attacks by the disease occurred in adult olive orchards [3] as well as in newly established ones [28]. Isolates of *V. dahliae* infecting olive can be classified as defoliating (D) and nondefoliating (ND) pathotypes according to their ability or inability, respectively, to defoliate the plant [26,29]. ND *V. dahliae* induces mild symptoms in susceptible olive cultivars, such as Arbequina and Picual, which may recover from disease when held for an extended period of time after inoculation [16,26]. Conversely, infections by D *V. dahliae* can be lethal to those same cultivars [15,26]. However, the two *V. dahliae* pathotypes were weakly virulent on the Acebuche-L olive genotype, which showed a resistant disease reaction in artificial inoculations with them [20]. Until recently, attacks by Verticillium wilt in olive orchards in Spain were caused primarily by ND *V. dahliae* [13]. However, the D pathotype has now spread from the area in southern Spain where it was first reported [4] to distant olive-growing areas in the same region, causing severe Verticillium wilt in newly established olive orchards [5,15, 19 and Jiménez-Díaz, unpublished work].

In previous studies, we developed a PCR procedure for the early and differential detection of both D and ND *V. dahliae* in infected olive plants [16,17]. Those studies revealed consistent differences in amount of the specific D and ND PCR products in agarose gels using as template total genomic DNA extracted from infected Picual plants. Usually, the most abundant PCR products were obtained with DNA from D *V. dahliae*-infected plants sampled just before expression of severe disease symptoms and that amount decreased shortly thereafter [17]. Although those PCR results were not quantitative in nature, we speculate that such a reduction in the amount of PCR products with DNA template sampled at the time the disease fully developed could indicate a decrease of the number of potential pathogen propagules within the infected plant. Such a speculation would agree with fluctuations of *V. dahliae* biomass in infected olive tissues found in an earlier study [26] while assessing *V. dahliae* colonization of olive plants by serial dilution plating of ground tissues. Also, similar findings were reported for *Verticillium albo-atrum* colonization in alfalfa and tomato [10,12] by means of a PCR-based quantitative approach.

The recently developed real-time PCR enables the quantification of nucleic acids in unknown samples by a direct comparison to standards amplified in parallel reactions [18] and promises to be useful for quantitative diagnosis of plant pathogens at low levels of infection. Real-time PCR is more accurate and less time consuming than conventional, end-point quantitative PCR because it monitors PCR products as they accumulate in the exponential phase, before reaction components become limiting. Additionally, real-time PCR is less affected by amplification efficiency, and it allows measurement of the melting profile of the PCR products [25].

Real-time PCR is being increasingly used in plant pathology for the accurate detection and quantification of pathogens in infected plants, even at very low levels of infection [2,7,14,30,31]. In addition to quantitative diagnostics, real-time PCR can also be an excellent tool for monitoring pathogen infection. So far, *Verticillium* spp. have been quantified in infected herbaceous hosts (alfalfa, potato, tomato) by assessing the amount of pathogen DNA using PCR-based quantitative procedures other than real-time PCR [8,10,12]. However, the biomass of D and ND *V. dahliae* in infected olive plants has not yet been quantified. Use of real-time PCR might provide accurate quantification of *V. dahliae* DNA in infected plants and thus of the number of potential pathogen propagules associated with disease reaction of olive genotypes to *V. dahliae* pathotypes. Therefore, the aim of this study was to test whether severity of disease and virulence of pathotypes are correlated with the amount of pathogen DNA in plant tissues. To achieve this aim, we carried out time-course infection bioassays of Verticillium wilt-susceptible and -resistant olives with D and ND *V. dahliae*, and monitored pathogen colonization in root and stem tissues by means of real-time PCR.

2. Materials and methods

2.1. Chemicals and media

Reagents used in this study were from Sigma Chemical Co, St Louis (MO) USA; Merck, Darmstadt, Germany; or Panreac, Barcelona, Spain, unless otherwise indicated. Media were made with deionized water and autoclaved at 121 °C for 20 min. Potato-dextrose agar (PDA) and potato-dextrose broth (PDB) were from Difco Laboratories, Detroit (MI) USA.

2.2. Fungal isolates and culture conditions

V. dahliae isolates V4I and V138I which are representatives of the ND and D pathotypes, respectively, were used. These isolates were characterised by biological and molecular pathotyping in previous studies [4,22] and are deposited in the fungal culture collection of the Departamento de Protección de Cultivos, Instituto de Agricultura Sostenible (C.S.I.C.), Córdoba, Spain. Single-spore cultures of isolates on plum-extract agar were stored by covering them with liquid paraffin [4], at 4 °C in the dark. Active

cultures of isolates were obtained on chlorotetracycline-amended (30 mg l^{-1}) water agar (CWA) and maintained by further subculturing on PDA. Cultures on PDA were grown for 7 days at 24 °C in the dark.

2.3. Plant material

Plants (3- to 8-month-old) of olive genotypes Acebuche-L, Arbequina and Picual were used. Plants were kindly provided by Cotevisa (L'Alcudia, Valencia, Spain). Arbequina and Picual are cultivars grown extensively throughout Spain [1]. Plants of those cultivars were obtained routinely by micropropagation techniques. Acebuche-L (commercial name) is a wild-olive genotype. Acebuche-L plants developed from seeds originating from the L'Emilia Romagna region in Italy (Lorenzo García, Cotevisa, personal communication).

2.4. Fungal and plant DNA extraction

Mycelia of *V. dahliae* isolates from cultures on PDA were lyophilised and ground to a fine powder using an autoclaved mortar and pestle, as previously described [22]. Fifty milligrams of powdered mycelia were used for DNA extraction according to Raeder and Broda [23]. Those DNAs were used as appropriate controls in PCR reactions.

Total genomic DNA was extracted from roots and stems (20 mg of freeze-dried tissue powder) of noninfected and *V. dahliae*-infected Acebuche-L, Arbequina, and Picual plants, using the commercially available DNeasy Plant Mini Kit (DNeasy) (Qiagen, Hilden, Germany). Previously, those plant tissues were manipulated, disinfested, freeze-dried, and ground to a fine powder as previously described [16,26]. Quality of extracted DNAs was verified by running aliquots (5 μl) in ethidium bromide stained agarose gels (0.7% w/v in TAE buffer) [27] and further visualization under UV light. Additionally, DNAs were also spectrophotometrically quantified in a BioPhotometer (Eppendorf AG, Hamburg, Germany).

2.5. Olive–Verticillium dahliae time-course of infection bioassays

The olive genotypes (Acebuche-L, Arbequina, and Picual) and *V. dahliae* isolates (V4I and V138I) used in this study were chosen to cover a range of plant/pathogen interactions differing widely in the level of disease reaction.

There were 98 plants of each olive genotype for each of the infection bioassays in the study. The group of 98 plants was divided into three treatments: (i) noninoculated control (16 plants); (ii) V4I-inoculated (41 plants); and (iii) V138I-inoculated (41 plants). Of the 41 plants inoculated with each of the *V. dahliae* isolates, 21 were sampled for isolations and in planta detection of the pathogen by specific nested-PCR, as well as quantification of D and ND *V. dahliae*, according to a time-course sampling schedule (three plants per each of seven sampling times between 0 and 75 days after inoculation, see below). The remaining 20 plants corresponding to each isolate were kept as a reference for symptom development and disease assessment. Of those latter plants, three plants were also randomly sampled at each of 76 and 100 days after inoculation (see below), at which times disease severity was also scored. Therefore, a total number of 27 inoculated plants were sampled for each olive genotype-*V. dahliae* pathotype interaction. In addition, two noninoculated plants were sampled at each of times 0, 52, and 100 days after inoculation to check for any accidental contamination both in the molecular and isolation analyses.

Plants were root-dip inoculated for 20 min in a suspension of 10^7 conidia ml^{-1} of *V. dahliae* V4I or V138I isolates. Conidia were obtained from 7- to 10-day-old cultures in PDB as previously described [16,17,26]. At this time, plants were transplanted into sterilised soil (sand:loam, 2:1, v/v) in sterilised clay pots (16.5 cm diameter × 15.5 cm height), and incubated in a growth chamber adjusted to 24/22 °C light/dark, 70–80% relative humidity, and a 12-h photoperiod of fluorescent light of 262 μE m^{-2} s^{-1}. Plants were watered every 1–2 days and fertilized weekly with 50 ml/pot of Hoagland's nutrient solution [11]. The experiment was arranged as a completely randomized design. Plants were observed for symptom development every 2–3 days. The disease reaction was assessed by severity of symptoms on a 0–4 scale according to the percentage of affected leaves and twigs (0 = no symptoms, 1 = 1–33%, 2 = 34–66%, 3 = 67–100%, and 4 = dead plant) at weekly intervals [16,17,26]. Data on disease severity were used to calculate a disease intensity index (*DII*) determined as $\mathrm{DII} = \sum(S_i N_i)/4N_t$, where S_i is the symptoms severity, N_i is the number of plants with S_i symptoms severity, and N_t is the total number of plants. DII data were plotted over time in days to obtain disease progress curves. Final DII values were subjected to analysis of variance using Statistix (NH Analytical Software, Roseville, MN). Treatment means were compared using Fisher's protected least significance difference (LSD) at $P < 0.05$.

