Upward movement of Verticillium dahliae *from soil to olive plants detected by qPCR*

Maria Teresa Ceccherini, Nicola Luchi, Ottorino-Luca Pantani, Judith Ascher, Paolo Capretti & Giacomo Pietramellara

World Journal of Microbiology and Biotechnology

ISSN 0959-3993
Volume 29
Number 10

World J Microbiol Biotechnol (2013)
29:1961-1967
DOI 10.1007/s11274-013-1342-0

Springer

World J Microbiol Biotechnol (2013) 29:1961–1967
DOI 10.1007/s11274-013-1342-0

SHORT COMMUNICATION

Upward movement of *Verticillium dahliae* from soil to olive plants detected by qPCR

Maria Teresa Ceccherini · Nicola Luchi · Ottorino-Luca Pantani · Judith Ascher · Paolo Capretti · Giacomo Pietramellara

Received: 11 December 2012 / Accepted: 25 March 2013 / Published online: 31 March 2013

Abstract Olive trees play an important role in cultural, ecological, environmental and social fields, constituting in large part the Mediterranean landscape. In Tuscany, an important economic activity is based on olive. Unfortunately, the Verticillium wilt affects this species and causes vascular disease. In the present study, a real-time quantitative PCR approach has been used to detect and quantify *Verticillium dahliae* in soil and in olive tree tissues both in micropropagated and in seedling olives. The minimum amounts of *V. dahliae* DNA sequences detected in soil were 11.4 fg which is equivalent to less than one fungal haploid genome. In micropropagated olive the pathogen was detected in the leaves after 43 days, showing a vertical upward movement of the fungus from the culture medium to stem and leaves. A similar fungal behaviour was observed in inoculated olive stem where after 15 days the fungal DNA was detected from symptomless stem tissue above 8 cm the inoculation site. The described molecular approach is expected to provide a more sensitive and less time-consuming alternative detection method for *V. dahliae* than plating assay procedures, which were traditionally proposed as an early diagnosis method for Verticillium wilt to farmers and tree nursery growers.

Keywords Olive · *Verticillium dahliae* · Real time PCR · Early diagnosis

M. T. Ceccherini (✉) · O.-L. Pantani · J. Ascher · G. Pietramellara
Dipartimento di Scienze delle Produzioni vegetali, del Suolo e dell'Ambiente agroforestale (DIPSA), sezione Scienza del Suolo e Nutrizione della Pianta, Università di Firenze, Piazzale delle Cascine, 28, 50144 Florence, Italy
e-mail: mariateresa.ceccherini@unifi.it

N. Luchi · P. Capretti
DISPAA, Sezione di Patologia Vegetale ed Entomologia, Università di Firenze, Piazzale delle Cascine 28, 50144 Florence, Italy

N. Luchi
Consiglio Nazionale delle Ricerche, Istituto per la Protezione delle Piante, Via Madonna del Piano 10, 50019 Sesto Fiorentino, Firenze, Italy

Introduction

Olive tree, *Olea europaea*, is an evergreen tree belonging to the family *Oleaceae*. It evolved from the wild type tree *O. europaea* var. *sylvestris*, which was cultivated in Palestine around 6000 years ago. Later it was spread westwards throughout the Mediterranean by Phoenicians, Etruscans, Greeks and Romans (Terral et al. 2004). At present time, olive trees shape most part of the Tuscan landscape, and therefore are of public interest both from a cultural, historical and ecological perspective.

In the Mediterranean basin one of the most serious vascular disease affecting olives is caused by *Verticillium dahliae* (Nigro et al. 2005). Several factors might account for the spread of the disease, including the use of infected planting material, the establishment of new orchards in infested soils or close to affected crops, and the establishment of irrigated orchards with a high tree density (Levin et al. 2003). For these reasons those regions characterised by Mediterranean climate like the Italian peninsula are at risk of infection. Severity of *V. dahliae* disease is strictly related to fungal virulence: the pathogen can indeed be classified into defoliating (D) and non defoliating (ND) pathotypes, according as they defoliate or not green leaves from shoots and twigs (Mercado-Blanco et al. 2003a; Cirulli et al. 2008). Although the detection of the

pathogen in plant tissues is improved by using polymerase chain reaction (PCR) methods compared with the isolation on growing media, quantification is still uncertain (Li et al. 1994). Alternatively, real time quantitative PCR (qPCR) allows a simpler quantification, in addition to an increased sensitivity compared with conventional PCR assays (Ceccherini et al. 2009; Luchi et al. 2006; Mercado-Blanco et al. 2003b). Sequences of the internal transcribed spacer region (ITS) of the ribosomal DNA (rDNA) are often used to design PCR primers in order to detect different fungal pathogens in host tissue, i.e., in potatoes (Mahuku et al. 1999; Atallah et al. 2007) and olive tissues (Markakis et al. 2009; López-Escudero and Mercado-Blanco 2011). However, the number of rDNA copies fluctuates with the age and the stage of growth of the organism. Such inconsistencies may affect the quantification of the pathogen number and potential comparisons among samples. On the other hand, single-copy genes would provide specific primers, allowing consistent pathogen quantifications regardless of age and growth stage (Atallah et al. 2007).

