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Characterization of resistance against the olive-defoliating *Verticillium dahliae* pathotype in selected clones of wild olive

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Verticillium wilt of olive is best managed by resistant cultivars, but those currently available show incomplete resistance to the defoliating (D) *Verticillium dahliae* pathotype. Moreover, these cultivars do not satisfy consumers' demand for high yields and oil quality. Highly resistant rootstocks would be of paramount importance for production of agronomically adapted and commercially desirable olive cultivars in D *V. dahliae*-infested soils. In this work, resistance to D *V. dahliae* in wild olive clones Ac-13, Ac-18, OutVert and StopVert was assessed by quantifying the fungal DNA along the stem using a highly sensitive real-time quantitative polymerase chain reaction (qPCR) protocol and a stem colonization index (SCI) based on isolation of *V. dahliae* following artificial inoculations under conditions highly conducive for verticillium wilt. Ac-13, Ac-18, OutVert and StopVert showed a symptomless reaction to D *V. dahliae*. The mean amount of D *V. dahliae* DNA quantified in stems of the four clones ranged from 3.64 to 28.89 pg/100 ng olive DNA, which was 249 to 1537 times lower than that in susceptible Picual olive. The reduction in the quantitative stem colonization of wild olive clones by D *V. dahliae* was also indicated by a sharp decrease in the SCI. Overall, there was a pattern of decreasing SCI in acropetal progression along the plant axis, as well as correlation between positive reisolation and quantification of pathogen DNA. The results of this research show that wild olive clones Ac-13, Ac-18, OutVert and StopVert have a valuable potential as rootstocks for the management of verticillium wilt in olive.

Keywords: *Olea europaea* L. subsp. *europaea* var. *sylvestris*, olive, qPCR, rootstocks, verticillium wilt

Introduction

Verticillium wilt caused by the vascular-colonizing, soilborne mitosporic ascomycete *Verticillium dahliae* (Inderbitzin *et al.*, 2011; www.mycobank.org) is the main soilborne disease affecting olive (*Olea europaea* subsp. *europaea* var. *europaea*) worldwide (Jiménez-Díaz *et al.*, 2012). The fungus is able to survive in the soil by means of melanized microsclerotia without a host for at least 14 years (Wilhelm, 1955). Microsclerotia in soil germinate multiple times in response to root exudates, giving rise to hyphae that can penetrate the olive root. Thereafter, hyphae grow across the root cortex, invade the xylem vessels and form conidia that spread upward in the olive stem by the transpiration stream, giving rise to extensive xylem colonization and functional impairment (Jiménez-Díaz *et al.*, 2012). As a result, attacks by the disease can cause severe losses of fruit yield as well as tree death (Levin *et al.*, 2003; Jiménez-Díaz *et al.*, 2012). Careful yield loss assessment in affected trees estimated 75–89% yield reduction in Picual olives in the third to fifth years after planting in an infested, saline soil (Levin *et al.*, 2003). This confers verticillium wilt in olive with a high socioeconomic significance because of the extent of olive cultivation, which spans over 10^7 ha in more than 20 temperate countries worldwide (FAO, 2012).

In Spain, the disease was first observed in 1979 in orchards in the south near Córdoba, Andalusia; subsequently, the pathogen has spread throughout that region causing severe infections and making verticillium wilt one of the main concerns for the Andalusian olive industry (Jiménez-Díaz *et al.*, 2012; Areal & Riesgo, 2014). Recent estimates of disease prevalence in Andalusia indicated that more than 50% of olive orchards were affected by verticillium wilt (Ruiz Torres, 2010) and surveys designed over 90 arbitrarily chosen orchards in this region indicated 71% disease prevalence with a mean incidence of 20% in the affected orchards (López-Escudero *et al.*, 2010).

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The increase in distribution and importance of verticillium wilt in olive in Andalusia has occurred concomitant with the spread of a highly virulent, defoliating (D) *V. dahliae* pathotype that appears to have displaced a previously existing nondefoliating (ND) one (Jiménez-Díaz *et al.*, 2011, 2012), but also with changes in cropping practices including the use of drip irrigation and self-rooted planting stocks to establish high-density plantings (Villalobos *et al.*, 2006). The D pathotype is characterized by its distinct ability to cause early drop of symptomless, green leaves from individual olive twigs and branches that eventually gives rise to complete defoliation and necrosis. Conversely, the ND pathotype can cause extensive dieback of olive twigs and branches without leaf shedding, as well as flower mummification and necrosis of inflorescences together with leaf chlorosis and necrosis (Navas-Cortés *et al.*, 2008; Jiménez-Díaz *et al.*, 2012).

Management of verticillium wilt in olive is made difficult by the long-term survival of microsclerotia in soil, broad host range of the pathogen and lack of success of fungicide treatments in infected trees (Pegg & Brady, 2002; Jiménez-Díaz *et al.*, 2012; Inderbitzin & Subbarao, 2014). Host resistance is the single most practical, sustainable and cost-efficient method for management of verticillium wilt in olive, but its effectiveness is curtailed by the widespread occurrence of the D pathotype in Spain and elsewhere (Jiménez-Díaz *et al.*, 2011, 2012). Compared with ND isolates, the D pathotype is consistently more virulent across olive cultivars (López-Escudero *et al.*, 2004; Dervis *et al.*, 2010) and can cause more severe wilt with much lower soil inoculum density (López-Escudero & Blanco-López, 2007). Thus, valuable resistance against the ND pathotype in some olive cultivars is overcome by the D pathotype, although valuable levels of incomplete resistance against D *V. dahliae* that appears to be quantitatively inherited have been found in some olive cultivars, of which Frantoio is considered among the most resistant (López-Escudero *et al.*, 2004; Martos-Moreno *et al.*, 2006; Bubici & Cirulli, 2011; Trapero *et al.*, 2015). However, none of these resistant cultivars are much used in commercial olive production; rather, choice of cultivar for establishing new olive orchards is guided mainly by edaphoclimatic conditions, denomination of geographic origin for oil production and market requirements. Also, most cultivars satisfying those conditions and requirements are susceptible to D *V. dahliae*. Thus, Arbequina and Picual, the most widely grown olive cultivars in the world and Spain, respectively (Barranco, 2010), are moderately and highly susceptible to D *V. dahliae* (Trapero *et al.*, 2013).

Resistant rootstocks would be of much interest for production of *Verticillium*-susceptible olive cultivars in soils infested with D *V. dahliae*, and efforts have been made in the last few decades to identify olive clones of use for that purpose (Wilhelm, 1981). Recently, grafting susceptible cultivars Coratina and Leccino onto resistant rootstock Frantoio provided excellent disease control to root dip inoculation with D *V. dahliae* under artificial conditions (Bubici & Cirulli, 2012). However, Frantoio and other resistant cultivars were disregarded for use as resistant rootstocks in heavily infested soils because local clones of those cultivars developed a noticeable amount of disease in a soil with moderate inoculum density of D *V. dahliae* (Trapero *et al.*, 2013). Wild olive (*O. europaea* subsp. *europaea* var. *sylvestris*) germplasm could be an alternative, valuable source of genotypes with resistance to D *V. dahliae* higher than that shown by olive cultivars. In previous research, wild olive clones Ac-13, Ac-18 (Spain) and OffVert, OutVert and StopVert (Italy) were highly resistant to a D *V. dahliae* isolate in a monocycle of infection by root dip inoculation with a conidial suspension (Jiménez-Díaz, 2008). In Italy, a screening for resistance of wild olive germplasm against D and ND pathotypes (Colella *et al.*, 2008) resulted in the identification of three resistant clones designated OffVert, OutVert and StopVert (M. Cirulli, unpublished data). That characterization was based on the assessment of foliar symptoms and vascular browning of stem cross-sections, as well as on the extent of reisolation of the fungus from inoculated plants, as practised regularly for the assessment of resistance in olive cultivars (López-Escudero *et al.*, 2004; Martos-Moreno *et al.*, 2006; Bubici & Cirulli, 2011; Trapero *et al.*, 2015). However, the absence or reduction of foliar symptoms in phenotypically resistant plants may not be necessarily associated with significant reduction in the growth of *V. dahliae* in the stem vascular tissues, which is the key feature of resistance and of paramount importance in selecting resistant rootstocks (Beckman & Talboys, 1981; Markakis *et al.*, 2009; Gramaje *et al.*, 2013). The development of real-time quantitative polymerase chain reaction (qPCR) protocols for the specific detection and quantification of *V. dahliae* in infected tissues has made possible a better understanding of the dynamics of *V. dahliae* biomass in infected olive tissues and its relationship with differences in susceptibility among olive cultivars (Mercado-Blanco *et al.*, 2003; Markakis *et al.*, 2009; Gramaje *et al.*, 2013). Use of these protocols would provide a better characterization of resistance phenotype to D. *V. dahliae* in wild olive rootstocks.