2.6. Isolation of Verticillium dahliae from inoculated plants

Vascular colonisation by *V. dahliae* was determined in each of the sampled olive plants by isolating the fungus on CWA. For each plant, three 5- to 10 mm-long stem and root pieces were thoroughly washed (25–30 min running water), surface-disinfested in NaClO (0.5% available chlorine) for 1.5 min (stems) or 2 min (roots), rinsed with sterile water, plated onto the medium, and incubated at 24 °C in the dark for 15–20 days [16,26]. The remaining stem and root tissues of each sampled plant were saved for PCR detection and real-time PCR quantification assays (see below). For stem pieces, the bark was removed with a clean scalpel before disinfestation. The plated stem pieces were representative of

the upper, middle and lower parts of the total length of the sampled stems. Root pieces were randomly chosen from the whole radical system of the sampled plants. Bark was not removed from root pieces.

2.7. In planta-PCR detection of D and ND Verticillium dahliae

The roots and stems of inoculated plants were sampled separately in a time-course after inoculation, for PCR detection, real-time PCR quantification, and isolation of *V. dahliae*. Three plants were destructively sampled at each of times 0, 2, 7, 15, 24, 41, 52, 76, and 100 days after inoculation with either of *V. dahliae* pathotypes. The entire stem and the whole radical system of each plant were used for extraction of total genomic DNA, except for tissue pieces previously removed for isolations in pure culture. All tissues were washed and disinfested as indicated above. The extracted DNA samples were used as template in the following PCR assays.

2.7.1. Random amplified polymorphic DNA-PCR

Preliminary random amplified polymorphic DNA (RAPD)-PCR assays were conducted to assess PCR-quality of total genomic DNA extracted from olive plants in the study. Reactions (final volume 25 μl) consisted of 0.5 μM OPH-20 primer (Operon Technology, Alameda, CA, USA), 200 μM each dNTPs, 2.5 μM 10 × reaction buffer (20 μM KCl, 10 mM Tris–HCl pH 9.0, 1% v/v Triton X-100), 0.75 U EcoTaq DNA polymerase (Ecogen, S.R.L., Barcelona, Spain), 2.5 mM $MgCl_2$, and 10–30 ng of template DNA. Reactions were conducted as follows: a denaturation step of 4 min at 94 °C, 30 cycles of 1 min denaturation (94 °C), 1 min annealing (37 °C), and 3 min extension (72 °C). A final extension step of 6 min (72 °C) was included.

2.7.2. Nested-PCR for in planta detection of Verticillium dahliae pathotypes

Nested-PCR assays were conducted to re-confirm the presence of D and ND *V. dahliae* in the inoculated olive plants. For detection of ND *V. dahliae*, nested-PCR assays were carried out according to Mercado-Blanco et al., [16] using primer pairs NDf/NDr (yielding a single-PCR product of 1410-bp) and INTND2f/INTND2r (yielding a nested-PCR product of 824-bp) which are specific for the ND pathotype. Primer pairs D1/D2 (single-PCR product of 548-bp) and INTD2f/INTD2r (nested-PCR product of 462-bp), specific for the detection of the D pathotype, were used in nested-PCR assays as previously described [17]. In addition to nested-PCR assays, single-PCR assays were also carried out using primer pairs INTND2f/INTND2r and INTD2f/INTD2r. This was done to detect possible differences in the abundance of pathogen DNA in infected olive plants throughout the time-course of infection bioassays.

RAPD and nested-PCR reactions were performed in a PTC-100™ Programmable Thermal Controller (MJ Research, Inc., Watertown, Mass., USA), or in a GeneAmp PCR System 9600 thermocycler (Perkin Elmer, Norwalk, CT, USA). Amplification products were separated on 1% agarose gels, stained with ethidium bromide, and visualised under UV light. The DNA size markers used for electrophoresis were from Roche Diagnostic S.L. (Barcelona, Spain). Reactions were repeated at least three times and always included negative controls (no DNA) and positive controls (DNA of *V. dahliae* isolates V4I and V138I DNA purified from pure mycelia).

2.7.3. Real-time quantitative PCR

Real-time PCR assays for quantification of D and ND *V. dahliae* DNA were conducted using primer pair DB19/DB22 [6]. DNA templates (100 ng of DNA extracted from roots and 200 ng of DNA extracted from stems) for the assays were first quantified spectrophotometrically as indicated above. Primer pair DB19/DB22 defines amplicons of 539-bp in D isolates and 523 bp in ND isolates of unknown genomic sequence (data not shown). We chose this primer pair to avoid any possible influence in amplification efficiency of the two different primer pairs used routinely for in planta detection of D and ND *V. dahliae* by nested-PCR. Real-time PCR was performed in an iCycler (BioRad, Hercules, CA, USA) apparatus and results were analysed with the manufacturer's software (Optical System Software v 3.0a). Reaction mixtures (30 μl) consisted of 100 nM of each primer, 1.2 mM of each dNTP, 2.5 mM $MgCl_2$, 3 μl of 10 × reaction buffer (50 μM KCl, 10 mM Tris–HCl pH 9.0 (25 °C), 1% v/v Triton XC-100), 0.9 U EcoTaq DNA polymerase (Ecogen, S.R.L., Barcelona, Spain), 3 μl Sybr-Green (Molecular Probes, Inc., Eugene, OR, USA) 1:15000 v/v in water (1:100) and DMSO (1:150), and 100 (200) ng of template DNA. Three simultaneous, replicated amplifications were carried out for each DNA sample, using 30 μl-aliquots from a 90 μl mixture. This was done to enhance intra-assay accuracy and overcome reaction-to-reaction differences resulting from factors such as variable PCR efficiency and measurements among wells, pipetting, etc. Amplification reactions were performed in 96-well microtiter plates (BioRad, Hercules, CA, USA). Each plate contained all DNA samples corresponding to a single olive genotype/*V. dahliae* pathotype interaction and tissue (roots, stems) assayed, and every reaction was made up using aliquots of the same master mix. Thus, all DNA samples (and their replicates) were submitted to the same experimental conditions. Additionally, each plate always contained known *V. dahliae* DNA samples that were used to develop the standard curve (see below), as well as DNA samples from noninoculated olive plants and a negative control reaction (no template DNA). For each *V. dahliae* pathotype/olive genotype interaction, the assay of DNA extracted from roots was repeated in two different plates to assess inter-assay variability. A shortage of extracted DNA for some stem

samples forced us to run one plate only, although we carried out six replicates per sample when possible. The real-time PCR program was: an initial step of denaturation (4 min, 95 °C) followed by 40 cycles of 1 min at 94 °C, 45 s at 54 °C, 45 s at 72 °C, and 25 s at 90 °C. Fluorescence emission of the target amplicon (T_m = 92.5 °C) was measured at 90 °C. A final extension step of 4 min at 72 °C was added. After that, a melting curve program was run for which measurements were made at 0.5 °C temperature increments every 10 s within a range of 60–100 °C. Finally, PCR products were also visualised in ethidium bromide-stained agarose gels (1% in TAE buffer) [27] under UV light, as an additional check of amplification.

A standard curve was developed by plotting the logarithm of known concentrations (10-fold dilution series from 10 ng to 1 pg/30 μl reaction) of *V. dahliae* isolates V176I (ND) or V138I DNA [22] against the Ct values (Fig. 2). Ct is the cycle number at which the fluorescence emission of the PCR product is statistically significant from the background. Ct value is inversely related to log of initial concentration, so that the lower Ct value the higher initial DNA concentration. To better simulate conditions of amplification of those serially diluted DNA samples to unknown samples, equal amounts (80 or 200 ng) of DNA from noninoculated olives were added to each sample in the dilution series. Those latter samples, together with standard samples run in parallel reactions in each of amplification runs (see above), served to develop a standard curve from which the DNA concentration of unknown samples could be derived.