In this study a *V. dahliae* quantification approach based on qPCR has been assessed after inoculation tests on micropropagated olive plants, seedlings, and soil samples.

The Aim of the present work was to compare the behaviour of the pathogen in planta both on micropropagates and seedlings, as well as in culture medium and in soil, during a time interval spanning from the initial stages of infection after inoculation, to development of symptoms in infected plants. The upward movement of the pathogen from the site of inoculum to the plant through the roots and the stem has been shown. The use of qPCR assay showed the ability to detect and quantify the fungal presence soon after infection and prior to the appearance of Verticillium wilt symptoms providing a tool to improve the diagnosis of the disease both in field and in nursery cultivation.

Materials and methods

Fungal isolates and culture conditions

Verticillium dahliae V138I isolate representative D pathotypes (Pérez-Artés et al. 2000), was grown on 2.0 % potato dextrose agar (PDA) for 10 days at 24 °C in the dark. Conidia from fruiting cultures were harvested by gently touching the colony surface by means of a sterile wet Pasteur pipette, and then they were suspended in quarter strength Ringer Solution (Oxoid, Ltd., Basingstoke, England) supplemented with 0.01 % Antifoam A and 0.02 % Tween 80 (Sigma Aldrich Co., Ltd.) in order to facilitate the suspension of the hydrophobic conidia. Suspension was adjusted to 10^7 conidia ml^{-1} by using a Bürker chamber and used for the subsequent inoculations.

Olive-*Verticillium dahliae* time-course in micropropagation

A set of olive *Arbequina* cultivar plantlets obtained through micropropagation techniques (Vivai Piante Battistini S.A., Cesena-Italy) were transplanted at three-to-four node stage from agar medium, as provided by the supplier, to transparent sterile pots containing 10 ml liquid Rugini Olive Medium—ROM (5.1 g l^{-1} Duchefa) supplemented with Na_2EDTA-$FeSO_4 \cdot 7H_2O$ (10 ml l^{-1}); L-Glutammine (2.194 g l^{-1}); M-Inositol (100 mg l^{-1}); D-Mannitol (36 g l^{-1}) at pH 5.7–5.8. Plantlets were kept in a glasshouse under natural photoperiod conditions.

Inoculation consisted in the addition of *V. dahliae* conidia (10^7 ml^{-1}) directly in the ROM (treated). Controls were treated with sterile water. Plantlets were observed for symptoms every 2–3 days and five replicates were sampled at intervals of 0, 7, 21, 43 and 93 days. DNA extraction was performed for each replicate, followed by qPCR detection of the target sequence. Besides, five untreated and five treated plants were kept as a reference until the end of the test for symptom development and disease assessment.

Olive-V. *dahliae* time-course in 18 months old plants

Olive seedlings (18 months old) of *Leccino* cultivar, one of the most extensively cultivated types in Tuscany, provided by Vivaio Trinci (Pistoia, Italy), were planted in a non sterile peat mould and coconut coir in plastic pots. Plants grew in a greenhouse under natural photoperiod conditions.

Olive seedlings were inoculated keeping root-dip for 10 min in a conidial suspension (10^7 ml^{-1}) of *V. dahliae* V138I isolate. After inoculation, seedlings were transplanted to non sterile soil in plastic pots (10 cm diameter × 15 cm height) and incubated in a growth chamber at 24/22 °C light/dark, 70–80 % relative humidity, and a 12 h photoperiod of fluorescent light. Plants were watered once a week, checked for symptoms every 2–3 days and five replicates were sampled at 0, 5, 15, 35 and 185 days. DNA extraction was performed on each replicate, followed by qPCR detection of the target sequence. The same number of untreated plants were used as controls.

