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Symptom development, pathogen isolation and Real-Time QPCR quantification as factors for evaluating the resistance of olive cultivars to *Verticillium* pathotypes

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Abstract Verticillium wilt is the most serious olive disease in the Mediterranean countries and worldwide. The most effective control strategy is the use of resistant cultivars. However, limited information is available about the level and source of resistance in most of the olive cultivars and there are no published data using microsclerotia, the resting structures of *Verticillium dahliae*, as the infective inoculum. In the present study, we correlated symptomatology and the presence of the fungus along with the DNA relative amount (molecules μl^{-1}) of a defoliating (D) and a non-defoliating (ND) *V. dahliae* strain in the susceptible cv. Amfissis and the tolerant cvs Kalamon and Koroneiki, as quantified by the Real-Time QPCR technology. The viability of the pathogen in the plant tissues was confirmed by isolating the fungus on PDA plates, while symptom assessment proved the correlation between the DNA relative amount of *V. dahliae* in plant tissues and cultivar susceptibility. It was further demonstrated that the D and ND strains were present at a significantly higher level in cv. Amfissis than in cvs Kalamon and Koroneiki. It was finally observed that the relative amount of the pathogen in roots was lower than in stems and shoots and declined in plant tissues over time. These data constitute a valuable contribution in evaluating resistance of olive cultivars or olive root-stocks to *V. dahliae* pathotypes.

Keywords Defoliating pathotype · Non-defoliating pathotype · *Olea europaea* · Vascular wilts

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Introduction

Olive (*Olea europaea*) belongs to the most vulnerable tree hosts of *Verticillium dahliae*. Verticillium wilt occurs throughout the range of olive cultivation, reducing the production of olive trees and potentially causing tree death (Jimenez-Diaz et al. 1998). Microsclerotia, the resting structures of *V. dahliae* which constitute the main potential infective inoculum of the pathogen in the field, persist in the soil for >20 years, and the use of fungicides has little effect (El-Zik 1985; Wilhelm 1955). *Verticillium dahliae* should be controlled by a combination of preventive measures; amongst these is the use of resistant or tolerant cultivars or root-stocks (Tjamos 1993). The use of host resistance is considered as the most effective and ecologically sound method for managing *V. dahliae* in olive orchards (Blanco-Lopez et al. 1989; Lopez-

Escudero et al. 2004). The olive-infecting *V. dahliae* pathotypes have been classified as defoliating (D) and non-defoliating (ND) according to their ability to defoliate the tree (Schnarthost and Sibbett 1971). The D pathotype is considered as more virulent than the ND pathotype. In Europe, the D pathotype of *V. dahliae* affecting commercial olive trees was first observed in Spain in 2001 (Lopez-Escudero and Blanco-Lopez 2005), whereas it has not yet been reported in Greece.

The Mediterranean countries are the major olive producers in the world; among them, Greece is the third major olive producer in world, producing 2,600,000 mt of olives in 2007 (FAOSTAT 2008). Therefore, olive pathogens and in particular *V. dahliae* constitute a serious threat for the income of the Mediterranean farmers and consequently the economy of these countries.

Although *V. dahliae* is a devastating pathogen of olive trees, limited information is available in the literature about the rate and pattern of colonisation of the D and ND pathotypes in resistant and tolerant olive cultivars. Mercado-Blanco et al. (2001), studied the colonisation of both pathotypes on one susceptible and two resistant Spanish olive cultivars. In this study, it was shown that the amount of fungal DNA quantified in each olive genotype correlated with susceptibility to Verticillium wilt; differences in the amount of fungal DNA measured *in planta* were influenced more by olive genotype than by the virulence of the infecting pathotype (D or ND), and the maximum amount of pathogen DNA in roots and stems of plants of all genotypes occurred before maximum disease expression. However, the infective inoculum used in this study was conidia and not microsclerotia, which is the potential inoculum under field conditions.

Recently, Antoniou et al. (2008), by using a stem-inoculation process, demonstrated that *V. dahliae* conidia could be translocated and could colonise the xylem of young olive trees at the same distance above and below the point of trunk injection, irrespective of cultivar susceptibility to *V. dahliae*. However, the pathogen was subsequently isolated at statistically significant percentages in susceptible cv. Amfissis compared to the tolerant cv. Kalamon, indicating operation of resistance mechanisms. Consequently symptom development in the susceptible cultivar was at least sixfold more intensive compared to the tolerant cultivar 6–11 months after trunk inoculation.

