Plant Disease • 2018 • 102:1559-1565 • https://doi.org/10.1094/PDIS-08-17-1189-RE

Long-Distance Spread of *Verticillium dahliae* Through Rivers and Irrigation Systems

Farah Baroudy, Università degli Studi di Bari–Aldo Moro, Dipartimento di Scienze del Suolo, della Pianta e degli Alimenti, Bari, Italy; and Lebanese Agricultural Research Institute, Laboratory of Mycology, Department of Plant Protection, Fanar, Lebanon; **Wassim Habib,** Lebanese Agricultural Research Institute, Laboratory of Mycology, Department of Plant Protection; **Ghassan Tanos,** Faculty of Agricultural and Veterinary Sciences, Department of Plant Protection, Lebanese University, Dekwaneh, Lebanon; **Elvis Gerges** and **Carine Saab,** Lebanese Agricultural Research Institute, Laboratory of Mycology, Department of Plant Protection; **Elia Choueiri,** Lebanese Agricultural Research Institute, Department of Plant Protection, Tal Amara, Lebanon; and **Franco Nigro,**† Università degli Studi di Bari–Aldo Moro, Dipartimento di Scienze del Suolo, della Pianta e degli Alimenti

Abstract

Verticillium dahliae Kleb. is a soilborne pathogen causing Verticillium wilt disease on several hosts. The pathogen survival structure (i.e., microsclerotia) can be efficiently spread by different dispersal methods. In the present study, the medium to long dispersal spread of the pathogen through rivers and irrigation canals was investigated. Samples of sediments (n = 29) were gathered from eight Lebanese rivers and three regional irrigation canals, in addition to samples of soil particles and plant residues (n = 14) from irrigation filters in commercial orchards. Specific conventional and real-time nested polymerase chain reaction assays detected the pathogen in six rivers—Al Kabir, Al Bared, Litani, Al Awali, Ostwan, and Litani South—and in all sampled canals—Ostwan, Al Bared, and Litani Canal 900. Starting DNA quantities ranged from 0.2 pg to 21.318 ng and an inoculum density, determined by a traditional plating method, varied between nondetectable and 0.2 microsclerotia/g. Viable *V. dahliae* microsclerotia were also found in residues collected from mesh-type irrigation filters of five commercial orchards. This study confirms that water is an important inoculum source of *V. dahliae*, being involved in the efficient spread of microsclerotia in Lebanese agricultural areas.

Verticillium dahliae Kleb. is a widely distributed soilborne pathogen, causing vascular wilt diseases in over 400 host plants and resulting in billions of dollars of damage annually in different parts of the world (Klosterman et al. 2009; Pegg and Brady 2002). The pathogen can survive in soil for nearly 15 years (Schnathorst 1981; Wilhelm 1955) as melanized, thick-walled resting structures, called microsclerotia (MS), which are resistant to environmental extremes and remain associated with organic and soil particles that can be spread for short and long distances. Several efficient dispersal methods have contributed to the actual worldwide distribution of Verticillium wilt diseases. The pathogen can be spread with planting material (Nigro et al. 2005; Vallad et al. 2005), soil and plant tissues debris (Navas-Cortés et al. 2008; Schnathorst and Sibbett 1971), cultivating machinery (Serrhini and Zeroual 1995), and organic amendments (López-Escudero and Blanco-López 1999).

Spread of *V. dahliae* by irrigation is believed to be a main dispersal method for the fungus. The effects of increased irrigation dosages and frequencies on wilt diseases incidence and severity have been reported worldwide in several crops, such as cauliflower (Xiao et al. 1998), cotton (El Zik 1985), guayule (Schneider 1948), olive (Pérez-Rodríguez et al. 2016), and potato (Cappaert et al. 1992). Also, wilt severity can vary among the irrigation methods (Davis and Everson 1986), and the shift from dry-land to irrigated systems might contribute to the expansion of Verticillium wilt diseases to new areas (Dervis et al. 2010). Irrigation can indeed affect several factors during Verticillium wilt life cycles, because it might not only increase root growth of the plant and, thus, the probability of pathogen–host contact (López-Escudero and Blanco-López 2005; Xiao and Subbarao 2000) but also affect the movement of pathogen propagules to the root surface and into the xylem tissues (Cappaert et al. 1994; Powelson and Rowe 1993). Furthermore, it was demonstrated that irrigation significantly increased *V. dahliae* soil inoculum densities in fields of cauliflower (Xiao et al. 1998) and olive (López-Escudero and Blanco-López 2005).