Quantification of *V. dahliae* sequence inside an inoculated olive stem

To assess if the chosen molecular approach could monitor the fungal movement inside the vascular systems, a 16 cm long stem was removed from a healthy olive plant and an agar plug colonized by *V. dahliae* was then inoculated by wounding in the basal portion of the stem. After 15 days at 24 °C, 0.5 cm long stem sections were cut, washed and

surface-sterilized in 5 % NaClO (for 3 min), then rinsed in sterile distilled water and used for DNA extraction.

Detection limit of *V. dahliae* sequences in non sterile soil

Diminishing amounts of pure V138I genomic (Table 1) DNA were added to 0.25 g of non sterile soil, in triplicate (Ascher et al. 2009). The soil was the same as that one used for the olive seedlings growth experiment. Total DNA was extracted from the inoculated soil samples and the pathogen specific sequence was quantified via qPCR.

DNA extraction from fungal mycelium, plant culture medium, soil and plant material

Verticillium dahliae V138I was grown on 2.0 % Potato Dextrose Broth (PDB) under shaking at 25 °C for 1 week; after centrifugation at 14,000 rpm for 10 min, a pellet of 200 mg of fresh mycelium was used for DNA extraction with the FastPrep Instrument Bio101 (Qbiogene) and appropriate Fast DNA Kit as suggested by the manufacturer. The extracted DNA was used as positive control in PCR reactions.

Total genomic DNA was extracted from: (a) plant tissues; (b) potted soil; (c) plant culture medium (ROM) inoculated with 10^7 conidia ml^{-1}, (d) ROM not inoculated. DNA extraction was carried out using the Fast DNA Kit according to Ceccherini et al. (2007), after grounding the samples in liquid nitrogen. One millilitre of fungal conidia suspension was centrifuged for 10 min at 14.000 rpm and the pellet was used for DNA extraction.

Quality of extracted DNAs was verified by means of running aliquots (5 µl) in ethidium bromide stained agarose gels (1 % w/v in 1 × TAE buffer) and further visualization by UV light in a Geldoc System (BioRad). DNAs were spectrophotometrically quantified (Picodrop™) and stored at −20 °C. until use.

Six-months micropropagated and eighteen-month old seedling plants were separately harvested. For each tissue (leaves, stem, collar and roots) five independent replicates were thoroughly surface sterilized by 5 % NaClO (for 3 min) and then rinsed in sterile distilled water.

Table 1 Soil samples inoculated with decreasing amounts of *Verticillium dahliae* genomic DNA

	DNA sample	ng seq ng^{-1} tot DNA
I	S+vert 24.5 ng	4.33E−04 (±1.35E−04) a
II	S+vert 2.45 ng	4.77E−05 (±1.30E−05) b
III	S+vert 0.0245 ng	1.81E−05 (±1.24E−05) c
IV	S+vert 0.001 ng	4.61E−06 (±7.57E−07) cd

The specific DB19–22 sequence was quantified via qPCR (regression coefficient 0.978, amplification efficiency 82.7 %; different letters indicate significant differences, alpha 0.01)

qPCR quantification assay

qPCR assays were conducted using the specific primer pair DB19/DB22 that generates amplicons of 539-bp in D isolates (Mercado-Blanco et al. 2003b), on 100 ng DNA templates for all the samples. Reactions were performed in an *i*Cycler (BioRad, Hercules, CA, USA) apparatus and the following results were analysed with the manufacturer's software (Optical System Software v 3.0a). Amplification was carried out in a 25 µl final volume containing: 2.5 pmol of each primer, 12.5 µl of iQ SYBR Green Supermix (2X) and sterile ddH_2O to reach the appropriate volume. Aliquots from a unique total mixture were used 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.

Three simultaneous replicates were carried out for each sample. Amplification reactions were performed in 96-well microtiter plates (BioRad, Hercules, CA, USA). The qPCR program was the following: 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 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. Additionally, each plate contained a known *V. dahliae* DNA used to develop the standard curve, as well as a negative control. The standard curve was developed by plotting the logarithm of known concentrations (tenfold dilution series from 10 to 10^{-4} ng in 25 µl reaction) of *V. dahliae* V138I DNA against the threshold Cycle (Ct) values (Ceccherini et al. 2003; Luchi et al. 2005).

Statistical analysis

Data of quantified *V. dahliae* sequence was assessed using the Bonferroni simultaneous confidence intervals (Sacchi and Meriggi 1995; Curtin and Schulz 1998).

Results

Quantitative detection of *Verticillium dahliae* sequences in non sterile soil and infected stem

In non sterile soil, the minimum *V. dahliae* amplified sequences were 4.61×10^{-6} ($\pm 7.64 \times 10^{-7}$) ng of

sequences ng^{-1} total DNA, corresponding to sample IV in Table 1. As the amount of specific sequences of the samples III and IV in Table 1 was at the edge of significance, their mean value ($1.14 \times 10^{-5} \pm 9.57 \times 10^{-4}$) was considered as the detection limit, which corresponded to 11.4 fg ng^{-1} total DNA. Lower concentrations of target DNA were not quantified consistently.