In the present study, we investigated the colonisation of *V. dahliae* in the susceptible cv. Amfissis and the tolerant cvs Kalamon and Koroneiki by using Real-Time QPCR technology and microsclerotia as the infective inoculum. However, this DNA-based quantification method does not ensure the presence of a metabolically-active fungus inside the xylem vessels of the tree; therefore, it was necessary to confirm the presence of an active form of the pathogen by isolating the fungus. In summary, the main objectives of this work were to: (1) evaluate the resistance of three most important Greek olive cvs Amfissis, Kalamon and Koroneiki to the D and ND *V. dahliae* pathotypes following symptom development and pathogen isolation, and (2) study the colonisation pattern of both pathotypes in different parts of the above cultivars over time.

Materials and methods

Plant material

Plant material consisted of 8 month-old rooted cuttings of cvs Amfissis, Kalamon and Koroneiki olive trees. Amfissis is susceptible to D and ND isolates, while Kalamon and Koroneiki are tolerant to both isolates (Antoniou et al. 2008; Martos-Moreno et al. 2006).

Verticillium dahliae isolates

A ND *V. dahliae* isolate (PA, Greek isolate) from a diseased olive tree and a defoliating isolate (A6, Spanish isolate) from cotton were used in the experiments. For short-term storage the fungi were maintained on potato dextrose agar (PDA, Merck) at 4°C, while for long-term they were stored at −80°C.

A multiplex PCR was performed to ensure that the two isolates belong either to the D or ND pathotypes. For this purpose, DNA of the *V. dahliae* isolates PA, A6 and the known D and ND pathotypes V117 (Blanco-Lopez et al. 1984) and V84 (Elena 1999) respectively, was extracted according to Lee and Taylor (1990). The primer pairs D1/D2 and NDr/NDf which are specific for the detection of the D and ND pathotypes respectively, were used in the multiplex PCR (Mercado-Blanco et al. 2001, 2002).

Inoculum preparation

Microsclerotia of PA (ND) *V. dahliae* strain were prepared in sucrose sodium nitrate (SSN) liquid culture according to Malandraki et al. (2008). A6 (D) strain microsclerotia were prepared in minimal medium (MM) agar (Puhalla and Mayfield 1974). Pieces of 7 day-old A6 strain grown in PDA were transferred to the MM agar and plates were incubated at 22°C for 4 weeks. Plates were flooded with sterile distilled water (SDW) and microsclerotia were detached from the plates with a sterile scalpel, centrifuged at 10,000 *g*, 20°C for 10 min to remove growth medium, dried, re-suspended in SDW and filtered through 70 μm mesh to select microsclerotia >70 μm. It is known that large microsclerotia have a stronger inoculum potential since they germinate easily and show high levels of pathogenicity (Hawke and Lazarovits 1994).

Olive–*Verticillium dahliae* bioassay

Forty-seven olive plants (8 months-old) of each of the three cultivars were transplanted in plastic pots containing 7 l soil infested with 20 microsclerotia g^{-1} of the *V. dahliae* D or ND pathotypes. Nine trees per cultivar were sampled at 2, 7 and 14 months (these time-points correspond to the dual growth cycle of olive trees based on active and rapid growth in spring and autumn; Wiesman and Lavee 1994) after transplanting, for pathogen isolation on PDA plates and QPCR quantification of the pathogen. In addition, 20 trees per cultivar were retained for recording symptom development and six trees per cultivar were transplanted to plastic pots containing sterilised soil and kept as negative controls. The experiment was performed in a randomised block design. Olive trees were maintained at 24±5°C with a 16-h light and dark cycle and neither herbicides nor pesticides were applied.