The main objective of this present study was to test if resistance in wild olive clones Ac-13, Ac-18, OutVert and StopVert is associated with significant reduction in the extent of stem colonization by the D *V. dahliae* pathotype. To that aim, a series of experiments was carried out under controlled conditions optimal for verticillium wilt development, using microbiological and qPCR protocols.

Materials and methods

Plant material

Certified, 6- to 10-month-old 40–52 cm high plants of wild olive clones Ac-13 and Ac-18, 10- to 12-month-old 30–50 cm high plants of OutVert and StopVert, and 6- to 12-month-old 45–56 cm high plants of Frantoio and Picual olives were used.

Frantoio and Picual olives are resistant and highly susceptible to D *V. dahliae*, respectively (López-Escudero *et al.*, 2004; Jiménez-Díaz *et al.*, 2012). Own-rooted plants of Ac-13, Ac-18, Frantoio and Picual were propagated by Plantas Continental, S.A. by rooting leafy stem cuttings in a pasteurized potting mixture (peat:sand, 2:1, v/v) under mist conditions in plastic tunnels (Caballero & Del Río, 2010). Plants of OutVert and StopVert were propagated for this work by Vitroplant Italia S.R.L. Società Agricola. These plants were axenically micropropagated from axillary buds and further rooted in the pasteurized potting mixture as above.

Verticillium dahliae isolate and inoculum production

Monoconidial *V. dahliae* isolate V138I from defoliated Coker 310 cotton in Córdoba (Spain), and representative of the D pathotype, was used in experiments. This isolate belongs to VCG 1A; it was proved highly virulent on cotton and olive and characterized to pathotype both by pathogenicity assays and DNA markers (Korolev *et al.*, 2001; Mercado-Blanco *et al.*, 2003; Collado-Romero *et al.*, 2006). Plum extract agar (900 mL distilled water, 20 g agar, 100 mL concentrated plum extract, 1 g yeast, 5 g lactose, pH 5.6–6.9; Talboys, 1960) cultures of this isolate covered with liquid paraffin were stored at 4 °C in the dark (Bejarano-Alcázar *et al.*, 1996) and 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 isolate were obtained by placing small agar plugs from stock cultures on chlortetracycline-amended water agar (CWA; 1 L distilled water, 20 g agar, 30 mg chlortetracycline) and further subculturing on potato dextrose agar (PDA; Difco Laboratories Inc.). Cultures on PDA were grown for 7 days at 24 °C in the dark and used for inoculum increase. For experiments, inocula consisted of conidial suspensions or infested cornmeal-sand mixture (CMS; sand:cornmeal:deionized water, 9:1:2, w/w) depending on the experiment (Nene & Haware, 1980). Conidia were from cultures in potato dextrose broth (PDB; Difco Laboratories Inc.) incubated at 125 rpm on an orbital shaker (Adolf Kühner AG) at 24 ± 1 °C in the dark for 7 days. Conidia in the liquid cultures were filtered through eight layers of sterile cheesecloth and inoculum concentration was adjusted to 10^7 conidia mL^{-1} with sterile water using a haemocytometer. Viability of conidia in the inoculum suspension was determined by dilution plating on PDA and incubating as above. Inoculum in CMS was produced in Erlenmeyer flasks containing 400 g autoclaved (twice at 121 °C for 1.5 h) mixture infested with twelve 5 mm diameter PDA discs from 7-day-old cultures and incubated at 24 ± 1 °C in the dark for 1 month. The infested CMS was homogenized, allowed to desiccate in an incubator adjusted to 33 °C for 3 days and thoroughly mixed with a pasteurized soil mixture (clay loam:peat, 2:1, v/v; pH 8.4, 24% water holding capacity) at a rate of 1:20 (w/w) (hereafter referred as infested soil mixture) to reach an inoculum density of 3×10^7 CFU g^{-1} soil of D *V. dahliae* V138I as determined by dilution-plating on semiselective NP-10 medium (Kabir *et al.*, 2004).

Resistance assays

Three experiments (I to III) were conducted to assess resistance of wild olive clones Ac-13, Ac-18, OutVert and StopVert to D *V. dahliae*. Frantoio and Picual olives were included in the experiments as D *V. dahliae*-resistant and susceptible controls, respectively, to ensure that virulence of inocula and incubation conditions were adequate for successful infection and disease on known olive cultivars. Plants were inoculated either by root dip in a conidial suspension or transplanting to the infested soil mixture, or in a sequence first by transplanting to the infested soil followed by root dipping in the conidial suspension, depending on the experiment.

In all experiments plants were grown in 1.5 L ($11 \times 11 \times 13$ cm^3) disinfested plastic pots (one plant per pot) in a growth chamber adjusted to 22 ± 2 °C, 60–80% relative humidity and a 14 h photoperiod of fluorescent light of 360 μmol m^{-2} s^{-1} for variable periods of time, depending on the experiment. These environmental conditions are optimal for infection by D *V. dahliae* and development of verticillium wilt in olive (Calderón *et al.*, 2014). Plants were watered every 1 – 2 days as needed and fertilized weekly with 100 mL Hoagland's nutrient solution (Hoagland & Arnon, 1950). Upon termination of experiments, the main stem of each plant deprived of leaves and lateral shoots was equally divided into three sections representative of the lowest (L, soil line to 12–25 cm height), medium (M, 12–25 to 23–51 cm height) and upper (U, 23–51 to 39–76 cm height) zones. These plant stem zones were used to quantitatively assess the extent of stem colonization by *V. dahliae* in each plant.

Experiment I aimed to determine if the possibility of recurrent infections, as compared to a monocycle of infection, would have an influence on the OutVert and StopVert resistance response. Plants of OutVert, StopVert, Frantoio and Picual were inoculated by either (i) root dip in a suspension of 10^7 conidia mL^{-1} of D *V. dahliae* V138I, or (ii) transplanting to infested soil mixture. For root-dip inoculation, plants were uprooted from the potting substrate, their roots washed free of substrate residues, slightly trimmed, and dipped in the conidia suspension for 20 min. Plants were then transplanted to pots filled with the pasteurized soil mixture. For transplanting inoculation, plants uprooted from the potting substrate were shaken to retain the rhizosphere soil and placed in pots filled with the infested soil mixture. Control plants were treated similarly with sterile distilled water (root-dip inoculation), or grown in non-infested CMS mixed with the pasteurized soil mixture at the same rate as infested CMS (transplanting inoculation). Inoculated and control plants were incubated in the growth chamber adjusted to the above conditions for 3 months.

Experiment II aimed to further confirm the resistance response of OutVert and StopVert clones shown in Experiment I after extended incubation and reinfection by the pathogen. Plants of OutVert and StopVert were inoculated twice in a sequence, first by transplanting to the infested soil mixture and incubating in the growth chamber for 12 weeks as for Experiment I, then by root dip in a suspension of 10^7 conidia mL^{-1} of D *V. dahliae* V138I for 20 min followed by transplanting to non-infested pasteurized soil mixture and incubating in the growth chamber as above for additional 15 weeks. Plants of susceptible Picual olives were inoculated either by transplanting to infested soil and incubating for 12 weeks, or by root dip in the conidial suspension at the time that wild olives were reinoculated by this procedure, and incubating for 15 weeks as above. Control plants were treated similarly as inoculated plants except for the absence of inoculum.