During a 15 days infection of a 16 cm long stem, the highest quantity of the sequences, 4.41×10^{-5} ($\pm 3.41 \times 10^{-5}$) ng ng^{-1} total DNA, was detected on the side where the *Verticillium* was inoculated; the quantity of sequences decreased (not significantly) along the stem and, at the centre, the sequences were 8.16×10^{-6} ($\pm 2.37 \times 10^{-6}$) ng ng^{-1} total DNA (Fig. 1).

Olive–*V. dahliae* time-course in micropropagation

In the ROM medium without plantlets, but inoculated with 10^7 conidia/ml, the amount of total extracted DNA was 2.23 µg. In inoculated plantlets, disease severity increased with time: first visible symptoms developed on leaves 7 days after inoculation (time T7); more serious symptoms developed by 21 and 43 days after inoculation, with maximal disease intensity by 93 days, when all leaves fell down (Fig. 2). In particular, at time T7 after the infection the highest amount of the target sequences was detected in the liquid culture (ROM) which contains the plantlet: i.e. $3.14 \times 10^{-2} \pm 4.43 \times 10^{-3}$ ng of sequence ng^{-1} total DNA. At the same time, the pathogen sequences were detected in roots and stem and the amount was significantly less than in ROM with plantlets. At time T43 (43 days after inoculation) the pathogen was detected also in leaves ($1.28 \times 10^{-3} \pm 1.21 \times 10^{-4}$) although in a lesser amount than in ROM with plant, but not significantly different from the other samples. At the end of 93 days incubation the pathogen sequence was detected in all the samples in different quantities (Table 2). No symptoms developed in non-inoculated plantlets.

Olive-*V. dahliae* time-course in 18 months old plants

Inoculated seedlings did not show symptoms of disease during the incubation period. The molecular assays, instead, allowed the quantification of the target sequence 5 days (T5) after the infection in the samples of non sterile soil in which the plant grew and in the collar. After 185 days (T185), when the plants showed necrosis in leaves, the pathogen sequence was consistently increased in soil with plant ($1.71 \times 10^{1} \pm 2.54 \times 10^{-1}$), and it was also well quantified in roots, collar and stem. The sequence was never detected in the leaves (Table 3).

Discussion

Climatic conditions of Tuscany made this region suitable for olive tree cultivation, which is a feature of its landscape. For the high quality of its product, Tuscany, with 73.000 hectares and a production of 20.000 olive oil tons, is on the top among Mediterranean olive areas. Its oil, which represent 50 % of the national production, is considerably appreciated on the world wide market (Guidotti and Ricciolini 2004; Vossen 2007).

Losses caused by the disease due to soil borne pathogens and particularly by Verticillium wilt, could have a significant economic impact on olive oil production. Consequently, quality of seedlings produced in nursery is remarkably important.

In Italy, the defoliating (D) pathotype has been isolated from cotton plants grown in experimental plots in Policoro, Matera, Italy (Cirulli et al. 2008). This pathotype has been used for inoculation tests on olive cultivars and 100 days