Few studies have confirmed that water is involved in the medium- to long-distance transport of *V. dahliae* infective propagules. The first evidence dates back to 1969, when MS were found in canals irrigating potato fields (Easton et al. 1969); later, in water stored in ponds for furrow-irrigated potato (Thanassoulopoulos et al. 1981); and, subsequently, in irrigation drippers and wells of irrigated olive orchards (Rodríguez-Jurado and Bejarano-Alcázar 2007). A more extended survey (García-Cabello et al. 2012) was recently carried out on watering systems in Guadalquivir Valley (Andalucía, Spain), where Verticillium wilt of olive has increased during the last two decades, particularly in irrigated groves, to reach a mean disease incidence in infested plots of 20.4% (López-Escudero et al. 2010). In this study, MS were detected in the main irrigation canal and reception tank, in irrigation sand filters and water storage ponds of commercial olive or cotton sites, and in water supplied to crops (García-Cabello et al. 2012). The detection of the pathogen in these facilities was done using a traditional wet-sieving technique (Huisman and Ashworth 1974), followed by plating on a semiselective medium (Butterfield and De Vay 1977), which unequivocally demonstrated the role of water in the spread of viable propagules of the pathogen in the whole region. Despite the availability of molecular protocols for the diagnosis of *V. dahliae* (Aljawasim and Vincelli 2015; Gramaje et al. 2013; Wang et al. 2013), polymerase chain reaction (PCR)-based techniques have not yet been applied for its specific detection in sediments and residues from watering systems. In addition, the role of natural perennial or seasonal rivers in transporting infested soil, organic residues, and MS was only hypothesized (García-Cabello et al. 2012) but never demonstrated.

In Lebanon, there are 17 perennial and about 23 seasonal rivers, which flow in several agricultural areas. Litani is considered one of the most important rivers in Lebanon, due to its involvement in the agricultural, electrical, and economic sectors (http://www.litani.gov.lb). Litani is the largest river, 80% of which is located in Bekaa Valley and 20% in South Lebanon, and reaches 170 km in length, with a water capacity of about 764 million m^3 per year. Its water resources have been harnessed for human uses since the 1960s through the construction of the Qaraoun dam, which provides an average flow of 4.5 m^3/s at a pumping height of 76 m, using the main propelling

†Corresponding author: F. Nigro; E-mail: franco.nigro@uniba.it

Accepted for publication 19 February 2018.

water pipe until Canal 900, which drains around 4.5 m^3/s over 18.5 km. The overall area of irrigable lands in the Litani basin and adjacent basins is 62,500 ha, distributed in Bekaa and South Lebanon. In North Lebanon, particularly in the main agricultural area of Akkar, several rivers flow west from their source in the mountains, the largest being Al Kabir that follows the northern border with Syria. Water from these natural streams is used for irrigation and, in particular, from Al Bared River, on which modern irrigation infrastructures are constructed.

In Lebanon, thousands of hectares of *Verticillium*-susceptible hosts are irrigated by means of regional pumping stations and canals or by direct use of river water. Recent nationwide surveys have highlighted the widespread occurrence of the pathogen and its potential impact on the agricultural sector (Baroudy et al. 2016; Habib et al. 2017). In fact, a high frequency (75.3%) of *V. dahliae* was reported in soils of the main Lebanese agricultural areas, coupled with a mean soil inoculum density (ID) of *V. dahliae* of 17.0 MS/g (Habib et al. 2017). The pathogen was also detected in 46.2% of olive orchards, infecting 25.7% of the total sampled trees (Habib et al. 2017), and in 77.5% of potato fields, with a frequency of 61.2% infected plants (Baroudy et al. 2016).

The high prevalence of the pathogen in agricultural areas together with the diversity of irrigation systems in Lebanon, where many rivers and irrigation canals pass through commercial fields, suggest that water could probably be a major source of MS dissemination to large areas. Therefore, the objective of this study was to evaluate the presence of *V. dahliae* propagules in natural water streams and in irrigation canals and water filters using a conventional nested-PCR protocol, and quantify the inoculum by both a real-time PCR (qPCR) protocol and a traditional plating technique.