Disease assessment

Verticillium wilt symptoms were recorded every week for 202 days after *V. dahliae* microsclerotia application. The disease index of each shoot was based on an arbitrary scale from 0–4 where 0=healthy shoot, 1=dull green leaves, 2=inward rolled leaves, 3=necrotic leaves, and 4=defoliated-dead shoot. The percentage disease index was calculated from the disease rating by the formula: Disease index(%)= $[\Sigma(\text{rating no.} \times \text{no. of shoots in the rating})/\text{Total no. of shoots} \times \text{highest rating}] \times 100$. Disease ratings were plotted over time to generate disease progress curves. Subsequently the area under the disease progress curve (AUDPC) was calculated by the trapezoidal integration method (Campbell and Madden 1990). Disease was expressed as a percentage of the maximum possible area with reference to the maximum value potential reached over the whole period of the experiment and it is referred to as relative AUDPC.

Plant DNA extraction

Root, stem and shoot tissues from the D and ND *V. dahliae*-inoculated trees were collected for DNA extraction. For each sampled plant, 10 cm-long pieces from the middle of the main stem or shoot, and 20 randomly selected 8–15 mm-long root pieces were collected. Bark was removed from the tissues and subsequently tissues were freeze-dried and ground to a fine powder by using an autoclaved mortar and pestle, in the presence of liquid nitrogen. Total DNA was isolated according to Dellaporta et al. (1983), with the appropriate modifications (incubation time at 65°C was increased to 1 h), and was quantified by spectrophotometry and agarose gel electrophoresis.

Pathogen quantification

QPCR assays for quantification of D and ND *V. dahliae* DNA were conducted using the primer pair ITS1-F 5'-CCGCCGGTCCATCAGTCTCTCTGTTTATAC-3' ITS2-R 5'-CGCCTGCGGGACTCCGATGCGAGCTGTAAC-3', designed on the ITS1 and ITS2 regions of the 5.8S ribosomal RNA gene (GenBank, accession number: Z29511), generating a 347 bp amplicon. QPCR was performed in a Stratagene Mx3005P™ thermocycler and for the amplification reactions the TAQuarate™ GREEN Real-Time PCR MasterMix (Epicentre, Madison, WI, USA) was used. The results were analysed with MxPro QPCR software. The expression levels of the olive actin gene (GenBank, accession number: AY788899), detected using the primer pairs OeACT-F 5'-ATCCTCACAGAGCGTGG-3' and OeACT-R 5'-CGATCATTGAAGGCTGG-3', generating a 229 bp amplicon, were used as internal standards to normalise differ-

ences in DNA template amounts. PCR cycling started with an initial denaturation step at 94°C for 2 min, followed by 40 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 30 s. The primer specificity and the formation of primer-dimers were monitored by dissociation curve analysis. All QPCR reactions were performed as duplicates. To quantify the relative DNA amount of *V. dahliae* isolates, a PCR product of the primer pair ITS1-F/ITS2-R was cloned into the pGEM-Teasy (Promega, Madison, WI, USA) vector and eight tenfold dilutions (10 ng–1 fg) of the plasmid were used to generate a standard curve (Fig. 1).

Pathogen isolation

Vascular colonisation by actively growing *V. dahliae* was determined in each of the sampled olive plants by isolating the fungus onto acidified PDA. For each tree, three 8–15 mm-long root pieces were thoroughly washed (60 min under running water), surface-disinfested by NaClO (0.5% commercial bleach) for 2 min, rinsed with SDW, and placed onto the medium, after the removal of the bark. In addition, ten wood chips from the stem and shoots of each tree were placed onto acidified PDA, after the removal of the bark. Plates were incubated at 22°C in the dark for 14 days and the emerging fungi were examined under a light microscope.

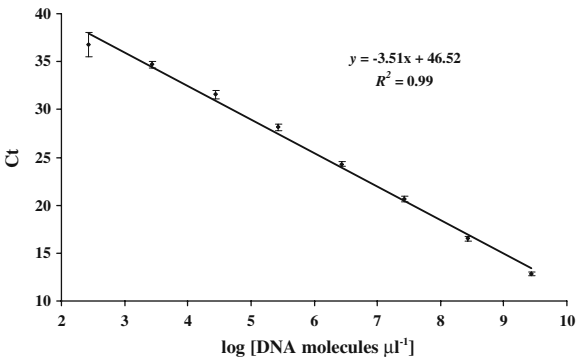

Fig. 1 Standard curve obtained from plotting the log of known DNA concentrations (10 ng–1 fg DNA), generated from cloned (into the pGEM-Teasy vector) PCR products of the primer pair *V. dahliae* ITS1-F/ITS2-R, against the Ct values obtained from QPCR. This curve served to calculate the relative amount of *V. dahliae* DNA in total genomic DNA samples extracted from infected olive tissues. *Vertical bars* indicate standard deviation