In addition to the RAPD-PCR assays indicated above, the quality of DNA templates was also assessed using primer pair Ias22f/Ias22r and total genomic olive DNA templates representative of sampling during the time-course of infections. Primer pair Ias22f/Ias22r amplifies a single sequence repeat (SSR) marker of ca. 130-bp in PCR assays using olive DNA as template [24]. Therefore, that amplicon was used as an internal positive control to distinguish uninfected olive tissues from PCR inhibitors. Results of quantification of the 130-bp amplicon were also used to calibrate the quantification of *V. dahliae* DNA. Since olive DNA could therefore be quantified, we could check for the real amount of DNA template added to each real-time PCR assay (which was determined spectrophotometrically). Reaction mixtures (30 μl) consisted of 100 nM of each primer, 1 mM of each dNTP, 2.5 mM $MgCl_2$, 3 μl of 10 × reaction buffer, 0.9 U EcoTaq, 3 μl Sybr-Green (prepared as above), and 33 ng of template DNA. The real-time PCR program was as follows: an initial step of denaturation (4 min, 95 °C) followed by 35 cycles of 1 min at 94 °C, 30 s at 64 °C, 30 s at 72 °C, and 15 s at 82 °C. Fluorescence emission of the target amplicon (T_m = 83.5–84.5 °C) was measured at 82 °C. A final extension step of 5 min at 72 °C was added. Data of quantified in planta *V. dahliae* DNA for each olive genotype-plant tissue-*V. dahliae* pathotype combination was subjected to analysis of variance according to a split-plot treatment arrangement in a completely randomized design with three replications (plants). Pathoytpes (D, ND) were assigned to whole plots and sampling time (nine) to subplots. Treatment means were compared using Fisher's protected least significant difference (LSD) test at $P \leq 0.05$.

3. Results

3.1. Development of Verticillium wilt on olive genotypes

No symptoms developed in noninoculated plants and *V. dahliae* was never isolated from them. Verticillium wilt in the inoculated plants was influenced by olive genotype and *V. dahliae* isolate (Fig. 1). Very low disease incidence and severity, and consequently low disease intensity index (DII), occurred in Acebuche-L olives over time, indicating that this genotype was resistant both to D and ND *V. dahliae*. First symptoms developed by 8 (time T_8) and 21 (time T_{21})

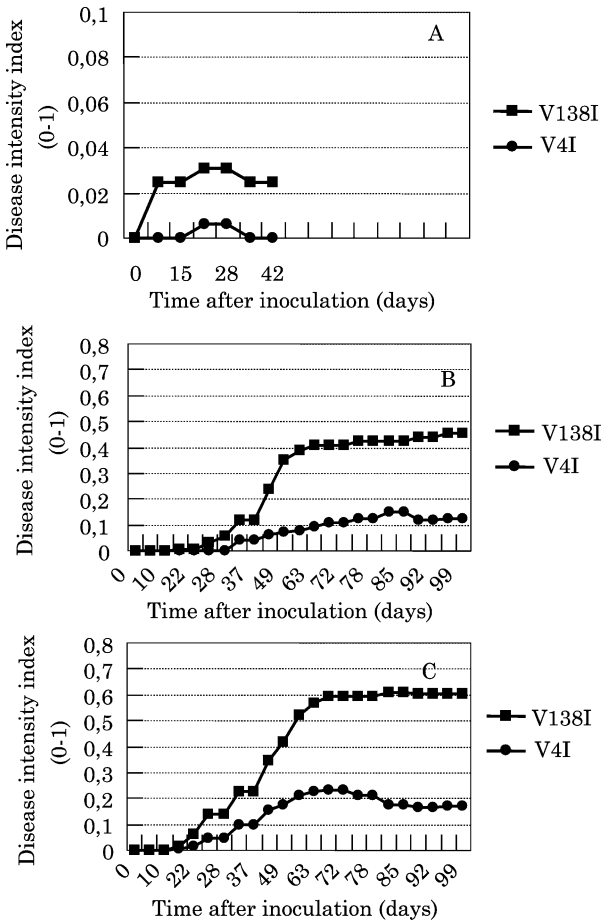

Fig. 1. Verticillium wilt disease progress curves on Acebuche-L (A), Arbequina (B), and Picual (C) olives inoculated with 10^7 conidia ml^{-1} of defoliating (V138I) and nondefoliating (V4I) *V. dahliae* isolates. Severity of symptoms was assessed on a 0–4 rating scale (0 = no symptoms, 4 = dead plant). A disease intensity index (DII = $\Sigma(S_iN_i)/4N_t$, which has maximum value of 1.0, see Section 2) was calculated and plotted over time. Each plotted point is the mean value of 20 plants. Note that vertical scales are different in A from B and C.

days after inoculation with *V. dahliae* isolates V138I (D) and V4I (ND) isolates, respectively. A maximum disease incidence of 10 and 5% for V138I- and V4I-inoculated plants, respectively, developed by 21 days after inoculation. The highest disease intensity for both *V. dahliae* isolates was also recorded at time T_{21}, with maximal disease intensity indices (DII_{max}) of 0.031 and 0.006 in V138I- and V4I-inoculated plants, respectively. Thereafter, DII values decreased so that there were very mild symptoms in 5% of the V138I-inoculated plants and no symptoms in V4I-inoculated ones at the end of the bioassay (Fig. 1A). Isolations from roots of Acebuche-L plants indicated that 11% of V138I-inoculated plants and 44% of V4I-inoculated ones were infected with *V. dahliae* (Tables 1 and 2).

Development of Verticillium wilt in V138I- and V4I-inoculated Arbequina plants occurred by times T_{14} (V138I-inoculated plants, 5% disease incidence) and T_{35} (V4I-inoculated plants, 30% disease incidence). All V138I-inoculated plants and 95% of V4I-inoculated ones were affected by time T_{76}. Disease intensity developed more rapidly in V138I-inoculated plants compared with V4I-inoculated ones (Fig. 1B). By time T_{98}, 20% of V138-inoculated Arbequina olives were dead and 40% developed symptoms in 67% or more of the aerial part (symptom severity score 3), whereas 70% of V4I-inoculated plants had no symptoms or symptoms in just 0–33% of the aerial part. The final DII in V138I-inoculated plants (0.454) was significantly ($P < 0.05$) higher than that of V4I-inoculated plants (0.123). Isolations from V138I- and V4I-inoculated Arbequina plants yielded *V. dahliae* from 33.3 and 37% of roots, and from 55.6 and 29.6% of stems, respectively (Tables 1 and 2).

First symptoms of Verticillium wilt in V138I(D)- and V4I(ND)- inoculated Picual plants developed by 14 days after inoculation (time T_{14}), at which time disease incidence was 10 and 5% in V138I- and V4I-inoculated plants, respectively. Maximal disease incidence was recorded at

Table 1
Comparative detection of defoliating *Verticillium dahliae* V138I in roots of Acebuche-L, Arbequina and Picual olives by means of isolation, nested-PCR, and real-time quantitative PCR (RT-QPCR) (see text for details)

Sampling time (days) (a)	Plant (no.)	Acebuche-L			Arbequina			Picual		
		Isolation (b)	Nested-PCR (c)	RT-QPCR (d)	Isolation (b)(e)	Nested-PCR (c)	RT-QPCR (d)	Isolation (b)(e)	Nested-PCR (c)	RT-QPCR (d)
0	1	+	+	0.0149	+ (0)	+	0.0015	− (0)	+	0.0023
	2	−	+	0.0008	+ (0)	+	0.0026	− (0)	+	0.0022
	3	−	+	0.0040	− (0)	+	0.0025	− (0)	+	0.0074
2	4	−	+	0.0226	− (0)	+	0.0174	− (0)	+	0.0871
	5	−	+	0.0376	− (0)	+	nq	− (0)	+	0.0331
	6	+	+	0.1084	+ (0)	+	0.0339	− (0)	+	0.0691
7	7	−	+	0.5889	+ (0)	+	0.9444	− (0)	+	1.2308
	8	+	+	0.2264	+ (0)	+	4.1244	+ (0)	+	1.5496
	9	−	+	2.4004	+ (0)	+	3.8490	− (0)	+	nq
15	10	−	+	0.1462	− (0)	+	0.4519	− (0)	+	1.4132
	11	−	+	0.3936	− (0)	+	0.1678	− (0)	+	0.0462
	12	−	+	0.6383	+ (0)	+	0.3388	− (0)	+	0.1333
24	13	−	+	0.0812	+ (0)	+	0.3891	+ (0.5)	+	1.1096
	14	−	+	0.1872	− (0)	+	0.0966	− (0.5)	+	0.7588
	15	−	+	0.0803	+ (0)	+	0.2630	− (0.5)	+	0.2673
41	16	−	+	0	− (0)	+	0.6041	− (0.5)	+	0.5334
	17	−	+	0.0452	− (0)	+	0.2344	− (3)	+	1.7589
	18	−	+	0.0491	− (3)	+	0.3350	− (3)	+	1.4970
52	19	−	+	0.0185	− (3)	+	0.1071	+ (3)	+	0.0977
	20	−	+	0.0305	− (1)	+	0.0501	+ (3.5)	+	0.2851
	21	−	+	0.0173	− (0)	+	0.0803	+ 0.5)	+	0.2722
76	22	−	+	0.0068	− (4)	+	nq	− (3)	+	0.0168
	23	−	+	0.0101	− (0)	+	0.0501	− (3)	+	0.0354
	24	−	+	0.0025	− (1)	+	0.0457	− (0)	+	0.0051
100	25	−	+	0.0013	− (3.5)	+	0.0127	− (2)	+	0.0475
	26	−	+	0.0008	− (3)	+	0.1022	− (4)	+	0
	27	−	+	0.0020	− (0.5)	+	0.0087	− (2)	+	0