Experiment III aimed to assess resistance in wild olive clones Ac-13 and Ac-18. Plants of Ac-13, Ac-18, Frantoio and Picual were inoculated by the root-dip method as for Experiment I. Control plants were treated similarly with sterile distilled water. Inoculated and control plants were incubated in the growth chamber adjusted to the above conditions for 3 months.

Experiment I consisted of a three-way factorial design with inoculation method, plant genotype and plant stem zone as factors. Experiments II and III consisted of a two-way factorial design with plant genotype and plant stem zone as factors. There were 10 and 6 replicated pots (one plant per pot) for inoculated and non-inoculated plants of each plant genotype, respectively, in a completely randomized design.

Disease assessment and isolation assays

The disease reaction in the plants was assessed by the incidence (percentage of plant showing disease symptoms) and severity of foliar symptoms. Symptoms were assessed on individual plants on a 0 to 4 rating 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 2- to 3-day intervals throughout the duration of the experiments (Mercado-Blanco *et al.*, 2003). The extent of stem colonization by *V. dahliae* in each plant was quantitatively assessed in the L, M and U zones by means of qPCR assays and isolations of the fungus in CWA upon termination of experiments. Also, vascular occlusion due to *V. dahliae* infection was estimated in stem microsections (Bubici & Cirulli, 2011).

From each of the L, M and U stem zones of a plant, four 10 mm long pieces were sampled and washed thoroughly under running tap water for 30 min. The bark was then aseptically removed and the exposed surface disinfected in 0.5% NaOCl for 1 min, rinsed twice in sterile water, and blotted dry between filter paper. Subsequently, each of the 10 mm long stem pieces was divided into halves. One half was plated onto CWA in Petri dishes and incubated at 24 ± 1 °C in the dark for 14 days and the other was stored at –80 °C until used for qPCR quantification assays. Colonies of *V. dahliae* were identified by microscopic observations of verticillate conidiophores and formation of microsclerotia (Inderbitzin *et al.*, 2011). Data from pathogen isolation from the stem were used to calculate the intensity of stem vascular colonization for each individual plant, according to a stem colonization index (SCI): $SCI = \sum N_i/N_j \times 100$, where N_i is the number of plated stem pieces yielding *V. dahliae* colonies and N_j is the total number of stem pieces plated.

Assessment of vascular occlusion

Stem segments, 5 cm long, were collected from the L and M stem zones of Ac-13, Ac-18, OutVert, StopVert and Picual plants 3 months after root dip inoculation in Experiments I and III. For each of those genotypes, five inoculated and two non-inoculated plants were sampled. Segments were stored in Farmer's fixative (absolute ethanol:glacial acetic acid, 3:1 v/v). Segments were hydrated by dipping in 30% ethanol followed by rinsing in distilled water, with 3 min immersion per step. Cross sections, 25 μm thick, were obtained from segments of each of the stem zones with a microtome. The microsections were then mounted on microscope slides in Canada balsam and examined under the light microscope (Axioskop 50; Carl Zeiss) using ×10 objective (Achroplan × 10/0.25 ∞/–; Carl Zeiss). Pictures of microsections were captured with a camera (TK-1280E; JVCKENWOOD Italia) mounted on the microscope. Pictures of several examined fields were merged to compose a unique image per microsection using PHOTOSHOP CS6 software (Adobe). Plugged and unplugged vessels were counted and the percentage of plugged vessels was calculated.

Real-time qPCR assays

Lyophilized mycelia of D *V. dahliae* V138I and 5 mm long stem segments from the L, M and U stem zones of OutVert, StopVert, Ac-13 and Ac-18 wild olives, and Picual and Frantoio olives sampled upon termination of experiments were used. The four 5 mm long pieces sampled from each zone of a plant genotype were pooled for DNA extraction, lyophilized and ground to a fine power. Mycelium of the fungus was obtained from 7-day-old cultures on a film of sterile cellophane layered over a plate of PDA and incubated at 24 ± 1 °C in the dark. Mycelia growing over the cellophane surface were scraped directly with a sterile scalpel, lyophilized and stored at −20 °C until use.

Fungal and plant DNA were extracted from 30–40 mg of lyophilized fungal mycelium of inoculated and non-inoculated stem tissues using the G-Spin IIp Plant Genomic DNA extraction kit (Intron Biotechnology) and the Fast Prep System Bio 101 (Qbiogene) according to Landa *et al.* (2007). DNA quality was assessed by gel electrophoresis and staining with ethidium bromide. Quantification of DNA samples was done according to Montes-Borrego *et al.* (2011) using the Quant-iT DNA Assay kit broad range fluorometric assay (Molecular Probes Inc.) and a Safire fluorospectrometer (Tecan). Efforts were made to ensure 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. Samples of DNA from stem tissues were diluted to approximately 20 ng μL^{-1}. Dilutions of DNA samples were carried out with sterile, ultrapure water (SUW).

DNA standard curve for qPCR was developed from 10-fold dilutions of *V. dahliae* isolate V138I. For this purpose, the fungal DNA (10 ng μL^{-1}) was serially diluted (1:10, $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$ and $1:10^6$) in a fixed background of DNA (20 ng μL^{-1}) extracted from olive stem according to Gramaje *et al.* (2013). The standard curve always included plant DNA and/or no template DNA as negative controls. Two independent DNA standard curves were obtained using separate plant and pathogen DNA sources that were treated as independent experiments. All qPCR amplifications were performed using the SYBR-4 qPCR protocol (Atallah *et al.*, 2007). This protocol has been shown to be more efficient, sensitive and reproducible for quantification of *V. dahliae* DNA in olive tissues compared with other protocols, with a detection limit of 18 fg *V. dahliae* DNA in infected, symptomless tissues (Gramaje *et al.*, 2013). Thus, PCR mixture consisted of 1 μL DNA sample, 1 × iQ SYBR Green Supermix (Bio-Rad), and 0.2 μM of the primer pair VertBt-F/VertBt-R in a final volume of 20 μL. The thermal cycling conditions consisted of an initial denaturation at 95 °C for 3 min; 40 cycles of 30 s at 95 °C, 30 s at 63 °C and 30 s at 72 °C; and a final extension step of 10 min at 72 °C. Fluorescence of the target amplicon was measured at 72 °C (Gramaje *et al.*, 2013). After the final amplification step, a melting curve temperature profile was obtained by heating to 95 °C, cooling to 55 °C, and slowly heating to 95 °C at 0.5 °C every 10 s to distinguish potential primer dimers and nonspecific amplification products. All qPCR assays were performed in an iCycler iQ3 (Bio-Rad) apparatus and results were analysed with the manufacturer's software (OPTICAL SYSTEM v. 3.0a).

Amplification reactions were performed in 96-well microtitre plates. Ten independent plant samples (biological replicates) and two simultaneous, replicated amplifications (technical replicates) were carried out for each DNA sample. Each plate contained all DNA samples of the *V. dahliae* standard curve as well as that of the stem tissues being 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 curve, as well as DNA samples from non-inoculated plants and a negative control reaction (no template DNA) that were used to test for inter-plate variability. Furthermore, to discard the occurrence of PCR inhibitors, a separate amplification control of an olive target DNA sequence was performed. In DNA samples from each stem zone and olive genotype, the olive β-actin gene was amplified using Act1-fw/Act1-rv primers (Schiliro *et al.*, 2012). Results from quantification of the β-actin were referenced to a DNA standard curve obtained as described above from six twofold dilutions of olive DNA (20 ng μL^{-1}) in SUW (Gramaje *et al.*, 2013).

Data analysis

Stem colonization index and plugging index data were analysed with the GENMOD procedure using the binomial distribution and the logit as link function in SAS (v. 9.4, SAS Institute Inc.). A likelihood ratio test was used to determine whether the plant genotype, plant stem zone or inoculation methods significantly affected ($P < 0.05$) stem colonization. The statistical significance ($P < 0.05$) of the likelihood ratio was determined by a chi-square test and the contrast statement was used to determine significant differences ($P < 0.05$) among treatments (Agresti, 2007). Orthogonal single-degree of freedom contrasts were computed to test the effect of selected experimental treatment combinations.