Statistics

Data on relative AUDPC, percentage of positive isolations and DNA amount were transformed with the $\sqrt{x+1}$ transformation before ANOVA was applied. When a significant ($P \leq 0.05$) F-test was obtained for treatments, data were subjected to means separation by Tukey's HSD test.

Results

Symptom development and virulence of isolates

Verticillium wilt symptoms, mainly chlorosis, started on day 83 in the ND infected cv. Amfissis trees, whereas cvs Kalamon and Koroneiki started to show chlorosis 38 days later, on day 121 (Fig. 2b). The relative AUDPC analysis demonstrated that symptom development in cvs Kalamon and Koroneiki was significantly lower than cv. Amfissis, proving their tolerance against the ND pathotype (Fig. 2d).

The D isolate proved to be more severe than the ND isolate in cv. Amfissis, whereas cvs Kalamon and Koroneiki showed the same level of tolerance for both pathotypes (Fig. 2c, d). The most common symptom, caused by the D isolate, in cv. Amfissis was defoliation and eventually plant death, whereas in cvs Kalamon and Koroneiki the most common symptom was chlorosis. Symptoms appeared earlier in cv. Amfissis (day 48) than cvs Kalamon and Koroneiki (day 114) and progressed rapidly reaching 55% on day 202, while the percentage of disease severity in cvs Kalamon and Koroneiki was 2% on day 202 (Fig. 2a). The relative AUDPC analysis confirmed the significant level of tolerance observed in cvs Kalamon and Koroneiki against the D pathotype (Fig. 2c).

Detection and quantification of D and ND *V. dahliae* pathotypes

The colonisation pattern of the D and ND pathotypes in the root, stem and shoots of cvs Amfissis, Kalamon and Koroneiki olive trees was studied over time by isolating the fungus onto acidified PDA and QPCR. The DNA extraction method (Dellaporta et al. 1983) yielded a DNA concentration of 200–600 ng μl^{-1}.