(a) Arbequina plants were sampled at times 0, 2, 7, 15, 23, 41, 53, 75, and 98 days after inoculation. (b) Growth of *V. dahliae* occurred from at least one out of the three root pieces per plant used for isolations. (c) Nested-PCR assays were performed with primers specific for both defoliating and nondefoliating *V. dahliae* [16,17]. (d) Mean *V. dahliae* DNA amount in ng /100 ng total genomic DNA extracted from roots of three sampled plants; nq = not quantified. An RT-QPCR value of 0 meant no quantification or values considered not suitable for quantification (< 1pg or with a Ct value ≥ 37) (see text for details). (e) In brackets, symptom severity (0-4) scored at the time that the plant was sampled. Acebuche-L plants sampled did not show any symptoms.

Table 2
Comparative detection of nondefoliating *Verticillium dahliae* V4I in roots of Acebuche-L, Arbequina, and Picual olives by means of isolation, nested-PCR, and real-time quantitative PCR (RT-QPCR) (see text for details)

Sampling time (days) (a)	Plant (no.)	Acebuche-L			Arbequina			Picual		
		Isolation (b)	Nested PCR (c)	RT-QPCR (d)	Isolation (b)(e)	Nested PCR (c)	RT-QPCR (d)	Isolation (b)(e)	Nested PCR (c)	RT-QPCR (d)
0	1	+	+	0	− (0)	−	0	− (0)	+	0.0011
	2	+	+	0.0009	− (0)	+	0.0018	− (0)	+	0.0027
	3	+	+	0.0038	+ (0)	+	0.0073	− (0)	+	0.0031
2	4	+	+	0.0033	+ (0)	+	0.1496	− (0)	+	0.0266
	5	+	+	0.0028	− (0)	+	0.0118	− (0)	+	0.0245
	6	+	+	0.0038	+ (0)	+	0.0881	− (0)	+	0.0018
7	7	+	+	0.0988	+ (0)	+	2.7881	+ (0)	+	0.7501
	8	+	+	2.4563	− (0)	+	0.7163	+ (0)	+	2.4848
	9	+	+	1.0355	+ (0)	+	2.5427	+ (0)	+	0.8514
15	10	+	+	0.6762	− (0)	+	0.0767	− (0)	+	1.3188
	11	+	+	0.2818	+ (0)	+	0.2482	+ (1)	+	2.9875
	12	+	+	0.0653	+ (0)	+	nq	+ (0.5)	+	2.5427
24	13	−	+	nq	− (0)	+	0.0653	− (0.5)	+	0.6239
	14	−	+	0.6311	+ (0)	+	0.3350	− (0.5)	+	0.8416
	15	−	+	0.9019	− (0)	+	0.0944	− (0.5)	+	1.0237
41	16	−	+	0.5189	− (0)	+	0.3126	− (0)	+	0.0518
	17	−	+	0.1995	+ (0)	+	0.5867	− (0)	+	0.5822
	18	−	+	0.0776	− (0.5)	+	0.1011	− (0)	+	0.0278
52	19	−	+	0.2570	− (0.5)	+	0.0582	+ (0)	+	0.1412
	20	−	+	0.2238	− (0)	+	0.0467	− (1)	+	0.0367
	21	−	+	0.0881	+ (0)	+	0.0359	− (0)	+	0.0831
76	22	−	+	0.0376	− (0.5)	+	0.1318	− (1)	+	0.0115
	23	−	+	0.0298	− (0.5)	+	0.0305	− (2.5)	+	0.0334
	24	−	+	0.0041	− (0.5)	+	0.0262	+ (1)	+	0.0064
100	25	−	+	0.0182	− (0)	+	0.0153	− (1)	+	0.0067
	26	−	+	0.0057	− (1)	+	0.1202	− (1)	+	0.0174
	27	−	+	0.0331	− (0.5)	+	0.0216	− (1)	+	0.0028

(a) Arbequina plants were sampled at times 0, 2, 7, 15, 23, 41, 53, 75, and 98 days after inoculation. (b) Growth of *V. dahliae* occurred from at least one out of the three root pieces per plant used for isolations. (c) Nested-PCR assays were performed with primers specific for both defoliating and nondefoliating *V. dahliae* [16,17]. (d) Mean *V. dahliae* DNA amount in ng /100 ng total genomic DNA extracted from roots of three sampled plants; nq = not quantified. An RT-QPCR value of zero meant no quantification or values considered not suitable for quantification (< 1 pg or with a Ct value ≥ 37) (see text for details). (e) In brackets, symptom severity (0–4) scored at the time that the plant was sampled. Acebuche-L, plants sampled did not show any symptoms.

times T_{50} for V138I-inoculated plants (90% incidence) and T_{64} for V4-inoculated ones (75% incidence). At the end of the bioassay, 100 days after inoculation, 75% of V138I-inoculated plants were affected in 34–66% of the aerial part and 25% of the plants were dead (final DII = 0.604) (Fig. 1C) compared with 65% of V4I-inoculated plants in which 0–33% of the aerial part showed symptoms. *V. dahliae* was isolated from roots of 18.5% of V138I-inoculated plants and 25.9% of V4I-inoculated ones, compared with 63 and 70.3% positive isolations from stems of same plants (Tables 1 and 2).

3.2. Detection of D and ND Verticillium dahliae in olive plants by nested-PCR

The yield of total genomic DNA extracted from roots was significantly ($P < 0.05$) higher than that from stems and it varied with olive genotypes; i.e. average yield (ng DNA/mg freeze-dried tissue) for Acebuche-L plants was (root:stem) 211.8:102.7, compared with 217.9:158.0 for Arbequina and 221.0:178.2 for Picual plants. All DNA samples were of PCR quality as indicated by results of RAPD assays using those samples as template DNA.

The 462-bp D-specific and 824-bp ND-specific markers were amplified in nested-PCR assays using the total genomic DNA from roots of each of the sampled V138I- and V4I-inoculated Acebuche-L plants, respectively. Those amplifications occurred from the very first sampling times (T_2, T_7) after inoculation despite a low disease incidence and severity in inoculated plants (Tables 1 and 2). Moreover, single-round PCR assays detected the 462-bp amplicon in roots of V138I-inoculated plants sampled at times T_7, T_{15} and T_{24}, and the 824-bp amplicon in roots of V4I-inoculated plants sampled from times T_7 to T_{52}, though as faint bands sometimes. On the contrary, those two markers were never amplified after one round of amplification using as template DNA extracted from stems of inoculated plants. However, the 462-bp D-specific and

824-bp ND-specific products were amplified in nested-PCR assays of 92.5% of stems sampled from V138I-inoculated plants and 26% of those sampled from V4I-inoculated ones, respectively. Although the 462-bp marker was amplified at all sampling times during the bioassay, the 824-bp one was not amplified from the sampling time T_{52} on.

Infection of Arbequina and Picual plants by D *V. dahliae* V138I and ND *V. dahliae* V4I-isolates was further confirmed by means of nested-PCR assays (Tables 1 and 2). In roots of Arbequina plants, the corresponding markers were amplified in all but one of the V4I-inoculated plants and 100% of V138I-inoculated ones (Tables 1 and 2). Detection of the pathogen in stem tissues was possible in 92.25% of V138I-inoculated plants and 44.4% of V4I-inoculated ones (two of three plants at each of sampling times T_7, T_{15}, T_{41}, T_{75}, and T_{98}). The D-specific (462-bp) and ND-specific (824-bp) markers were visualised as faint bands after one round of amplification using DNA from roots of a variable number of plants sampled from time T_7 on. However, those markers were never visualized in single-round PCR-assays using DNA from stem as template. Results from Picual-inoculated plants were similar to those from Arbequina. Thus, all plants yielded the markers specific for the corresponding D or ND pathotype from DNA extracted from roots. Conversely, in stem DNA the pathogen was detected in 70% of V138I-inoculated plants and 37% of V4I-inoculated ones, and never before 15 days after inoculation. A high number of potential pathogen propagules in roots was suggested by amplification of the D- and ND-specific markers, even as faint bands in some cases, in single-round PCR from each plant sampled from time T_7 on. Conversely, one round of amplification allowed detection of the D pathotype in the stem of some V138I-inoculated plants sampled at times T_{24}, T_{41}, T_{76} and T_{100}, but it failed to detect the pathogen in stems of V4I-inoculated plants. Neither D- nor ND-specific amplicons were ever detected in extracts of noninoculated plants.