The cycle threshold (C_t) values for each qPCR reaction was 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 v. 3.0a (Bio-Rad). Moreover, to compare and establish relationships among the different DNA standard curves generated from different plates, the threshold position was manually defined and fixed at the same position for all treatments and experiments (Vaerman *et al.*, 2004). The amplification efficiency (AE) was calculated from the slopes of the standard curves using the equation $AE = 10^{(-1/\text{slope})} - 1$ (Higuchi *et al.*, 1993; Adams, 2006). 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 (ng). Data of *V. dahliae* DNA quantification in stem tissues were subjected to standard analysis of variance (ANOVA) with the GLM procedure in SAS to determine whether the plant genotype, plant zone or inoculation methods significantly affected ($P < 0.05$) *V. dahliae* DNA concentration in the stem zones. Data fulfilled the assumptions for ANOVA according to appropriate statistics. Orthogonal single-degree of freedom contrasts were computed to test the effect of selected experimental treatment combinations.

Results

Development of verticillium wilt on wild and cultivated olive genotypes

No symptoms developed in non-inoculated plants of wild olive clones Ac-13, Ac-18, OutVert or StopVert, or Picual and Frantoio olives, in all three experiments. Severe disease symptoms developed in Picual plants inoculated with D *V. dahliae* V138I either by root dipping in a conidial suspension or transplanting to infested soil mixture in all experiments. Symptoms consisted of early dropping of symptomless, green leaves from individual twigs that eventually resulted in complete defoliation, necrosis and death of the plant, which are typical for the defoliating syndrome caused by the D. *V. dahliae* pathotype (Jiménez-Díaz *et al.*, 2012). Symptoms started to develop by 27–37 days after inoculation depending on the inoculation method and experiment. At the end of Experiment I, mean severity of symptoms in root dip-inoculated Picual plants (3.9 on the 0–4 rating scale) was significantly higher ($P < 0.05$) than that in plants inoculated by transplanting to infested soil mixture (2.7), and disease incidence was 100 and 90%, respectively. Similarly, the final disease incidence and severity in Picual plants in Experiments II and III ranged from 80 to 100% and 3.0 to 3.6, respectively, depending on the method of inoculation.

Symptoms did not develop in OutVert and StopVert plants inoculated by root dip in a conidial suspension or transplanting to infested soil mixture (Experiment I) or after 15 weeks of inoculation by root dip following 12 weeks of growth in infested soil mixture (Experiment II), nor in wild olive clones Ac-13 and Ac-18 inoculated by root dip in the conidial suspension (Experiment III). Incipient leaf chlorosis and necrosis without defoliation developed in Frantoio olives inoculated by transplanting to infested soil mixture and/or root dipping in a conidial suspension in Experiments I and III. In Experiment I, 20–40% of plants were affected at the end of the experiment, the mean symptom severity (0.4–0.5) being significantly ($P < 0.05$) lower than that in susceptible Picual olive. Similar incipient foliar symptoms with 0.7 mean severity developed in 60% of Frantoio plants inoculated by root dip in a conidial suspension by the end of Experiment III.

Characterization of resistance in OutVert and StopVert clones of wild olive to single challenge inoculation with defoliating *Verticillium dahliae*

Verticillium dahliae was reisolated from all stem zones sampled from plants of the two wild olive clones and Picual (data not shown) and Frantoio olives (Fig. 1). Overall, results from inoculation by root dip in a conidial suspension or transplanting to infested soil mixture were nearly identical ($\chi^2 < 0.01$, $P = 0.9556$). However, the intensity of stem colonization assessed by the stem colonization index (SCI) was significantly influenced by plant genotype ($\chi^2 = 177.58$, $P < 0.0001$) and sampled stem zone ($\chi^2 = 42.66$, $P < 0.0001$), as well as by the inoculation method × plant genotype ($\chi^2 = 7.97$, $P = 0.0186$) and plant genotype × sampled zone ($\chi^2 = 13.14$, $P = 0.0106$) interactions (Table 1). The mean SCI value in OutVert wild olive (10.83 ± 4.73%) was not significantly different from that in StopVert (11.25 ± 4.86%) inoculated either by root dip in a conidial suspension ($\chi^2 = 0.29$, $P = 0.5912$) or transplanting to the infested soil mixture ($\chi^2 = 2.29$, $P = 0.1302$) (Fig. 1; Table 1). However, the SCI level in those clones

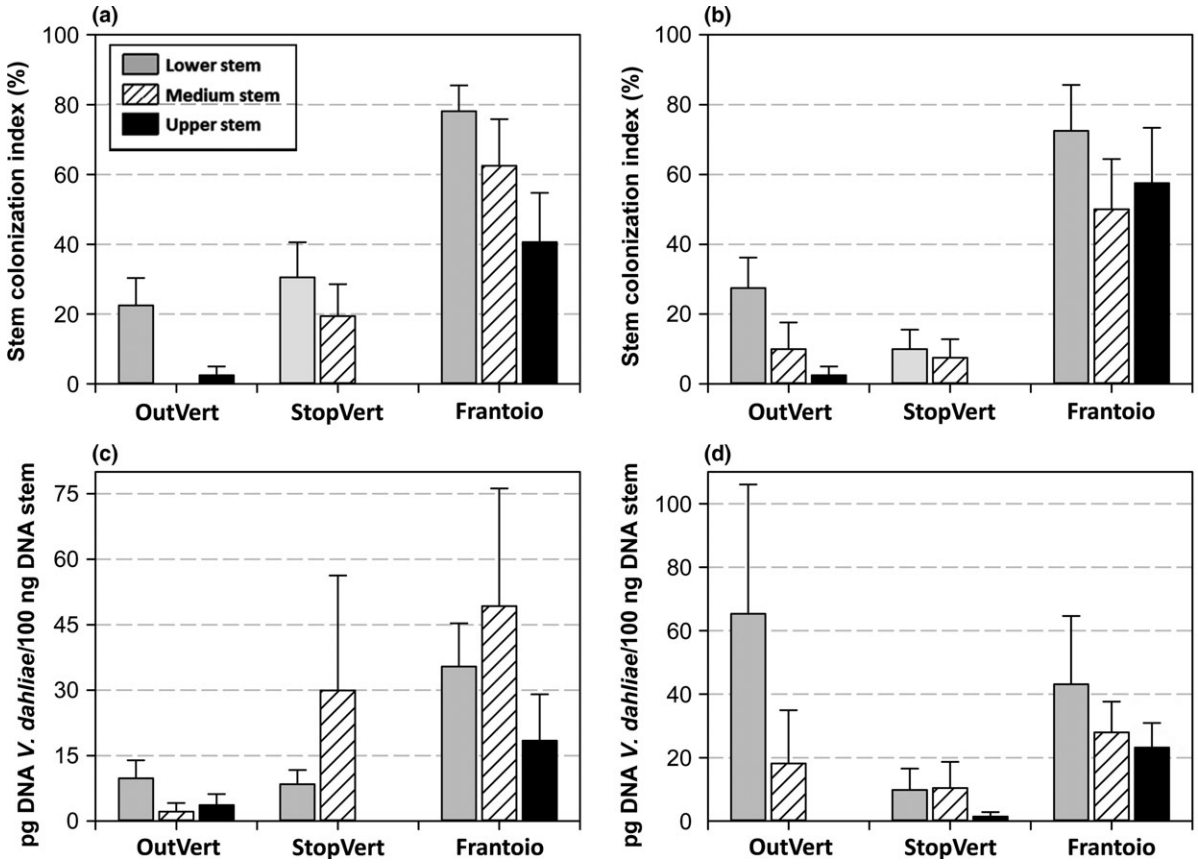

Figure 1 Effects of plant genotype and stem zone sampled on the intensity of colonization (a,b) by, and DNA concentration (c,d) of, defoliating (D) *Verticillium dahliae* in OutVert and StopVert wild olives and Frantoio olive in Experiment I. Plants were inoculated by root dip in a suspension of condia (a,c) or by transplanting to an infested soil mixture (b,d). A stem colonization index was calculated using results from isolations to comparatively assess the extent of colonization by D *V. dahliae*. Each bar represents the mean value of 10 replications (plants) and error bars indicate the standard error of the mean.

was significantly lower ($P < 0.05$) than that in Frantoio olive (60.21 ± 5.69%) that served as the reference for resistance (Fig. 1; Table 1). A pattern of decreasing acropetal colonization along the stem axis occurred in plants of OutVert wild olive and Frantoio olive, the mean SCI being higher ($P < 0.05$) in the lowest stem zone compared with that in the medium and upper zones, which did not differ significantly ($P \geq 0.05$; Fig. 1; Table 1). However, for StopVert wild olive the mean SCI value was higher in the lowest stem zone, but the mean SCI in the medium stem zone was significantly higher ($\chi^2 = 11.30$, $P = 0.0008$) than that in the upper stem zone (Fig. 1; Table 1). As expected, the mean SCI in susceptible Picual olive inoculated by transplanting to infested soil mixture reached 100% (data not shown).