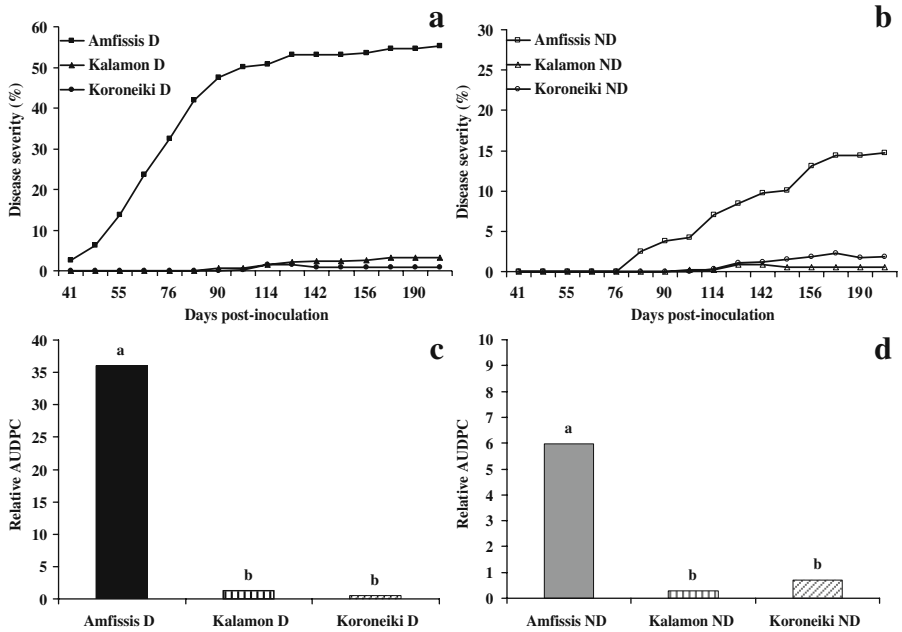

Fig. 2 Verticillium wilt disease severity on cvs Amfissis, Kalamon and Koroneiki infested with microsclerotia of the defoliating (*D*) or non-defoliating (*ND*) *V. dahliae* pathotypes (**a**, **b**). Subsequently, disease ratings were plotted over time to generate the disease progress curves. The area under the disease progress curve (AUDPC) was calculated by the trapezoidal integration method (Campbell and Madden 1990). Disease severity was expressed as a percentage of the maximum possible AUDPC for the whole period of the experiment and is referred to as relative AUDPC (**c**, **d**). Columns with different *letters* differ significantly according to the Tukey HSD test ($P \leq 0.05$)

The percentage of positive D and ND isolations was significantly higher in the stems and shoots of cv. Amfissis than cvs Kalamon and Koroneiki 2 months after transplanting into the infested soil (Fig. 3a, b). However, the percentage of positive D and ND isolations was statistically the same in the roots of the three cultivars (Fig. 3a, b). These results coincide with the QPCR results, which demonstrated a significantly higher D and ND DNA amount in stems and shoots of cv. Amfissis than cvs Kalamon and Koroneiki (Fig. 4a, b). In addition, the QPCR results revealed that the D pathotype was present in the tissues of cv. Amfissis at a higher amount than the ND pathotype (Fig. 4a, b). The D pathotype was present three-, seven-, and ninefold more than the ND pathotype in the stem, roots and shoots of cv. Amfissis respectively (Fig. 4a, b). Moreover, it was demonstrated that the D and ND pathotypes were present at lower levels in the roots than the stem and shoots in the susceptible cv. Amfissis, whereas there was no difference among the tissues in cvs Kalamon and Koroneiki (Fig. 4a, b).

Seven months after transplanting into the infested soil, the percentage of positive D and ND isolations in stem and shoots was higher in cv. Amfissis than cvs Kalamon and Koroneiki (Fig. 3c, d); however, this percentage had decreased compared to the previous sampling time (Fig. 3a, b). These results are generally in agreement with the QPCR results, although sometimes QPCR detected a low amount of D or ND DNA (cvs Kalamon and Koroneiki transplanted to the ND and D infested soil respectively) where the fungus was not isolated in the acidified PDA (Fig. 4c, d).

Fourteen months after transplanting into the D-infested soil, cv. Amfissis trees were dead; therefore, neither the method of positive isolations nor QPCR was attempted in these trees. In cvs Kalamon and Koroneiki, a further decrease in the percentage of positive isolations and the DNA amount of the D pathotype compared to the previous sampling times was demonstrated (Fig. 3e, 4e). This decrease was also observed in the ND-infested trees, where cv. Amfissis showed a higher percentage of positive