Materials and Methods

To investigate the presence of *V. dahliae* MS in watering systems in Lebanon, samples were collected from different sites from July 2015 until the end of June 2016. The types of samples were either sediments from rivers and canals or soil and organic residues from farmers' filters (Table 1).

Sampling from rivers and canals. The first sampling was conducted during summer 2015 to screen the presence or absence of MS in rivers. The selected rivers were surrounded by agricultural fields planted with *V. dahliae*-susceptible crops, and distributed in the main Lebanese agricultural regions: the Bekaa Valley, North Lebanon, and South Lebanon. In order to define the sampling areas and the number of samples from each river, the locations of infested potato and olive fields, identified in previous studies (Baroudy et al. 2016; Habib et al. 2017), were plotted on a map in which the main rivers are shown (Fig. 1). Accordingly, eight rivers were sampled: four in North Lebanon (Al Kabir, Al Bared, Ostwan, and Arka), Litani river in Bekaa region, and three in South Lebanon (Al Awali, Hasbani, and Litani South).

In general, one or two samples of sediments were collected from rivers, except for Litani River in Bekaa, from which seven samples were taken. Sampling was done during July to August 2015, when the river flow was low. The number of samples was proportional to the length of the river part passing through agricultural lands and the number of surrounding sites of olive or potato infested with *V. dahliae*. In general, the sampled stretches were in proximity to agricultural areas. In total, 17 samples of sediments were taken from the bottom of the rivers with the help of a shovel; each sample (approximately 1.5 kg) consisted of at least eight soil pellets (approximately 200 g) collected from a 1-km stretch of the river. Furthermore, a second sampling covered irrigation canals following the same methodology for river sampling and was carried on during March to April 2016, shortly after the canals were emptied. In particular, modern cemented canals transporting water from MS-infested rivers used to irrigate *Verticillium*-susceptible hosts were considered. Consequently, 12 samples of sediments were gathered from Al Bared and Ostwan Canals in Akkar, Northern Lebanon, and Canal 900, receiving water from Litani River in Bekaa (Table 2).

Samples of sediments from rivers and canals were air dried at room temperature for at least 5 weeks and then subjected to the wet-sieving technique described by Harris et al. (1993), slightly modified (Nigro et al. 2005). Briefly, each sample was passed through a 2-mm sieve to remove organic debris and large soil particles, and homogenized by careful mixing. Each sample (25 g) was suspended in 100 ml of sterile distilled water and shaken at 270 rpm for 1 h. The resulting suspension was passed through nested sieves with 150- and 20-μm openings. Residues retained on the 20-μm sieve were collected on a sterile filter paper and allowed to dry for 24 h at room temperature, before storage at 4°C for subsequent use. From each sample, two replicates of 25 g were processed.

Sampling from irrigation filters. Fourteen farmers benefiting from Litani Canal 900 and Al Bared Canal for irrigating *Verticillium*-susceptible hosts were selected for further sampling, in order to check whether or not *V. dahliae* MS can reach the agricultural fields through the irrigation water (Table 3). Two types of filters, sand and mesh, were sampled. From sand filters, 1 kg of sand was collected from the cylindrical tank of the filter at the end of an irrigation cycle. As for the mesh filters, the soil particles and plant debris retained on the screens after an irrigation cycle were collected by washing them into a large bucket during two different days. Each two subsamples from the same site were mixed to form one sample.

Samples from sand filters consisting of sand, soil residues, and debris were air dried for 3 weeks; then, two replicates of 250 g were each suspended in 300 ml of sterile distilled water and shaken at 270 rpm for 1 h. The suspensions were then filtered through nested sieves (150 and 20 μm) and processed as described above. On the other hand, the whole volume of the suspensions resulted from washing the mesh filters were passed through sterile filter papers to remove excess water. The filtrates were suspended in 100 ml of sterile distilled water and the mix was shaken at 270 rpm for 1 h. Suspensions were then filtered again on sterile filter paper and residues were collected after 24 h at room temperature. Two replicates per site were analyzed.

Detection and inoculum quantification of *V. dahliae*. The presence and quantification of the pathogen inoculum was carried out using two molecular protocols, conventional nested PCR and nested qPCR (qnPCR), in addition to a traditional plating method.