Total genomic DNA extracted from stem samples of each of the wild olive clones and olive cultivars in Experiments I–III was appropriate for qPCR assays (Fig. S1). There were no significant differences ($P \geq 0.05$) among slopes and intercepts of the *V. dahliae* standard curves of plates, thus allowing comparison of data from the different experiments. The quantification limit for SYBR-4 protocol was fixed at a C_t of 36 (18 fg *V. dahliae* DNA) as described by Gramaje *et al.* (2013). The mean amount of D *V. dahliae* DNA in stem tissues of susceptible Picual olive ranged from 4574 to 5532 pg *V. dahliae* DNA/100 ng olive DNA depending on the experiment, which was >90 times higher than the highest mean value reached in the two wild olive clones and Frantoio olive. Therefore, data for Picual olive were excluded from statistical analyses.

Results of DNA quantification in Experiments I and II are shown in Figures 1 and 2. Overall, the amount of D *V. dahliae* DNA in stem tissues in Experiment I did not differ significantly ($P < 0.05$) among inoculation methods, sampled stem zones and interactions between any of the experimental factors. Conversely, there was a significant effect of plant genotype ($F = 4.27$, $P = 0.0158$). Thus, across inoculation methods, the mean amount of D *V. dahliae* DNA in StopVert wild olive (10.71 ± 5.07 pg *V. dahliae* DNA/100 ng olive DNA) was significantly ($P < 0.05$) smaller than that in OutVert (16.81 ± 7.78 pg *V. dahliae* DNA/100 ng olive DNA) (Table 1; Fig. 1). The mean amount of D *V. dahliae*

Table 1 Effects of genotype and stem zone sampled on the intensity of colonization by, and DNA concentration of, defoliating (D) *Verticillium dahliae* in plants of OutVert and StopVert wild olives and Frantoio olive inoculated by root dip or transplanting to infested soil in Experiment I

Contrast	Source of variation	Intensity of stem colonization			Concentration of DNA in stem		
		d.f.	χ^2	$P > \chi^2$	d.f.	F	$P > F$
	Inoculation method (IM)	1	<0.01	0.9556	1	0.02	0.8948
	Genotype (G)	2	177.58	<0.0001	2	4.27	0.0158
	IM × G	2	7.97	0.0186	2	2.01	0.1374
	Plant stem zone (PZ)	2	42.66	<0.0001	2	2.65	0.0737
	IM × PZ	2	3.49	0.1743	2	1.44	0.2397
	G × PZ	4	13.14	0.0106	4	0.84	0.5022
	IM × G × PZ	4	4.31	0.3660	4	0.61	0.6583
Genotype	Frantoio versus OutVert	–	–	–	1	4.90	0.0284
	Frantoio versus StopVert	–	–	–	1	0.38	0.5383
	Outvert versus StopVert	–	–	–	1	7.75	0.0061
Genotype × Inoculation method	Root dip: Frantoio versus OutVert	1	80.01	<0.0001	–	–	–
	Root dip: Frantoio versus StopVert	1	51.47	<0.0001	–	–	–
	Root dip: Outvert versus StopVert	1	0.29	0.5912	–	–	–
	Infested soil: Frantoio versus OutVert	1	65.80	<0.0001	–	–	–
	Infested soil: Frantoio versus StopVert	1	92.83	<0.0001	–	–	–
	Infested soil: Outvert versus StopVert	1	2.29	0.1302	–	–	–
Plant zone × Genotype	Frantoio: Lower versus Medium & upper zones	1	10.44	0.0012	–	–	–
	Frantoio: Medium versus Upper zone	1	0.75	0.3875	–	–	–
	OutVert: Lower versus Medium & upper zones	1	26.63	<0.0001	–	–	–
	OutVert: Medium versus Upper zone	1	0.07	0.7896	–	–	–
	StopVert: Lower versus Medium & upper zones	1	11.65	0.0006	–	–	–
	StopVert: Medium versus Upper zone	1	11.30	0.0008	–	–	–
Genotype × Plant zone	OutVert versus StopVert: Lower zone	1	1.00	0.3139	–	–	–
	OutVert versus StopVert: Medium zone	1	4.86	0.0275	–	–	–
	OutVert versus StopVert: Upper zone	1	1.64	0.2009	–	–	–

DNA in resistant Frantoio olive (37.24 ± 7.09 pg *V. dahliae* DNA/100 ng olive DNA) was significantly higher ($F = 4.90$, $P = 0.0284$) than that in OutVert but it was not significantly different ($F = 0.38$, $P = 0.5383$) from that in StopVert (Table 1). However, this mean value of D *V. dahliae* DNA in Frantoio olive was 8.6- and 3.2-fold higher, respectively, than in OutVert and StopVert plants inoculated by root dip, and 1.1- and 4.2-fold higher respectively than in OutVert and StopVert plants in infested soil mixture (Fig. 1).

Characterization of resistance in OutVert and StopVert clones of wild olive to sequential inoculations with defoliating *V. dahliae*

Verticillium dahliae was not reisolated from any of the stem zones sampled from StopVert plants or from the medium and upper stem zones of OutVert plants, after incubation for 12 weeks in infested soil mixture and an additional 15 weeks following root dip inoculation in a conidial suspension. Isolations from the lower stem zone of OutVert plants resulted in a mean SCI value of 32.5% (Fig. 2a). Frantoio olive was not included in this bioassay. As in Experiment I, the mean SCI in susceptible Picual olive inoculated by root dip or transplanting to infested soil mixture reached 100% (data not shown).

Overall, the amount of pathogen DNA in stem tissues did not differ significantly ($F > 1.96$, $P > 0.1002$) among plant genotype or sampled stem zone, but a significant ($F = 3.30$, $P = 0.0443$) interaction occurred between them (data not shown). This was due to differences in the pattern of DNA detection and quantification between the two wild olive clones (Fig. 2b). *Verticillium dahliae* DNA was detected and quantified only in the lower stem zone of OutVert plants (35.21 ± 20.58 pg *V. dahliae* DNA/100 ng olive DNA), and in the medium and upper stem zones of StopVert plants, although at a much lower amount (0.45 ± 0.45 and 5.24 ± 4.47 pg *V. dahliae* DNA/100 ng olive DNA, respectively; Fig. 2b).