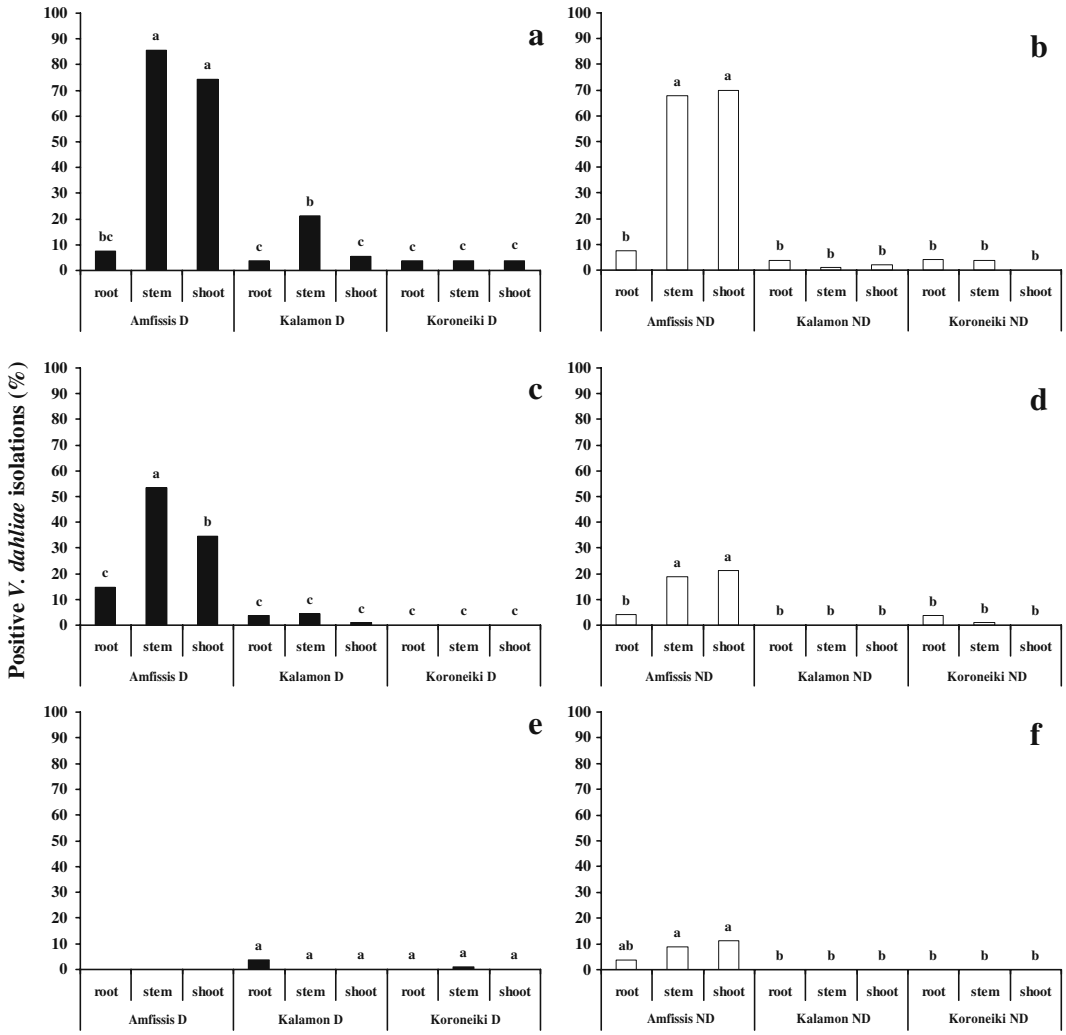

Fig. 3 Percentage of positive isolations of the defoliating (*D*) or non-defoliating (*ND*) *V. dahliae* pathotypes from roots, stems and shoots of cvs Amfissis, Kalamon and Koroneiki, sampled at 2, (**a**, **b**), 7 (**c**, **d**) and 14 (**e**, **f**) months after transplanting to infested soil. *Columns with different letters* differ significantly according to the Tukey HSD test ($P \leq 0.05$)

isolations and amount of DNA than cvs Kalamon and Koroneiki (Fig. 3f, 4f).

Discussion

Verticillium wilt has been often reported as the main phytopathological problem in olive tree orchards in the Mediterranean countries and California (Blanco-Lopez et al. 1984; Snyder et al. 1950), causing serious economic losses, as there are no chemical treatments to control it. Thus, *V. dahliae* management strategies in olive tree orchards are focused on the use of tolerant rootstocks or varieties. The existence of two pathotypes of the fungus, a defoliating and a non-defoliating, causes further problems to the growers; however Verticillium wilt attacks in Greek olive orchards are caused solely by the non-defoliating pathotype, since the D pathotype has not yet been reported in Greece.

In the present study, we selected three olive Greek cultivars with different degrees of tolerance against the D and ND pathotypes of *V. dahliae*. Based on previous resistance-screening studies (Antoniou et al. 2008, Martos-Moreno et al. 2006), and the importance for the Greek olive industry we selected the susceptible cv. Amfissis and the tolerant cvs Kalamon and Koroneiki. Furthermore, we