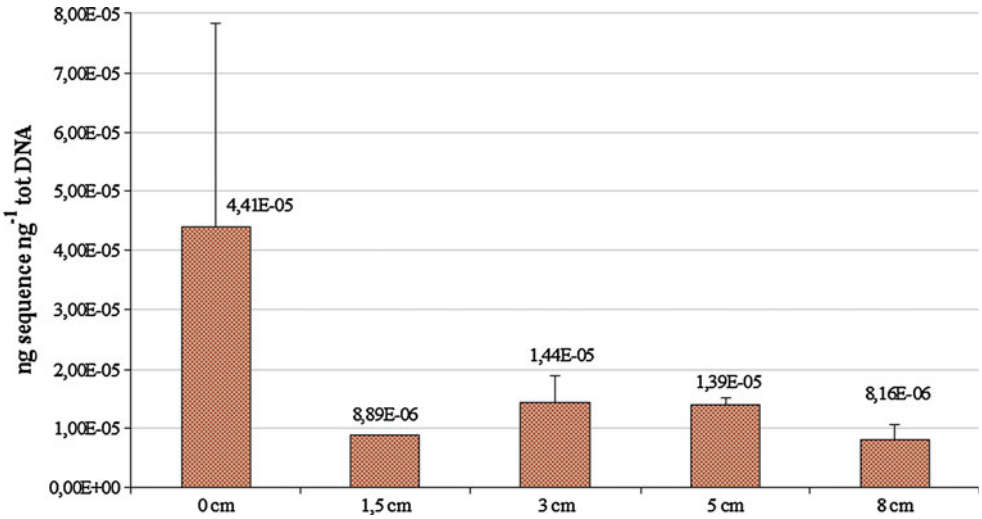

Fig. 1 Quantification of the amount of the DB19/22 sequence in an infected stem. *Verticillium* was applied at the left side and was detected along the stem (regression coefficient 0.996, amplification efficiency 70.3 %

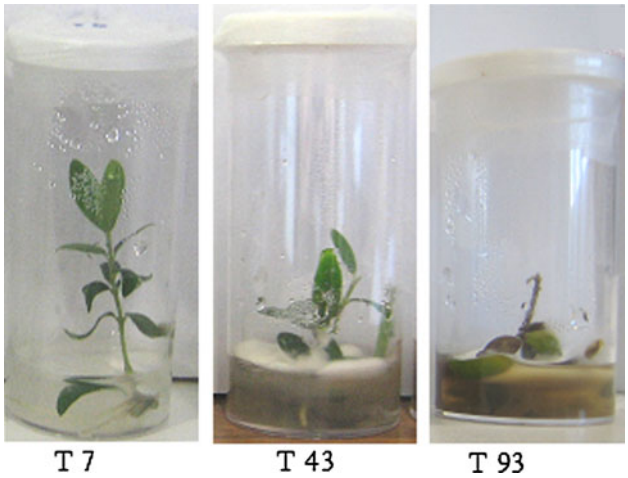

Fig. 2 Infected micropropagated plantlets. Disease severity increased with the time of incubation: first symptoms developed 7 days after inoculation (T7); more serious diseases were evident at T43 and T93 days

after inoculation the pathogen on the susceptible cv. Leccino, it caused severe defoliation on 100 % of tested seedlings and death in 70 % (Cirulli et al. 2008; Bubici and Cirulli 2012). In addition, one can suppose that the spreading of the fungus could be due to a 'host-shifting' (the capacity of the fungus to infect a new host species, becoming adapted specifically to that new host in a new country), in this case from cotton to olive in Italy. At present, control of Verticillium wilt of olive is based on precautionary measures, such as the use of pathogen-free plants and non-infested soil in case of new orchards. Also the use of resistant or tolerant cultivars can help avoid economic damage in already infested orchards; chemicals have so far failed to exert effective control under commercial conditions and soil solarization of grown up/permanent trees, alone or followed by organic amendments or antagonists, has proven only slightly effective (Tsror 2011). Thus, early, rapid and reliable detection of *Verticillium dahliae* pathotypes can help prevent pathogen-infected olive from being planted and thus facilitate the management of Verticillium wilt in olive. Therefore both cultivators and nursery growers could take advantage from a better knowledge of the relationship between the time of detection and the effect of the disease.

Verticillium dahliae forms microsclerotia, resting structures, agglomerates of thick-walled, melanized cells that can survive for many years in soil, which can have devastating effects since even low numbers of microsclerotia can lead to high levels of disease (Debode et al. 2011). Currently methods for determining inoculum density involve soil plating onto semiselective media and counting colonies of *V. dahliae*, but this means incubating the plates for several weeks, and then microscopically analyzing the fungal colonies on the medium.

A DNA-based detection soil assay technique, such as real time PCR, may offer an alternative for more specific and rapid detection of Verticillium species in soil. Specific and accurate quantitative measurements in host tissues are fundamental to detect pathogenic sequences soon after infection and prior to the appearance of wilt, providing a tool to accelerate the diagnosis (Atallah et al. 2007). Suggestions on the methods to approach the quantitative detection of pathogen sequences in soil and plants have been published by Bilodeau et al. (2012). As expansion of the cited work we developed an application based on qPCR, able to detect and quantify DNA sequences belonging to *V. dahliae* (D), in a short time-course, after the inoculation of the fungal conidia both in micropropagation and in planta conditions.

Total amounts of DNA templates used for qPCR assays were highly uniform (100 ng), and thus confirmed that every single amplification reaction contained equalized amounts of total DNA (Ceccherini et al. 2003).