Characterization of resistance in Ac-13 and Ac-18 clones of wild olive to single challenge, root dip inoculations with defoliating *V. dahliae*

Verticillium dahliae was reisolated from all stem zones sampled from plants of Ac-13 wild olive and susceptible Picual olive. The pathogen was not isolated from the medium stem zone sampled from Frantoio olives or from any of stem zones samples from plants of Ac-18 wild olive (Fig. 3). Across stem zones, the mean SCI in Ac-13 wild olive was not significantly ($\chi^2 = 2.60$, $P = 0.1067$) different from that in Frantoio olive. In these latter genotypes, the mean SCI had a similar pattern of

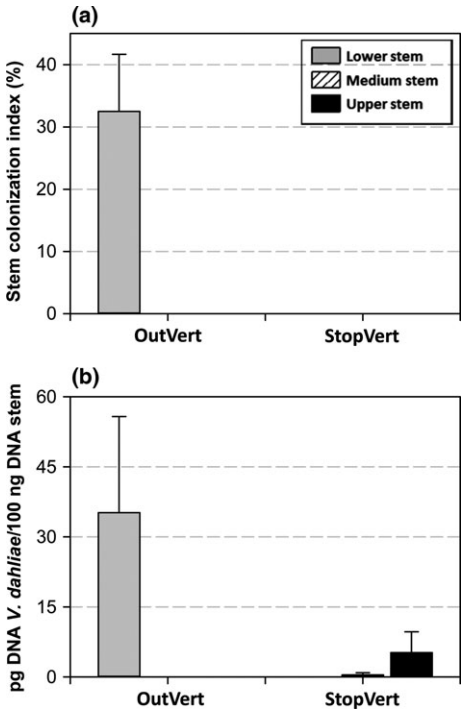

Figure 2 Effects of plant genotype and stem zone sampled on the intensity of colonization (a) by, and DNA concentration (b) of, defoliating (D) *Verticillium dahliae* in OutVert and StopVert wild olives in Experiment II. A stem colonization index was calculated using results from isolations to comparatively assess the extent of colonization by D *V. dahliae*. Each bar represents the mean value of 10 replications (plants) and error bars indicate the standard error of the mean.

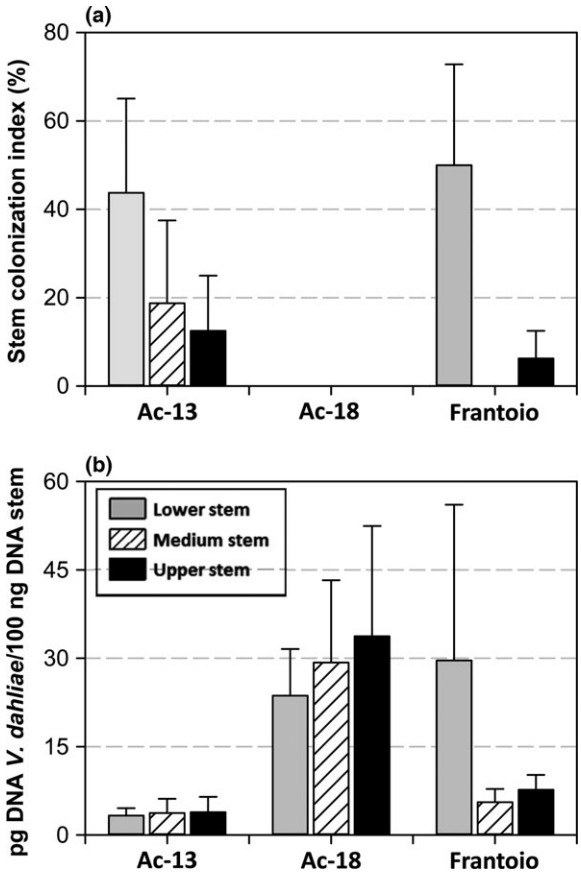

Figure 3 Effects of plant genotype and stem zone sampled on the intensity of colonization (a) by, and DNA concentration (b) of, defoliating (D) *Verticillium dahliae* in Ac-13 and Ac-18 wild olives in Experiment III. A stem colonization index was calculated using results from isolations to comparatively assess the extent of colonization by D *V. dahliae*. Each bar represents the mean value of 10 replications (plants) and error bars indicate the standard error of the mean.

decreasing acropetal colonization along the plant stem ($\chi^2 = 18.89$, $P < 0.0001$) as described for StopVert and OutVert wild olives. Thus, the mean SCI in Ac-13 and Frantoio reached a highest value of 43.75 and 50.00%, respectively, in the lower stem zone, and decreased to 12.50 and 6.25%, respectively, in the upper stem zone (Table 2; Fig. 3a). In this experiment, the mean SCI in susceptible Picual olive ranged from 43.75 to 12.50% in the lower and upper stem zones, respectively.

There were no significant differences ($F \leq 3.26$, $P \geq 0.0540$) among the quantified D *V. dahliae* DNA across plant genotypes ($F = 3.26$, $P = 0.0540$) or sampled stem zones ($F = 0.19$, $P = 0.8308$; Table 2), except for the amount of D *V. dahliae* DNA in Ac-13 wild olive that was significantly lower ($F = 4.35$, $P = 0.0465$) than that in Frantoio olive (Fig. 3b). Across stem zones, the mean amount of D *V. dahliae* DNA was higher in Ac-18 wild olive (28.89 ± 7.54 pg *V. dahliae* DNA/100 ng olive DNA) than in Frantoio olive (14.30 ± 8.67 pg *V.*

Table 2 Effects of genotype and stem zone sampled on the intensity of colonization by, and DNA concentration of, defoliating (D) *Verticillium dahliae* in plants of Ac-13 and Ac-18 wild olives inoculated by root dip in Experiment III

	Intensity of stem colonization			Concentration of DNA in stem		
Source of variation	d.f.	χ^2	$P > \chi^2$	d.f.	F	$P > F$
Genotype	1	2.60	0.1067	2	3.26	0.0540
Plant stem zone	2	18.89	<0.0001	2	0.19	0.8308
Genotype × Plant stem zone	2	3.58	0.1670	4	0.59	0.6694
Contrasts for Plant stem zone						
Lower versus Medium & upper zones	1	18.89	<0.0001	–	–	–
Medium versus Upper zone	1	0.43	0.5142	–	–	–

Table 3 Effects of genotype and stem zone sampled on the intensity of plugging of xylem vessels in OutVert and StopVert wild olives from Italy, Ac-13 and Ac-18 wild olives from Spain, and susceptible Picual olive inoculated with defoliating *Verticillium dahliae* in Experiments I and III

	Italian wild olives				Spanish wild olives		
	d.f.	χ^2	$P > \chi^2$		d.f.	χ^2	$P > \chi^2$
Source of variation							
Genotype	2	257.04	<0.0001	Genotype	2	144.22	<0.0001
Plant stem zone	1	68.93	<0.0001	Plant zone	1	49.33	<0.0001
Genotype × Plant stem zone	2	6.73	0.0345	Genotype × Plant stem zone	2	15.18	0.0005
Contrasts for Genotype							
Picual versus OutVert	1	49.51	<0.0001	Picual versus Ac-13	1	138.22	<0.0001
Picual versus StopVert	1	20.30	<0.0001	Picual versus Ac-18	1	12.38	0.0004
Outvert versus StopVert	1	158.73	<0.0001	Ac-13 versus Ac-18	1	54.34	<0.0001
Contrasts for Plant stem zone by genotype							
Picual: Lower versus Medium zone	1	25.54	<0.0001	Picual: Lower versus Medium zone	1	25.54	<0.0001
Outvert: Lower versus Medium zone	1	50.11	<0.0001	Ac-13: Lower versus Medium zone	1	9.73	0.0018
Stopvert: Lower versus Medium zone	1	0.01	0.9190	Ac-18: Lower versus Medium zone	1	29.24	<0.0001

dahliae DNA/100 ng olive DNA) and lowest in Ac-13 wild olive (3.64 ± 1.14 pg *V. dahliae* DNA/100 ng olive DNA). No clear pattern of variation along the plant stem was found in the amount of D *V. dahliae* DNA quantified in the different stem zones for the different plant genotypes (Fig. 3b).