3.3. Adjustment of conditions for real-time quantitative PCR assays

A standard curve was established by plotting the log of known concentrations of *V. dahliae* DNA against Ct values (Fig. 2). Theoretically, Ct values should decrease by 1 unit as the number of DNA molecules in the reaction doubled (i.e. 100% efficiency of the amplification reaction). The Ct increment calculated from the equation in Fig. 2 was 0.953, indicating the high-efficiency of the PCR assay in our study. Ct values resulting from assays with unknown samples were plotted onto this curve and the inferred concentration of *V. dahliae* DNA was calculated. A Ct of 37 (0.7 pg of DNA according to the standard curve) was considered to be the threshold value in the standard curve suitable for quantification, as PCR efficiency at a higher number of cycles is far from the value 1 (100% efficiency). Therefore, the minimum amount of pathogen DNA that could be accurately quantified in our assays was 1 pg, which corresponded to a Ct value of 36.3 ± 0.69. Nevertheless, amplification of *V. dahliae* DNA in samples that were not quantified could still be detected in agarose gels (carried out after each real-time quantitative PCR assay) (Fig. 3) or after nested-PCR assays.

Preliminary runs of real-time PCR assays were conducted with the program indicated in Section 2, but excluding the 90 °C, 25 s step. Melting profiles obtained from those runs showed an occasional presence of nonspecific amplified products or primer-dimers with a $T_m \leq 90$ °C (data not shown). That would have disturbed quantification of the target amplicon ($T_m = 92.5$ °C) since it makes it difficult to differentiate each dsDNA–Sybr-Green complex produced in the reaction. Therefore, the reading step for fluorescence emission was set at 90 °C (25 s) and included in the PCR program. At that temperature, the nonspecific products will be denatured and fluorescence emission would result only from the target amplicon bound to Sybr-Green. This step did not

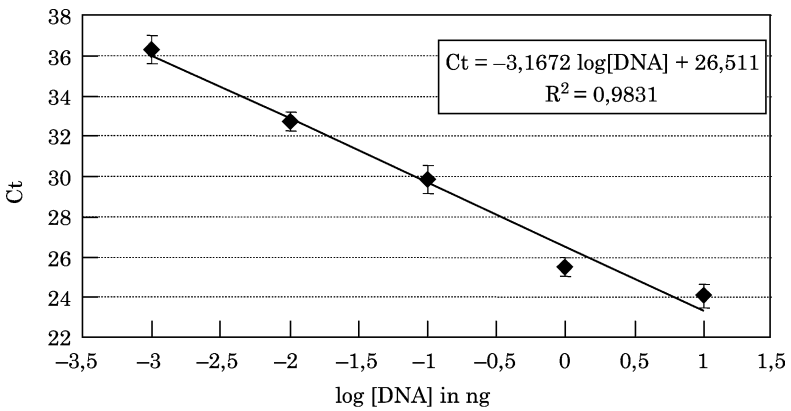

Fig. 2. Standard curve obtained from plotting log of known DNA concentrations of defoliating (V138I) and nondefoliating (V176I) *V. dahliae* isolates (10 ng–1 pg DNA) against the Ct values obtained from real-time quantitative PCR assays, (see Section 2 for conditions in the assays). This curve served to calculate the amount of *V. dahliae* DNA in total genomic DNA samples extracted from infected olive tissues. Error bars represent the standard deviation.

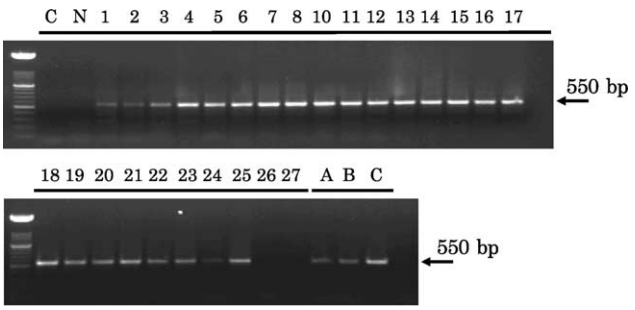

Fig. 3. Agarose gels (1% w/v) showing the end-point PCR product (amplicon of ca. 550-bp defined by primer pair DB19/DB22) obtained after a real-time quantitative PCR run. Lanes corresponded to total genomic DNA samples extracted from roots of Picual plants inoculated with defoliating isolate *V. dahliae* V138I. Numbers above gels indicate the number of plant sampled (see Table 1 for time of sampling of each plant). Results shown correspond to one of the real-time quantitative PCR assays carried out in the study. Mean DNA amounts from all assays are represented in Fig. 7A. DNA in plant n°9 could not be quantified due to shortage of DNA template. C: negative control (no template DNA). N: negative control (noninoculated Picual plant). The lower gel also contained results of a real-time PCR assays carried out with aliquots of known DNA concentration of nondefoliating *V. dahliae* 176I isolate used to develop the standard curve showed in Fig. 2 (A: 100 pg; B: 1 ng; C: 10 ng). Note that differences in band fluorescence observed in the gel (end-point result of the PCR assays) do not correlate with quantified DNA levels recorded during the log phase of amplification (real-time PCR quantification assay).

avoid amplification of nonspecific products and/or primer-dimers, but it allowed the single quantification of the amplicon defined by primer pair DB19/B22. The same was done for quantification of olive DNA, by setting the reading step at 82 °C (15 s).

Real-time quantitative assays carried out to estimate (and corroborate) the actual amount of total DNA in the reaction, indicated a high degree of uniformity in all samples (Fig. 4). This was a reassuring evidence that the DNA samples used for quantification of *V. dahliae* DNA actually contained essentially equal amounts of olive genomic DNA. However, differences were found among some DNA samples in each of the olive genotype/*V. dahliae* pathotype bioassays, and also among some DNA templates sampled at a given point in the time-course of infection for the three independent bioassays. An example is shown in Fig. 4, for DNA extracted from V4I-inoculated Picual plant 19 sampled at time T_{52}, for which we obtained a mean Ct value of 19.43. This Ct value is significantly different from those obtained for DNAs from V4I-inoculated Acebuche-L (21.87) and Arbequina (22.2) plants sampled at same time, as well as DNA from inoculated Picual plants sampled at other times throughout the bioassay. Although those differences might affect the actual quantification of *V. dahliae* DNA in an infected sample, they did not influence the overall fluctuations in *V. dahliae* DNA content observed during the disease progress (see below).

3.4. Quantification of D and ND Verticillium dahliae DNA in infected olive genotypes

Results of real-time quantitative PCR using samples of total genomic DNA from roots of D *V. dahliae* V138I- and ND *V. dahliae* V4I-inoculated Acebuche-L plants are shown in Fig. 5A and B, and Tables 1 and 2. Maximal