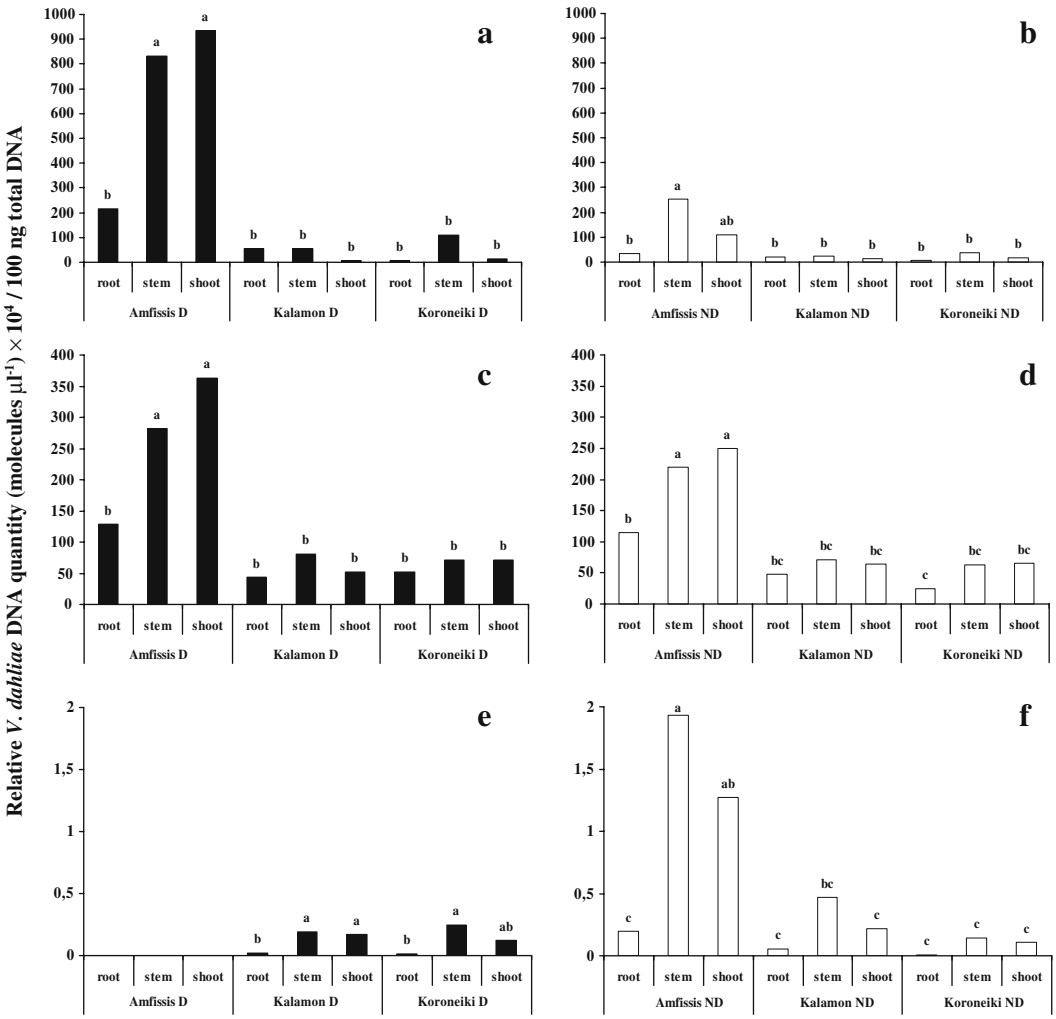

Fig. 4 Relative DNA amount (molecules μl^{-1}) of the defoliating (*D*) or non-defoliating (*ND*) *V. dahliae* pathotypes from roots, stems and shoots of cvs Amfissis, Kalamon and Koroneiki, sampled at 2, (**a**, **b**), 7 (**c**, **d**) and 14 (**e**, **f**) months after transplanting to infested soil. *Columns with different letters* differ significantly according to the Tukey HSD test ($P \leq 0.05$)

attempted to simulate the natural infection process of olive trees in the field by using microsclerotia as the infective inoculum, in contrast to previous studies where the root system of olive trees was dipped into a conidial suspension of the fungus (Martos-Moreno et al. 2006; Mercado-Blanco et al. 2003). This is a critical difference between this and previous studies since conidia are short-lived in soil (Schreiber and Green 1962) and do not exhibit the repeat germination that is observed with microsclerotia (Menzies and Griebel 1967).