In order to obtain basic information on the minimum quantity of the target sequence that could be detected, diminishing amounts of pure V138I genomic DNA were added to 0.25 g of non sterile soil. Then, total DNA was extracted from the inoculated soil samples and the specific sequence of the pathogen was quantified via qPCR. In this way, it could be possible to detect 11.4 fg of sequence per ng of total DNA, a value comparable with data in literature. Atallah et al. (2007) reported a limit of 148 fg of pathogen DNA using the single-copy gene β-tubulin to detect *V. dahliae* in potato, and Debode et al. (2011) reported a limit of 10 fg DNA for *V. trisorpus* and 100 fg DNA for *V. longisporum*.

In our case, *V. dahliae* DNA at T 0, was not detectable in any tissue, both in micropropagated tissues and in planta,

Table 2 *Verticillium dahliae* sequence quantified in micropropagated plantlets (regression coefficient 0.998, amplification efficiency 72.5 %; different letters indicate significant differences, alpha 0.01)

	T 7	±SD	T 21	±SD	T 43	±SD	T 93	±SD
Leaves	0.00E+00	0.00E+00 b	0.00E+00	0.00E+00 b	1.28E−03	1.21E−04 b	2.65E−04	3.95E−05 d
Stem	9.97E−05	3.41E−05 b	3.32E−04	7.48E−05 b	3.00E−05	5.02E−06 b	3.78E−04	6.54E−05 cd
Root	1.04E−03	9.48E−06 b	1.02E−03	2.29E−05 b	1.13E−03	8.70E−06 b	1.06E−03	2.10E−05 bc
OM with pl	3.14E−02	4.43E−03 a	1.87E−03	3.23E−03 ab	5.15E−03	9.56E−04 a	4.35E−03	4.10E−04 a
OM no pl	0.00E+00	0.00E+00 b	5.91E−03	8.33E−04 a	1.06E−03	3.21E−05 b	6.35E−04	1.39E−04 bc

Table 3 *Verticillium dahliae* sequence quantified in 18 month old plants (regression coefficient 0.963, amplification efficiency 76.6 %; different letters indicate significant differences, alpha 0.01)

	T 5	±SD	T 15	±SD	T 35	±SD	T 185	±SD
Leaves	0.00E+00	0.00E+00 **b**	0.00E+00	0.00E+00 **b**	0.00E+00	0.00E+00 **b**	0.00E+00	0.00E+00 **c**
Stem	0.00E+00	0.00E+00 **b**	0.00E+00	0.00E+00 **b**	0.00E+00	0.00E+00 **b**	3.01E−05	0.00E+00 **c**
Root	0.00E+00	0.00E+00 **b**	0.00E+00	0.00E+00 **b**	0.00E+00	0.00E+00 **b**	7.63E−01	0.00E+00 **b**
Collar	8.00E−06	7.97E−06 **b**	5.03E−06	2.00E−06 **a**	3.64E−05	1.88E−05 **a**	4.98E−05	0.00E+00 **c**
Soil with pl	3.62E−05	0.00E+00 **a**	0.00E+00	0.00E+00 **b**	0.00E+00	0.00E+00 **b**	1.71E+00	2.54E−01 **a**
Soil no pl	0.00E+00	0.00E+00 **b**	0.00E+00	0.00E+00 **b**	1.48E−05	6.24E−06 **ab**	4.18E−05	3.41E−06 **c**

this show that the number of potential pathogen propagules was very low. Considering that 148 fg of DNA is equivalent to approximately five haploid genomes of *V. dahliae* (Collins et al. 2003), the sensitivity of our real time assays allow the detection of as few as 0.38 haploid genomes of *V. dahliae* (Mahuku and Platt 2002).

In micropropagation conditions, in liquid Rugini Olive Medium, in the presence of plants, the detection of the DB19/DB22 sequence was observed after 7 days (T7). Although we didn't monitored infection in control plants only irrigated, the findings suggest that both the nutrients contained in the OM medium and plant secretions could stimulate the growth of the pathogen (Broeckling et al. 2008). The sequences showed a decreasing trend probably because nutrients exhausted. Similar amounts of *V. dahliae* sequences were detected at T7 in roots and stems. In the leaves the pathogen sequences were detected later, after 43 days, which indicates a vertical upward movement of the fungus from the culture medium to stem and leaves through roots, diffusion that, 93 days later, caused the death of the plantlets (Fig. 2).