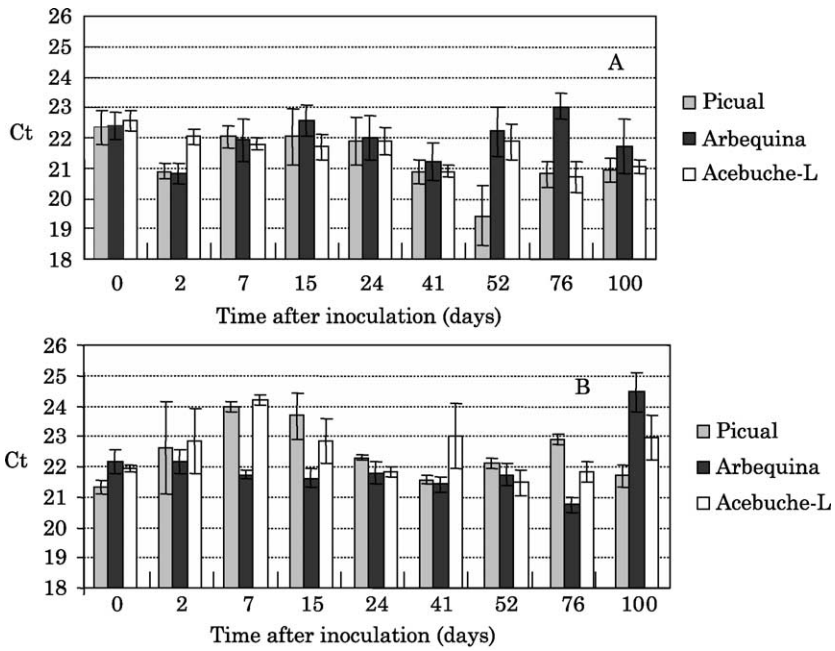

Fig. 4. Results of quantification of olive genomic DNA in total genomic DNA from infected olive plants sampled at different times after inoculation. Results from one *V. dahliae* V4I-inoculated plant (A) and one *V. dahliae* V138I-inoculated (B) plant are shown for each olive genotype and sampling time after inoculation. Amplifications were done using primer pair Ias22f/Ias22r [24] which amplifies a sequence of the olive genome, and 33 ng (spectrophotometrically quantified) of template DNA and repeated three times. Values obtained were useful to corroborate whether or not the amount of total genomic DNA in a real-time PCR assay was really equalized and to correct data on quantified *V. dahliae* DNA. Error bars represent the standard deviation.

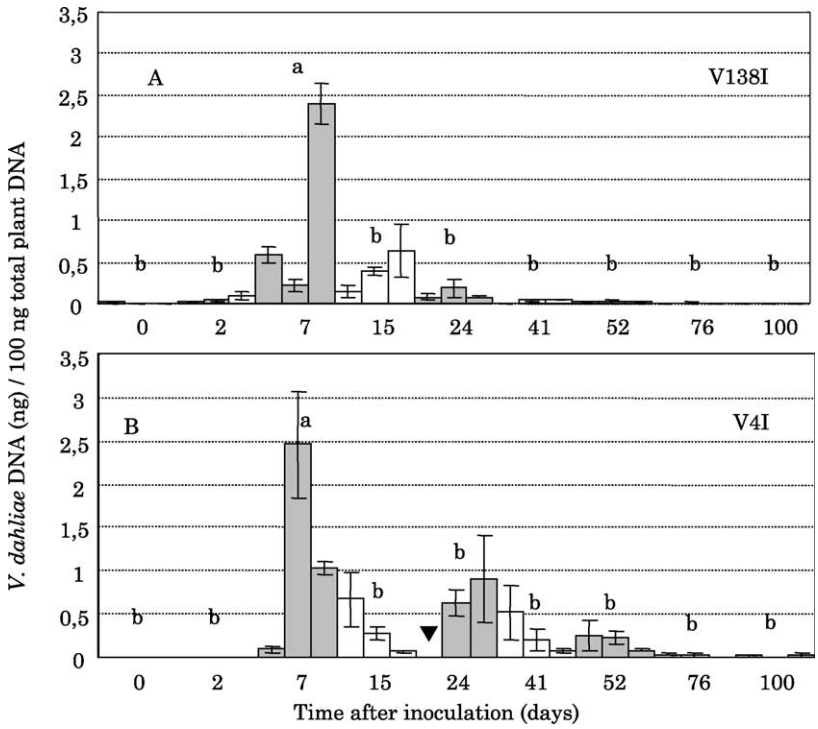

Fig. 5. Results of in planta *V. dahliae* DNA quantification in roots of (A) defoliating *V. dahliae* V138I- and (B) nondefoliating *V. dahliae* V4I-inoculated Acebuche-L plants during a time-course of infection bioassay. DNA content is expressed in ng per 100 ng of total genomic DNA extracted from roots. Each bar is the mean value of two independent real-time quantitative assays that included three replications for each DNA sample (see text for details). An inverted solid triangle (▼) indicates that the DNA sample (plant n°13) could not be quantified. Pathogen DNA could not be quantified in stems of Acebuche-L. The same letters above bars indicate that there were no significant differences ($P < 0.01$) among sampling times for each of the *V. dahliae* isolates (V138I or V4I). Error bars represent the standard deviation.

amounts of pathogen DNA in the roots of V138I-infected plants occurred at times 7 (T_7) (mean 1.07 ng/100 ng) and 15 (T_{15}) (mean 0.39 ng/100 ng) days after inoculation. The pattern for the amount of *V. dahliae* V4I DNA in Acebuche-L plants during the bioassay differed slightly from that shown for V138I DNA. While the maximum amount of V4I DNA was also found at time T_7 (plant n° 8, 2.46 ng), V4I DNA content in most plants sampled at time T_{24} (mean DNA content 0.77 ng/100 ng), T_{41} (0.27 ng/100 ng) and T_{52} (0.19 ng/100 ng) was higher than that of V138I DNA found in plants sampled at those same times (Fig. 5A and B), although due to plant-to-plant variability, the differences were not significant at $P < 0.01$. Quantification of *V. dahliae* DNA in Acebuche-L plants was possible ahead of development of symptoms in V4I-inoculated plants (occurring at time T_{21}), and just before that in V138I-inoculated ones (occurring at time T_8). Furthermore, maximal amounts of *V.dahliae* DNA were detected 2 weeks earlier than the maximum disease incidence caused by each of the pathotypes (occurring at time T_{21}). At that sampling time, DII_{max} values (within a range of 0–1) were 0.006 for V4I-inoculated plants and 0.031 for V138I-inoculated ones. Quantification of D and ND *V. dahliae* DNA in stems of Acebuche-L plants inoculated with the corresponding pathotype was not possible because of low fungal DNA amount harboured in the infected tissues. Nevertheless, the presence of a PCR product with $T_m = 92.5$ °C in some of DNA samples from stems was confirmed after obtaining the melting curve profiles (data not shown).

Results of real-time PCR quantification of *V. dahliae* DNA in Arbequina- plants showed a similar pattern for the D- and ND-pathotypes (Fig. 6A–D, Tables 1 and 2). The amount of *V. dahliae* DNA in roots of V138I-inoculated plants was maximum (mean DNA content of 2.97 ng/100 ng) at time $T_{7;}$ thereafter, that amount decreased progressively, i.e. 0.32 ng/100 ng at T_{15}, 0.25 ng/100 ng at T_{24}, and 0.39 ng/100 ng at T_{41}, to reach a level lower than 0.08 ng/100 ng (Fig. 6A). There were no significant differences in the actual amount of *V. dahliae* DNA in roots of V4I-inoculated plants compared with that in V138I-inoculated ones at the same sampling times (Fig. 6C). *V. dahliae* DNA in stem tissues of Arbequina plants was much lower compared with that in root and did not vary significantly over time (Fig. 6B and D). Maximal amounts of pathogen DNA in stem were recorded at time T_{41} (mean DNA content 10 pg/200 ng total DNA) for V4I-inoculated plants and at time T_{98} (164 pg/200 ng) for V138I-inoculated ones. Overall, *V. dahliae* DNA in stems of V138I-inoculated plants was significantly ($P < 0.05$) higher compared with that in V4I-inoculated ones (Fig. 6B and D). That pattern correlated with DII_{max} values throughout the bioassay, that was significantly higher ($P < 0.05$) for V138I-inoculated plants (DII = 0.454) than for V4I-inoculated ones