Assessment of stem vascular occlusion

Plugging of xylem vessels in the stem of non-inoculated plants was observed occasionally and only in the lower zone of Ac-13 plants (not shown). Conversely, a variable degree of plugging in stem xylem vessels was observed in inoculated plants of Ac-13, Ac-18, OutVert and StopVert wild olives, as well as Picual olive, depending on plant genotype and sampled stem zone (Fig. 4). Overall, the incidence of vessel plugging in stems of susceptible Picual plants (1.57 ± 0.61%) was significantly lower ($P < 0.05$) than that in plants of any wild olive genotype (>2.58 ± 0.94%), except for StopVert that showed the lowest ($P < 0.05$) incidence of vessel plugging (0.23 ± 0.09%) in the study. The incidence of plugging of xylem vessels in stem of wild olives Ac-13, Ac-18 and OutVert was significantly higher ($P < 0.01$) in the lowest zone compared with that in the medium zone. There was no significant difference ($\chi^2 = 0.01$, $P = 0.9190$) between the incidence of xylem vessel plugging in the lower and medium stem zones of StopVert wild olive (Table 3; Fig. 4). When the wild olive genotypes were compared separately by their geographic origin, the incidence of plugging in stem xylem vessels of OutVert plants was higher ($\chi^2 = 158.73$, $P < 0.0001$) than that in plants of StopVert (Italian clones); and similarly, the incidence of vessel plugging in stem of Ac-13 plants was higher ($\chi^2 = 54.34$, $P < 0.0001$) than that in plants of Ac-18 wild olive (Spanish clones; Table 3; Fig. 4).

Discussion

Wild olive genotypes highly resistant to D *V. dahliae* used as rootstocks can provide an improved means for the management of verticillium wilt in grafted susceptible olive cultivars that are agronomically adapted and commercially desirable (Jiménez-Díaz *et al.*, 2012; Trapero *et al.*, 2013, 2015; Arias-Calderón *et al.*, 2015). In previous studies, it was concluded that wild olive clones Ac-

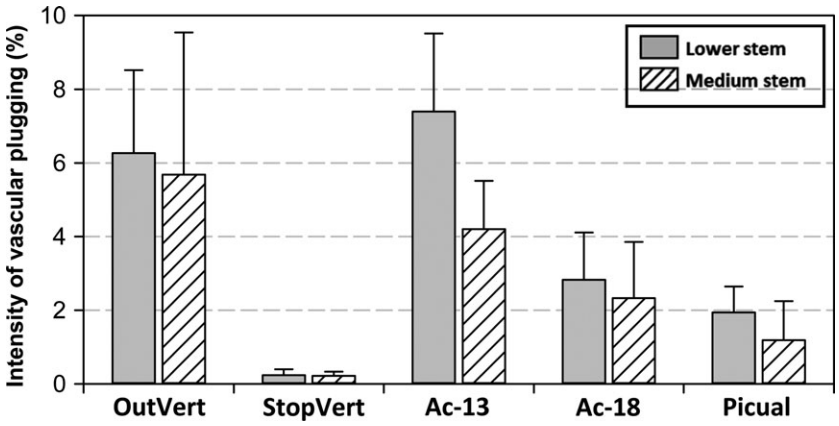

Figure 4 Effects of plant genotype and stem zone sampled on the intensity of plugging of xylem vessels in wild olives OutVert and StopVert from Italy, and Ac-13 and Ac-18 from Spain, and susceptible Picual olive inoculated with defoliating (D) *Verticillium dahliae* in Experiments I and III. Each bar represents the mean value of five replications (plants) and error bars indicate the standard error of the mean.

13, Ac-18, OutVert and StopVert are highly resistant to D *V. dahliae* based on the development of incipient or no foliar disease symptoms, together with reduced stem vascular browning and reisolation of the fungus (Colella *et al.*, 2008; Jiménez-Díaz, 2008; Bubici & Cirulli, 2011). However, reduction of the pathogen growth and vascular spread during the systemic phase of colonization, which is the basis of resistance against *V. dahliae* in the plant (Beckman & Talboys, 1981; Pegg & Brady, 2002; Eync *et al.*, 2009), was not assessed in those studies. Moreover, a symptomless reaction in tolerant (but not resistant) cultivars is associated with an extent of systemic colonization comparable to that in susceptible cultivars (Chen *et al.*, 2004). This present study showed that the amount of D *V. dahliae* DNA quantified in stems of symptomless Ac-13, Ac-18, OutVert and StopVert wild olives by a highly sensitive and efficient qPCR protocol (Gramaje *et al.*, 2013) was on average 249 to 1537 times lower than that in severely affected Picual olive, depending on the clone and inoculation procedure. Because the amount of pathogen DNA in the plant should be representative of the number of potential fungal propagules, the symptomless reaction in Ac-13, Ac-18, OutVert and StopVert found in this and previous studies corresponds to expression of resistance, rather than tolerance, against D *V. dahliae* in them. Although previous studies have identified wild olive genotypes with a resistant phenotype to D *V. dahliae* (Colella *et al.*, 2008; Arias-Calderón *et al.*, 2015; Trapero *et al.*, 2015), to the authors' knowledge this is the first time that symptomless resistance in wild olive has been demonstrated to correlate with the plant's ability to quantitatively reduce the extent of stem colonization by the pathogen. Mercado-Blanco *et al.* (2003) were unable to quantify the D *V. dahliae* DNA in stems of a wild olive resistant clone (Acebuche-L) and suggested that the number of potential pathogen propagules in their tissues must be very low. However, the qPCR protocol used by these authors was less sensitive than that used in the present study, because the maximum amount of D *V. dahliae* DNA in susceptible Picual plants was over 14 times lower than that found in the same cultivar in the present study (mean = 5532 pg/100 ng olive DNA), using the same isolate and inoculation procedure as those of Mercado-Blanco *et al.* (2003).

This study addressed quantifying the pathogen colonization in different zones along the plant stem of Ac-13, Ac-18, OutVert and StopVert wild olives because, if used as rootstocks, grafting will be made between the lowest and the medium stem zones and thus infection of the scion would eventually take place from them (Markakis *et al.*, 2009; Bubici & Cirulli, 2012; Jiménez-Díaz *et al.*, 2012). Results indicate that D *V. dahliae* can systemically spread from roots to stem in all clones because its DNA could be quantified in the three stem zones sampled from them. There was no clear pattern in the DNA quantification along the stem, except for lack of detection in the uppermost zone of OutVert and StopVert wild olives inoculated by transplanting to infested soil or root dip in a conidial suspension, respectively. Other authors have reported a similar pattern of systemic pathogen spread and reduction of D. *V. dahliae* DNA in stems of resistant olive cultivars with reduced severity of foliar symptoms (Mercado-Blanco *et al.*, 2003; Markakis *et al.*, 2009; Gramaje *et al.*, 2013). Regardless of the inoculation method, the degree of resistance against D *V. dahliae* in StopVert was higher than that in OutVert as indicated by the significantly ($P < 0.05$) lower amount of *V. dahliae* DNA in stems of the former (Table 1; Fig. 1). On average, the amount of D *V. dahliae* DNA in stems of root-dip inoculated susceptible Picual olive was 391 and 249 times higher than in StopVert and OutVert, respectively. Similarly, the degree of resistance against D *V. dahliae* in Ac-13 and Ac-18 wild olives challenged by root dip inoculation was higher in Ac-13 than in Ac-18, the mean amount of fungal DNA in stem tissues being 1537 and 191 times lower than that in stems of susceptible Picual olive, respectively.

This study also aimed to confirm whether resistance in OutVert and StopVert to a monocycle of infection determined by Colella *et al.* (2008) and Bubici & Cirulli (2011) would hold under a more stringent scenario of potential recurrent infections that may occur under natural conditions. The necessity for this was suggested by results of Trapero *et al.* (2013). These authors found that the symptomless resistance phenotype in some olive cultivars to root dip inoculation with D *V. dahliae* under artificial conditions was not exhibited when the cultivars were planted in highly infested natural soil. In this present study, that scenario was simulated by growing plants in highly infested soil (3×10^7 CFU g^{-1} soil) and for an extended time period following sequential root dip inoculation. Results under these conditions did not significantly differ from those in a monocycle of infection by root dip inoculation in 10^7 conidia mL^{-1}, which is an inoculum concentration over twice that used by Colella *et al.* (2008). Moreover, extending the incubation period from 12 weeks for single inoculations to 27 weeks for sequential inoculations was associated with an apparent reduction in the amount of *V. dahliae* DNA in stems, which could be quantified only in the lowest stem zone of OutVert plants but not in that of StopVert plants (Table 2; Fig. 2). A similar pattern of decrease in the amount of D *V. dahliae* DNA in stem and root tissues over time was reported in Acebuche-L wild olive, as well as in resistant olive cultivars with reduced severity of foliar symptoms (Mercado-Blanco *et al.*, 2003; Markakis *et al.*, 2009; Gramaje *et al.*, 2013). This decrease in the amount of pathogen DNA may correspond with a general, non-specific defence reaction in verticillium diseases, whereby mycelium of *Verticillium* spp. within the xylem vessels in both compatible and incompatible interactions undergoes lysis, which in the susceptible host operates too late to be effective (Pegg, 1985; Heinz *et al.*, 1998).