Molecular protocols. For each replicate of the processed dried residues of sediments from rivers and canals and from samples of soil and organic filters residues, two aliquots (0.5 g) were subjected to the extraction of total nucleic acids (TNA) by the cetyltrimethylammonium bromide (CTAB) method (Cullen et al. 2001), slightly modified as described by Habib et al. (2017). Briefly, extraction was made in 1,000 μl of extraction buffer (0.12 M Na_2HPO_4, 1.5 M NaCl, and 2% CTAB) with 50 mg of acid-washed glass beads (425 to 600 μm; Sigma-Aldrich, St. Louis) and two 5-mm steel balls, and the suspension was thoroughly agitated using the mixer mill Retsch MM301 (Retsch GmbH, Haan, Germany). After centrifugation, the supernatant was added to 1 volume of chloroform and agitated for 3 min and the nucleic acids, collected by centrifugation, were precipitated with 2 volumes of isopropanol and 0.1 volume of 3 M sodium acetate at −80°C for 30 min and recovered by centrifugation. The pellet was then washed with 200 μl of absolute ethanol (70%), dried, and suspended in 50 μl of nuclease-free water.

TNA was used as template in two separate protocols: (i) a conventional nested-PCR protocol developed as a quick screening method for the detection of the pathogen (Nigro et al. 2002; Schena et al. 2004a) and (ii) the qnPCR protocol, founded on the previous one reported by Schena et al. (2004b) and recently optimized on soil samples, as detailed by Habib et al. (2017).

In the conventional nested-PCR protocol, the first step of amplification using primer pair Ver2/Ver3 (Ver2: 3′-ATCGGCAAAATTTTAGGA-5′ and Ver3: 3′-CGGAATTGGTTCAGTGTA-5′) was done in a final volume of 25 μl containing 20 ng of DNA, 0.2 μM each primer, and 1× GoTaq Colorless Master Mix (Promega Corp., Madison, WI); the second step, using primer pair Vd7B/Vd10 (Vd7B: 3′-GACCGTCTGCAGCT CATCT-5′ and Vd10: 3′-ATTAGTCATAGGCACTGGA-5′), was done by adding 1 μl of the first amplification product to 1× GoTaq Colorless

Master Mix (Promega Corp.) and 0.2 μM each primer in a final volume of 25 μl. The conventional nested-PCR were performed in a T100 Thermal cycler (Bio-Rad Laboratories, Singapore) programmed with the same cycling conditions as the qnPCR protocol (Habib et al. 2017).

Following the second amplification step, 8.4 μl of each PCR product was added to 1.6 μl of UView loading dye (Bio-Rad Laboratories, Hercules, CA), loaded on 1% agarose gel (Bio-Rad Laboratories, Hercules, CA) and then run in Tris-boric acid-EDTA buffer (Bio-Rad Laboratories, Hercules, CA) at 110 V for 45 min in a horizontal gel-submerged electrophoretic cell. Gel images were visualized and captured on Geldoc (Bio-Rad Laboratories, Hercules, CA). The expected length of the amplified DNA fragments (131 bp) was estimated by comparison with a 100-bp DNA Step Ladder (Bio-Rad Laboratories, Hercules, CA). All PCR included a sample of DNA extracted from an MS-inoculated soil sample as a positive control together with a no-template DNA as a negative control. A sample was considered positive when at least one of its replicates gave a band of 131 bp on agarose gel.

Table 1. Origin, type, and number of samples collected during 2015 to 2016 from rivers and irrigation systems

Region	Sampling date	Source	Sample type	Number of samples
North	30 July 2015	River Al Bared	Sediments	2
	30 July 2015	River Arka	Sediments	2
	1 August 2015	River Al Kabir	Sediments	1
	1 August 2015	River Ostwan	Sediments	1
	30 March 2016	Canal Ostwan	Sediments	2
	11 April 2016	Canal Al Bared	Sediments	5
	11 June 2016	Irrigation filter, mesh type	Soil and organic debris	8
Bekaa	4 August 2015	River Litani	Sediments	7
	7 March 2016	Canal Litani Canal 900	Sediments	5
	23 June 2016	Irrigation filter, mesh type	Soil and organic debris	1
	23 June 2016	Irrigation filter, sand type	Soil and organic debris	5
South	8 August 2015	River Al Awali	Sediments	1
	8 August 2015	River Litani South	Sediments	2
	27 August 2015	River Hasbani	Sediments	1