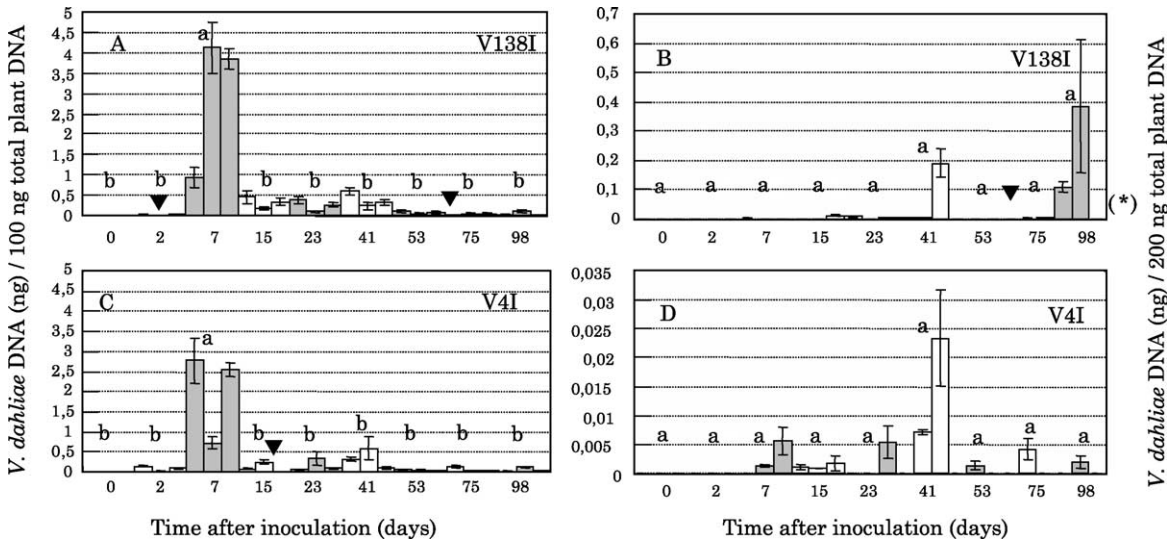

Fig. 6. Results of in planta *V. dahliae* DNA quantification in roots (A and C) and stems (B and D) of (A and B) defoliating *V. dahliae* V138I- and (C and D) nondefoliating *V. dahliae* V4I-inoculated Arbequina plants during a time-course of infection bioassay. Note that the DNA content is expressed in ng per 100 ng of total genomic DNA extracted from roots, and in ng per 200 ng of total genomic DNA extracted from stems. For roots, results are the mean of two independent real-time quantitative assays that included three replications for each DNA sample. Results for stems correspond to one run with three to six replications (see text for details). Inverted solid triangles (▼) indicate DNA samples (roots of plants n°5 and 22, stem of plant n°22 inoculated with *V. dahliae* V138I, and root of plant n°12 inoculated with *V. dahliae* V4I) that could not be quantified. Same letters above bars indicate that there were no significant differences ($P < 0.01$) among sampling times for each of *V. dahliae* isolates (V138I or V4I) and plant tissue. The asterisk (*) indicates that the DNA amount quantified in stems of V138I-inoculated plants (B) was on average significantly higher than that in V4I-inoculated plants. Note the difference in the *y*-axis scale between figures B and D. Error bars represent the standard deviation.

(DII = 0.125). The maximum amount of *V. dahliae* V4I DNA in stems of inoculated Arbequina plants occurred 6 days later than the appearance of first symptoms but before maximum disease incidence (occurring at time T_{76}). At that time (T_{41}), the V4I DNA content in stems was more than 60 times less than in roots. Conversely, maximum DNA content in stems of V138I-inoculated plants occurred at the time (T_{98}) when the highest DII (0.454) was recorded. At T_{98}, the mean amount of V138I DNA in stems was two times higher than that in roots of the same plants. Compared with *V. dahliae* DNA content in stems, the maximum amount of pathogen DNA in root was found at time T_7, i.e. much earlier than appearance of first symptom in plants inoculated with either of the *V. dahliae* pathotypes.

Unlike the situation described for Acebuche-L and Arbequina olives, the amount of *V. dahliae* DNA in roots of V138I-inoculated Picual plants did not display a clear maximum throughout the time-course of sampling (Fig. 7A, Table 1). However, as for Acebuche-L and Arbequina the first significant amount of pathogen DNA in root tissues of Picual plants was detected at time T_7, 1 week earlier than development of symptoms, and much sooner than maximum disease incidence (occurring at time T_{50}). From 7 days after inoculation (T_7), the amount of pathogen DNA in root tissues did not vary significantly ($P < 0.01$) (mean DNA content 0.62 ng/100 ng) until T_{41}, before maximum disease incidence, at which time a mean DNA amount of 1.25 ng/100 ng was recorded. The amount of *V. dahliae* V138I DNA in stems of Picual plants also was highest at time T_{41} (mean DNA content 0.39 ng/100 ng) (Fig. 7B, Table 2), but this amount was about three times lower than that in roots. From that time point, the pathogen DNA in olive tissue decreased progressively and significantly until the end of the bioassay. Compared with isolate V138I, *V. dahliae* V4I DNA in roots of inoculated Picual plants reached high levels from time T_7 to T_{41}, a period shorter than that in V138I-inoculated plants (time T_7 to T_{52}) (Fig. 7A and C). Maximum *V. dahliae* V4I DNA in root tissues was quantified at time T_{15} (2.28 ng/100 ng) followed by a steady decrease. First symptoms in V4I-inoculated plants occurred by time T_{14}. Despite low DII in those plants at time T_{15}, the amount of pathogen DNA in roots was significantly higher ($P < 0.01$) than that in V138I-inoculated plant at the same time. On the other hand, the amount of *V. dahliae* DNA in stems of V4I-inoculated plants at T_{41}, once symptoms were expressed, was significantly lower ($P < 0.01$) than that in V138I-inoculated plants.

4. Discussion

Objective, accurate quantitative measurement of pathogen colonization in host tissues is of importance for a better understanding of the disease reaction of olive genotypes to *V. dahliae* pathotypes. In this present study, we assessed the reaction of three olive genotypes (Acebuche-L, Arbequina and Picual) to infection and disease caused by defoliating (D) and nondefoliating (ND) *V. dahliae* pathotypes by monitoring the amount of pathogen DNA in a time-course after inoculation using a real-time quantitative PCR assay.

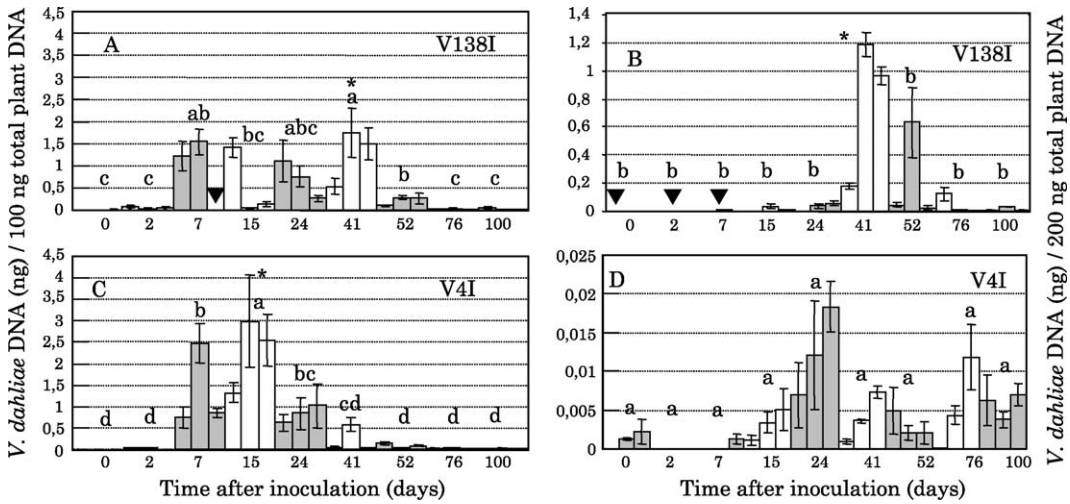

Fig. 7. Results of in planta *V. dahliae* DNA quantification in roots (A and C) and stems (B and D) of (A and B) defoliating *V. dahliae* V138I- and (C and D) nondefoliating *V. dahliae* V4I-inoculated Picual plants during a time-course of infection bioassay. Note that DNA content is expressed in ng per 100 ng of total genomic DNA extracted from roots, and in ng per 200 ng of total DNA extracted from stems. For roots, results are mean of two independent real-time quantitative assays that included three replications for each DNA sample. Results for stems correspond to one run with three to six replications (see text for details). Inverted solid triangles (▼) indicate DNA samples (root of plant n°9, and stems of plants n°1, 5, and 10 inoculated with *V. dahliae* V138I) that could not be quantified. Same letters above bars indicate that there were no significant differences ($P < 0.01$) among sampling times for each of *V. dahliae* isolates (V138I or V4I) and plant tissue. The asterisks (*) indicate significant differences between *V. dahliae* isolates at the same sampling time. Note the difference in the *y*-axis scale between figures B and D. Error bars represent the standard deviation.

In planta quantification of *Verticillium* spp. was achieved in herbaceous hosts by end-point PCR-based procedures [8,10,12]. To our knowledge, this is the first report on the application of real-time PCR technology to quantify different pathotypes of a Verticillium pathogen.