The reduction in the quantitative stem colonization by D *V. dahliae* in symptomless Ac-13, Ac-18, OutVert and StopVert wild olives was also indicated by a sharp

decrease in the extent of pathogen reisolation assessed by a SCI, the mean of which ranged from 10.83 to 24.90%, compared with 100% in the susceptible Picual olive. Overall, there was a pattern of decreasing SCI in acropetal progression along the plant axis, as well as correlation ($r = 0.1690$, $P = 0.0043$; data not shown) between positive reisolation and quantification of pathogen DNA by qPCR, with few exceptions. Thus, *V. dahliae* could not be isolated from the medium and upper stem zones of root-dip inoculated OutVert and transplanted-inoculated StopVert, respectively, whereas the pathogen DNA was quantified in them. More importantly, a complete failure of positive reisolation of the pathogen occurred from stems of root-dip inoculated Ac-18 and sequentially inoculated StopVert, despite D *V. dahliae* DNA being quantified from all or some of the sampled stem zones, respectively. This partial or total failure of pathogen reisolation has also been reported in D. *V. dahliae*-resistant olive cultivars that developed limited foliar symptoms (Markakis *et al.*, 2009; Gramaje *et al.*, 2013; Arias-Calderón *et al.*, 2015) as well as in resistant wild olives (Mercado-Blanco *et al.*, 2003; Colella *et al.*, 2008; Arias-Calderón *et al.*, 2015). This failure of reisolations is consistent with the aforementioned reduction in the amount of D. *V. dahliae* DNA, and may result from loss of pathogen viability and/or degradation in infected tissues over time, determined by expression of a non-specific defence reaction (Pegg, 1985; Heinz *et al.*, 1998).

This study used Frantoio olive as a resistance reference because of its resistance to D. *V. dahliae* and current commercial interest (López-Escudero *et al.*, 2004; Barranco, 2010; Bubici & Cirulli, 2011; Arias-Calderón *et al.*, 2015; Trapero *et al.*, 2015). Unlike the wild olive clones, incipient symptoms developed in Frantoio olives, with incidence varying from 20 to 60% depending on inoculation methods and experiments, which agrees with reports by other authors (Martos-Moreno *et al.*, 2006; Cirulli *et al.*, 2008; Bubici & Cirulli, 2012; Gramaje *et al.*, 2013; Arias-Calderón *et al.*, 2015). Correspondingly, D *V. dahliae* DNA was quantified in all Frantoio stem zones, the mean amount being 129 to 339 times lower than the mean amount of *V. dahliae* DNA quantified in susceptible Picual olive, depending on the method of inoculation and experiment. Also, the mean amount of *V. dahliae* DNA quantified in stems of Frantoio olive was 1.4- to 3.7-fold greater than that reported by Gramaje *et al.* (2013) (approximately 10 pg *V. dahliae* DNA/100 ng olive DNA) in root dip-inoculated symptomless plants grown for 35 days, which was about a third of the time period in the present study. Compared with the studied wild olive clones, the mean amount of D. *V. dahliae* DNA quantified in Frantoio stem tissues was 2-fold lower than that in Ac-18, but 3.9-, 2.2- and 3.5-fold greater than that in Ac-13, OutVert and StopVert, respectively. The statistical significance of these differences at $P < 0.05$ was obscured by plant-to-plant variability in some cases. The degree of stem colonization in Frantoio assessed by the SCI was similar to that reported by Gramaje *et al.* (2013), and similar or significantly higher than that in Ac-13 and OutVert and StopVert, respectively. The mean SCI in Frantoio decreased with an acropetal pattern along the plant axis that correlated with quantification of pathogen DNA by qPCR, except for lack of pathogen isolation from the medium stem zone in one of experiments, as discussed above for Ac-18 and StopVert wild olives.

Resistance against D *V. dahliae* in Ac-13, Ac-18, OutVert and StopVert wild olives may involve defence mechanisms that lessen root penetration and lateral spread of the fungus in extravascular root tissues (e.g. cell wall reinforcement through callose deposition, increased lignification, lignin cross-linking), as well as those operating within the xylem that reduce systemic colonization by the pathogen (e.g. build-up of vessel occlusions by gums, gels or tyloses; phenolics accumulation) (Beckman & Talboys, 1981; Talboys, 1984; Nicholson & Hammerschmidt, 1992; Eync *et al.*, 2009). Artificial wounding for root dip inoculation, and micro- or macrobreakages that may have occurred during transplanting to infested soil, might have overcome defence mechanisms in resistant wild olive clones operating during the penetration phase of pathogenesis. Nevertheless, the method of inoculation did not have an influence on the quantitative degree of resistance of Frantoio, OutVert and StopVert, suggesting that the defence mechanisms in stems involved in resistance must operate during the colonization phase within the xylem. Bubici & Cirulli (2012) found that plugging of xylem vessels in stems of D *V. dahliae*-inoculated Frantoio plants was higher compared with that in susceptible olives and suggested that plugging may contribute to resistance in Frantoio. In this present study, plugging of xylem vessels in stems of infected Ac-13, Ac-18 and OutVert was significantly higher than in susceptible Picual olive, and occurred to a larger extent in the lower stem zone, suggesting that it may contribute to reduce systemic spread of D *V. dahliae* along the stem. However, that increased vessel plugging did not occur in StopVert, suggesting that defence mechanisms underlying resistance to D *V. dahliae* may vary depending on plant genotypes. Vessel plugging may occur together with formation of tyloses in the same plant as a response to vascular infection, but this was not assessed in this work. Quantitative reduction in the extent of vascular colonization by the pathogen in the wild olive clones may also be due to accumulation of phenolic compounds, as suggested by Markakis *et al.* (2010) who attributed resistance to D *V. dahliae* in Koroneiki olive to the phenolic compound verbascoside.

In conclusion, results of this research show that wild olive clones Ac-13, Ac-18, OutVert and StopVert have a level of resistance to D *V. dahliae* higher than that occurring in cultivated olive germplasm and have valuable potential as rootstocks for the management of verticillium wilt in olive. Several authors found that Frantoio rootstock provided partial or complete verticillium wilt control in grafted susceptible olive cultivars root-dip inoculated with 1×10^7 or 5×10^6 conidia mL^{-1} of D *V. dahliae* under controlled conditions, respectively (Porras-Soriano *et al.*, 2003; Bubici & Cirulli, 2012).

However, Trapero *et al.* (2013) cautioned about the use of Frantoio as a resistant rootstock because verticillium wilt did develop in this cultivar in field soils infested with a moderate amount of D *V. dahliae* microsclerotia. The authors are confident about the consistency and durability of the observed resistance in the four clones of wild olive in this study. This is based on the fact that resistance was challenged with an extremely high inoculum density of 3×10^7 CFU mL^{-1} of D *V. dahliae* under conditions suitable for infection that gave rise to full development of the disease in susceptible Picual olive by 8–10 weeks after inoculation, depending on the experiment.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1: Standard regression line of a six-point twofold serial dilution of *Olea europaea* DNA (20 ng μL^{-1}) diluted in sterile ultrapure water extracted from olive stems obtained for the β-actin gene using Act1-fw/Act1-rv primers in qPCR protocol as described in Schiliro *et al.* (2012). Threshold cycles (C_t) were plotted against the log of genomic DNA standard curves of known concentrations (upper panel). Threshold cycles (C_t) of quantification of olive genomic DNA from wild olive (StopVert, OutVert, Ac-13 and Ac-18) or olive cultivar (Frantoio and Picual) samples obtained from stems of plants infected with defoliating *Verticillium dahliae* sampled at different stem zones (lower panels).