The molecular approach on the inoculated stem (Fig. 1) allowed monitoring the DB19–22 sequence. After 15 days the fungus was detected from symptomless stem tissue at 8 cm from the inoculation site. The amount of signal decreased from the infected portion to the centre of the stem, following the movement of the pathogen in woody tissues. The progression of infection was similar to the vascular disease caused by *Ophiostoma novo-ulmi* and *O. ulmi*. After 60 days from inoculation the presence of the pathogen, as CU protein, was found in symptomless leaves remotely located from inoculation site (Scala et al. 1997). In 18-month old olives, maximal absolute quantity of the pathogen DNA was quantified in soil and roots, 1.71 and 0.763 ng ng^{-1} of total DNA, respectively, at the death, 185 days after the infection; at the same time lesser amounts of pathogen DNA were quantified in collar and stem tissues. Interestingly, at T5 the pathogen sequences were detected in soil (3.62×10^{-5} ng) and in the collar (8.00×10^{-6} ng) when the plants were apparently healthy (no wilt symptoms). Since we experienced from the investigation that after 185 days olives suddenly withered to death, we could consider the quantity of pathogen sequences in soil and collar, as a "threshold of alert" for wilt, at least in the conditions of the experiment.

A difficulty in predicting Verticillium wilt in trees is that the plant dies suddenly without a gradual development of symptoms. Considering that in most cases symptoms occur under the same environmental conditions that favour plant growth (Goud 2003) and that *V. dahliae* has been found in asymptomatic tissues of infected plants and seedlings in nursery, the application of an early diagnostic method could allow to detect the pathogen before the presence of symptoms, certifying the phytosanitary quality of seedlings. Therefore, a rapid method to extract DNA directly from soil and plant tissue samples, combined with qPCR to effectively detect specific soil pathogens, is desirable. This would offer the best benefits for early detection of latent infections in plant tissues in which the pathogen could be biologically active.

Acknowledgments This research was supported by grants from the Fondazione Monte dei Paschi di Siena. Thanks are due to Rafael M. Jiménez-Dìaza (Departamento de Protección de Cultivos, CSIC, Instituto de Agricultura Sostenible (IAS) Córdoba (Spain) for providing V. *dahliae* isolates V138I. Particular thanks we due to Maurizio Lambardi and Anna De Carlo (IVALSA-Trees and Timber Institute-lorence, CNR of Italy) for their irreplaceable help in micropropagation technique.

References

Ascher J, Ceccherini MT, Guerri G, Nannipieri P, Pietramellara G (2009) "e-motion" of extracellular DNA (e-DNA) in soil. Fresen Environ Bull 18:1764–1767

Atallah ZK, Bae J, Jansky SH, Rouse DI, Stevenson WR (2007) Multiplex real-time quantitative PCR to detect and quantify *Verticillium dahliae* colonization in potato lines that differ in response to Verticillium Wilt. Phytopathology 97:865–872

Bilodeau GJ, Koike ST, Uribe P, Martin FN (2012) Development of an assay for rapid detection and quantification of *Verticillium dahliae* in soil. Phytopathology 102:331–343

Broeckling CD, Broz AK, Bergelson J, Manter DK, Vivanco JM (2008) Root exudates regulate soil fungal community composition and diversity. Appl Environ Microbiol 74:738–744

Bubici G, Cirulli M (2012) Control of verticillium wilt of olive by resistant rootstocks. Plant Soil 352:363–376

Ceccherini MT, Potè J, Kay E, Van Tran V, Maréchal J, Pietramellara G, Nannipieri P, Vogel TM, Simonet P (2003) Degradation and transformability of DNA from transgenic leaves. Appl Environ Microbiol 69:673–678

Ceccherini MT, Ascher J, Pietramellara G, Mocali S, Viti C, Nannipieri P (2007) The effect of pharmaceutical waste-fungal biomass, treated to degrade DNA, on the composition of eubacterial and ammonia oxidizing populations of soil. Biol Fertil Soils 44:299–306

Ceccherini MT, Ascher J, Guerri G, Pietramellara G (2009) In-field detection and quantification of extracellular DNA. J Plant Nutr Soil Sci 172:626–629

Cirulli M, Colella C, D'Amico M, Amenduni M, Bubici G (2008) Comparison of screening methods for the evaluation of olive resistance to Verticillium dahliae Kleb. J Plant Pathol 90:7–14

Collins A, Okoli C, Morton A, Parry D, Edwards SG, Barbara DJ (2003) Isolates of *Verticillium dahliae* pathogenic to crucifers are of at least three distinct molecular types. Phytopathology 93:364–376

Curtin F, Schulz P (1998) Multiple correlations and bonferroni's correction. Biol Psychiatry 44:775–777

Debode J, Van Poucke K, França SC, Maes M, Höfte M, Heungens K (2011) Detection of multiple *Verticillium* species in soil using density flotation and real-time polymerase chain reaction. Plant Dis 95:1571–1580