In our study, the use of unspecific Sybr-Green binding to dsDNA for quantifying the target amplicon (ca. 530 bp, $T_m = 92.5$ °C) required a 90 °C fluorescence emission reading step in the amplification program that eliminates any interference caused by accumulation of unspecific PCR products. We achieved a threshold of 1 pg DNA for accurate quantification of *V. dahliae* DNA in our assay; although lower amounts of pathogen DNA were still present in tissues of some infected, but symptomless plants, as indicated by nested-PCR assays. In addition, quantifying olive DNA by amplification of a SSR olive marker [24] provided an internal positive control (DNA quality test) as well as an additional check of the quantified *V. dahliae* DNA. Total amounts of DNA templates for PCR assays were highly uniform thus confirming that every single amplification reaction contained equalized amounts of total DNA. Total genomic DNA extracted from infected olives and used for assays consisted of olive DNA (which was the overwhelming major DNA species in the mixture), *V. dahliae* DNA (a minor DNA species, e.g. maximum amount fungal DNA quantified was 4.7 ng in 100 ng total DNA), and other unidentified DNA species (likely negligible amounts). Therefore, it can be assumed that DNA templates containing (and amplifying) equivalent amounts of olive DNA had an equivalent amount of total DNA in the reaction.

In the bioassays, development of Verticillium wilt in Arbequina and Picual olives agreed with that reported earlier [15–17,26] and confirmed that those cultivars are susceptible or very susceptible to D *V. dahliae*, respectively, and moderately susceptible to ND *V. dahliae*.

Conversely, Acebuche-L was resistant to disease caused by either of the two pathotypes as found previously [20]. Detection of the pathogen was unreliable by isolating from infected plants but consistent using the nested-PCR procedure, which validated previous results [16,17]. Thus, all root samples but one used in the study were infected by D or ND *V. dahliae*. The nested-PCR procedure fulfilled all expectations for qualitative diagnosis but gave no information about the actual amount of pathogen propagules (that can be inferred from the amount of pathogen DNA) in infected plant tissues.

Results in this study led to four main conclusions. We show that the amount of pathogen DNA quantified in each olive genotype correlated with susceptibility to Verticillium wilt. Maximal absolute amounts of pathogen DNA quantified in roots ranged from 1.76 ng/100 ng in V138I-inoculated Picual to 4.12 ng/100 ng in V138I-inoculated Arbequina, with actual measurements of *V. dahliae* DNA varying significantly both among sampling times for a given olive genotype/*V. dahliae* pathotype interaction and, in some cases, between *V. dahliae* pathotypes for a given olive genotype (see below). Lesser amounts of pathogen DNA were quantified in stem tissues compared with those in root tissues, so that it was necessary to double the amount of total genomic DNA template (up to 200 ng) in real-time PCR assays to measure amounts of pathogen DNA in stems of

infected olives. Despite that, we could not quantify *V. dahliae* DNA in stems of Acebuche-L plants indicating that the number of potential pathogen propagules in them was very low. However, both *V. dahliae* V4I- and V138I-isolates infected Acebuche-L stems as indicated by positive nested-PCR assays and, in a few cases, by isolations. As might be expected, the number of plants in which pathogen DNA could be effectively quantified correlated with incidence and severity of Verticillium wilt in olive genotypes. Thus, the amount of pathogen DNA (either V4I or V138I isolates) quantified in roots and in stems during the time-course of infection was higher in Picual plants (the most severely diseased genotype) and lesser in Arbequina and Acebuche-L plants, in that order. Those two latter genotypes also produced fewer diseased plants, although all roots were infected as indicated by nested-PCR assays. Therefore, the results of quantitative analysis of pathogen DNA in Arbequina plants correspond to reduced disease incidence and severity in Arbequina compared with Picual, and the virtual absence of disease symptoms in Acebuche-L.

A second conclusion from this study was that differences in the amount of pathogen DNA measured in planta were influenced more by the olive genotype than by the virulence of the infecting pathotype (D or ND) except for some cases. Thus, the amount of *V. dahliae* DNA in roots of resistant Acebuche-L was rather similar for D V138I (highly virulent)- or ND V4I (mildly virulent)-inoculated plants when maximal DNA amounts were measured 1 week after inoculation (mean values of 1.072 ± 0.951 ng/100 ng for V138I-inoculated plants and 1.036 ± 0.969 ng/100 ng for V4I-inoculated ones; absolute maximal values of 2.456 ng for V4I and 2.400 for V138I). Comparatively, a similar pattern was found in roots of Arbequina plants, but the average amount of fungal DNA in stems was higher for V138I-infected plants than V4I-infected ones. On the contrary, significantly higher amounts of *V. dahliae* DNA were measured in V4I-infected Picual roots compared with V138I-infected ones at time $T_{15,}$ and in V138I-infected roots and stems compared with that in V4I-infected ones at time $T_{41.}$ In terms of pathogen persistence in the infected tissues, it seemed clear that the more susceptible was the olive genotype and the more virulent was the *V. dahliae* pathotype in the interaction (indicated by higher DII values, i.e. Picual/V138I or V4I), the longer was the time period for which significant amounts of pathogen DNA were measured in them. In Picual stems, that period was longer for V138I-inoculated plants (times T_7 to T_{52}) than for V4I-inoculated ones (times T_7 to T_{41}). A distinguishing difference between D and ND pathotypes in Picual olives was the significant variation in the amount of *V. dahliae* DNA at times T_{41} and T_{15} mentioned above. In that cultivar, in the most compatible interaction (V138I-inoculated plants), the amount of pathogen DNA in roots remained at high level until time T_{41}, after symptom development, and there was a significant increase in the amount of *V. dahliae* DNA in stems at the same time. This suggests that a boost in the amount of the D pathotype took place in stems of Picual plants around T_{41}, at which time there was still a high level of potential pathogen propagules in the roots, related to development of severe symptoms or plant death. Significant increases in pathogen amount did not take place in less compatible interactions, i.e. Picual/*V. dahliae* V4I or even Arbequina/*V. dahliae* V138I or V4I, for which the amount of pathogen DNA in the stems was lower and remained rather constant over time. Therefore, the difference in virulence of D and ND *V. dahliae* on Picual, and possibly on Arbequina, could be attributed to differences in pathogen amount in stem of the infected plants, among other factor(s).

The third conclusion in this present study was that the maximum amount of pathogen DNA in roots of plants of all olive genotypes and stems of Picual occurred before maximum disease expression. Thereafter, the amount of pathogen DNA in root decreased sharply (Arbequina and Acebuche-L) or varied slightly during a time period that spanned from 7 to 52 days after inoculation (Picual). Comparatively, the pathogen DNA quantified in stems of Arbequina and Picual plants was much less and occurred later than in roots. That tissue difference suggests a temporal sequence of systemic colonization by the pathogen. It is interesting to note that severely affected plants, e.g. V138I-inoculated Picual plants sampled at times T_{76} and T_{100}, did not harbour high amounts of pathogen DNA in root tissues, suggesting that the number of potential pathogen propagules in them decreased at later stages after full development of disease at the time that translocation of the fungus to the stem took place. Some of the higher amounts of pathogen DNA found in our assays were measured in asymptomatic plants sampled at earlier times. Therefore, maximal disease incidence and severity followed a boost in pathogen amount that took place first in root tissues and subsequently in stems. Results of pathogen DNA quantification in roots of Picual plants (and to a lesser extent in Arbequina and Acebuche-L plants) in this study confirm the implications of earlier work using comminutes of infected tissues; these suggested that pathogen biomass may decrease once the disease is fully developed [26]. In our study, we did not observe clear evidence of the cyclical *V. albo-atrum* colonization pattern reported in alfalfa and tomato [10,12]. It might be possible that hyphal lysis underlying that process [21] takes place to a lesser degree in *V. dahliae* colonization of olives. Alternatively, putative cycles of colonization in olive may require a time period longer than the time duration (100 days) of our bioassays.

A last conclusion from our study was that real-time quantitative PCR can better assess the reaction of olive genotypes to *V. dahliae* pathotypes. In this present study, real-time PCR quantification of D and ND *V. dahliae* DNA in stem adequately correlated with the level of susceptibility to disease (as indicated by incidence and severity of symptoms) of Arbequina and Picual olives. Similarly, the disease resistance reaction of Acebuche-L was

correlated with resistance to the pathogen, as indicated by failure to quantify pathogen DNA in stem. Conversely, the amount of pathogen DNA in root tissues of Acebuche-L was comparable to that in susceptible Arbequina, but lower than that in the highly susceptible Picual. Dan et al. [8] used an end-point PCR-based procedure to differentiate between tolerance and resistance to *V. dahliae* in potato, and recommended that accurate quantification of the pathogen biomass in potato should be assessed in resistance breeding. Based on results from the present study, we concur with the recommendation of those authors on the necessity of accurate quantification of *V. dahliae* biomass in olive genotypes during breeding programs for resistance to Verticillium wilt. The real-time quantitative PCR approach presented here can help in pursuing such an aim. In addition, this work has proved that this technique is an excellent tool for quantitative pathogen diagnosis as well as for monitoring pathogen colonization and disease development.

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