Despite previous reports (Martos-Moreno et al. 2006), in which cv. Koroneiki was classified as moderately resistant, in the present study it was demonstrated that cvs Kalamon and Koroneiki exhibit a high degree of tolerance against both *V. dahliae* pathotypes (Fig. 2). The results of symptom development and fungal isolation along with QPCR support these levels of tolerance, since symptom severity, percentage of positive isolations and the fungal DNA amount were significantly higher in the stem and shoots of cv. Amfissis than cvs Kalamon and Koroneiki (Fig. 3, 4). In contrast to stem and shoot colonisation, the roots of the three cultivars showed the same level of colonisation, implying that restriction of systemic spread rather

than inhibition of penetration is responsible for the observed resistance of cvs Kalamon and Koroneiki. Eynck et al. (2007), reached the same conclusion by investigating the non-host resistance of *Brassica napus* against *V. dahliae*. In addition, the level of the fungal DNA amount and the percentage of positive isolations were higher in stem and shoots than roots, irrespective of the cultivar (Figs. 3, 4). This can possibly be explained by the fact that conidia, produced in the xylem, are carried away from the roots to the stem and shoots through the transpiration stream (Emechebe et al. 1975; Garber and Houston 1966; Presley et al. 1966; Talboys 1962). However, Mercado-Blanco et al. (2003) reached the opposite conclusion (lower DNA content in the stem tissues than in root tissues) by studying the colonisation pattern of the D and ND pathotypes in three Spanish olive cultivars. This discrepancy between the two studies can possibly be attributed to the different infection methods and sampling time-points. Mercado-Blanco et al. (2003) dipped the root system of olive trees in a conidial suspension taking samples >100 dpi, while in the present study olive trees were transplanted to microsclerotia-infested soil and the colonisation process of *V.dahliae* pathotypes was followed for over a year.

The QPCR along with the symptom development and fungal isolation results revealed the high virulence of the D compared to the ND pathotype against cv. Amfissis (Figs. 2, 3, 4). On the other hand, cvs Kalamon and Koroneiki remained tolerant to both pathotypes by exhibiting a low level of fungal DNA content and percentage of positive isolations. This indicates that the D *V. dahliae* isolates may not constitute a lethal threat for two of the most important Greek cultivars; however, it is obvious that further experimentation is required under field conditions before reaching a final conclusion about their level of resistance.

It is also worth mentioning that the susceptible cv. Amfissis exhibited the maximum level of the D-*V. dahliae* DNA relative amount and percentage of positive isolations before maximum disease expression (Figs. 2, 3, 4). This observation has also been made by Mercado-Blanco et al. (2003) in olive trees and Pantelides et al. (2009) in eggplants infected by *V.dahliae*, suggesting that in non-tolerant responses, maximum disease severity follows an increase in pathogen growth and probably reflects early maximum sporulation in the xylem, causing reductions in leaf water potential, stomatal conductance, rate of carbon assimilation and accumulation of proline, soluble sugars and ABA (Goicoechea et al. 2000; Tzeng and DeVay 1985). These physiological and biochemical changes in the host due to pathogen proliferation in the xylem contribute to the consequent appearance of Verticillium wilt symptoms.

Furthermore, it was observed that the amount of DNA steadily declined with time in all cultivars (Fig. 4). This decrease can be attributed to the natural phenomenon of recovery associated with mechanisms that allow trees to overcome injury and decay, and can be activated after infections caused by vascular pathogens such as *V. dahliae*. Lopez-Escudero and Blanco-Lopez (2005), studied the phenomenon of recovery in susceptible and resistant olive cultivars to the D and ND pathotypes of *V. dahliae* and concluded that recovery depends on the virulence of the *V. dahliae* isolate, the level of symptom severity shown by the plant and the level of cultivar resistance. Several factors have been recognised as being responsible for the phenomenon of recovery. Among these is the capability of the tree to occlude infected vessels, inactivate the viable fungus in the xylem, impede new infections and increase the levels of certain phenolic compounds (Baidez et al. 2007). Further study is therefore needed to determine the biochemical basis of resistance in order to elucidate the mechanisms underlying the observed *V. dahliae*-resistance of cvs Kalamon and Koroneiki.

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