Goud JC (2003) Verticillium wilt in trees detection, prediction and disease management PhD thesis, Wageningen Universiteit, Wageningen, The Netherlands With references With summary in English and Dutch xii+98 pp

Guidotti A, Ricciolini M (2004) The phytosanitary defence of the olive in tuscany regional agency for development and innovation in the forest-agricultural sector. Informatore Agrario 17:67–70

Levin AG, Lavee S, Tsror LL (2003) Epidemiology of Verticillium dahliae on olive (cv Picual) and its effect on yield under saline conditions. Plant Pathol 52:212–218

Li KN, Rouse DI, German TL (1994) PCR primers that allow intergeneric differentiation of ascomycetes and their application to verticillium spp. Appl Environ Microbiol 60:4324–4331

López-Escudero FJ, Mercado-Blanco J (2011) Verticillium wilt of olive: a case study to implement an integrated strategy to control a soil-borne pathogen. Plant Soil 344:1–50

Luchi N, Capretti P, Pinzani P, Orlando C, Pazzagli M (2005) Real–time PCR detection of Biscogniauxia mediterranea in symptomless oak tissue. Lett Appl Microbiol 41:61–68

Luchi N, Capretti P, Vettraino AM, Vannini A, Pinzani P, Pazzagli M (2006) Early detection of Biscogniauxia nummularia in symptomless European beech (*Fagus sylvatica* L) by TaqMan^{TM} real-time PCR. Lett Appl Microbiol 43:33–38

Mahuku GS, Platt HW (2002) Quantifying Verticillium dahliae in soils collected from potato fields using a competitive PCR assay. Am J Potato Res 79:107–117

Mahuku GS, Platt HW, Maxwell P (1999) Comparison of polymerase chain reaction based methods with plating on media to detect and identify Verticillium wilt pathogens of potato. Can J Plant Pathol 21:125–131

Markakis EA, Tjamos SE, Antoniou PP, Paplomatas EJ, Tjamos EC (2009) Symptom development, pathogen isolation and real-time qPCR quantification as factors for evaluating the resistance of olive cultivars to Verticillium pathotypes. Eur J Plant Pathol 124:603–611

Mercado-Blanco J, Rodríguez-Jurado D, Parrilla-Araujo S, Jiménez-Díaz RM (2003a) Simultaneous detection of the defoliating and nondefoliating verticillium dahliae pathotypes in infected olive plants by duplex, nested polymerase chain reaction. Plant Dis 87:1487–1494

Mercado-Blanco J, Collado-Romero M, Parrilla-Araujo S, Rodríguez-Jurado D, Jiménez-Díaz RM (2003b) Quantitative monitoring of colonization of olive genotypes by Verticillium dahliae pathotypes with real-time polymerase chain reaction. Physiol Mol Plant Pathol 63:91–105

Nigro F, Gallone P, Romanazzi G, Schena L, Ippolito A, Salerno MG (2005) Incidence of Verticillium wilt on olive in Apulia and genetic diversity of Verticillium Dahliae isolates from infected trees. J Plant Pathol 87:13–23

Pérez-Artés E, García-Pedrajas MD, Bejarano-Alcázar J, Jiménez-Díaz RM (2000) Differentiation of cotton-defolianting and nondefoliating pathotypes of Verticillium dahliae by RAPD and specific PCR analyses. Eur J Plant Pathol 106:507–517

Sacchi O, Meriggi A (1995) Habitat requirements of the stone marten (Martes Foina) on the tyrrhenisn slopes of the northen apennines. Hystrix 7:99–104. Proc. I1 It. Symp. on Carnivores

Scala A, Pattuelli M, Coppola L, Guastino M, Tegli S, Del Sorbo G, Mittempergher L, Scala F (1997) Dutch elm disease progression and quantitative determination of cerato-ulmin in leaves, stems and branches of elms inoculated with Ophiostoma novo-ulmi and O ulmi. Physiol Mol Plant Pathol 50:349–360

Terral JF, Alonso N, Capdevila RBI, Shatt N, Fabre L, Fiorentino G, Marinval P, Jorda GP, Pradat B, Rovira N, Alibert P (2004) Historical biogeography of olive domestication (Olea europaea L) as revealed by geometrical morphometry applied to biological and archaeological material. J Biogeogr 31:63–77

Tsror A (2011) Epidemiology and control of Verticillium wilt on olive Israel. J Plant Sci 59:59–69

Vossen P (2007) Olive oil: history production, and characteristics of the world's classic oils. HortScience 42:1093–1100