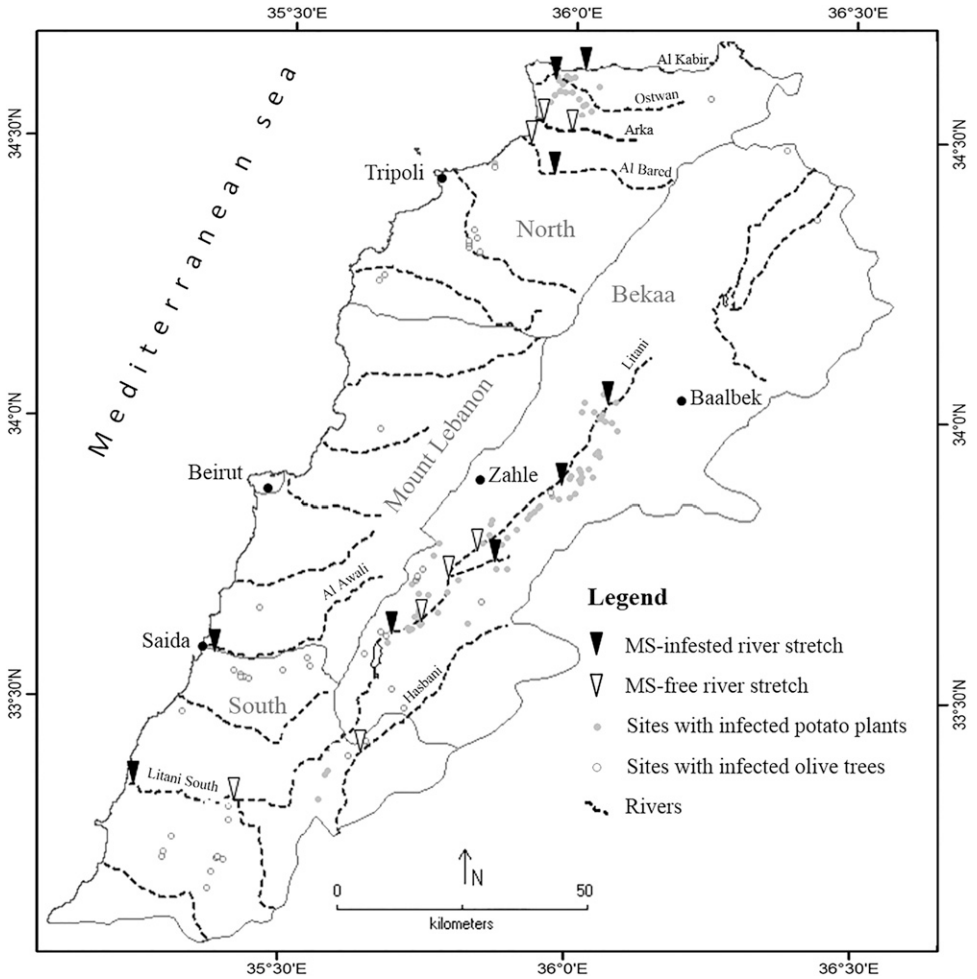

Fig. 1. Distribution of sediment samples gathered from Lebanese rivers during July to August 2015. Sites with infected potato plants and olive trees revealed by previous national surveys (Baroudy et al. 2016; Habib et al. 2017) are shown on the map to highlight the potential role of rivers in pathogen dissemination. MS = microsclerotia.

The qnPCR was conducted as recently described by Habib et al. (2017). A standard curve was generated by plotting the logarithm of known DNA concentrations of a 10-fold dilution series of DNA (25 ng μl^{-1}) of *V. dahliae* isolate Vd323 against the threshold cycle (Ct) obtained in the qPCR assays. A DNA quantity of 0.000025 ng (25 ft), corresponding to a Ct of 37 according to the standard curve, was considered the threshold value suitable for quantification. To determine the quantity of *V. dahliae*, a standard curve was generated by plotting the logarithm of known DNA concentrations of a 10-fold dilution series of DNA (25 ng μl^{-1}) of *V. dahliae* isolate V323 against the Ct obtained in the qPCR assays. A DNA quantity of 0.000025 ng (Ct of 37 according to the standard curve) was considered the threshold value suitable for quantification. The efficiencies (E) of the PCR were calculated using the slope of the standard curve, according to the formula $E = 10^{-1/slope}$, and percentage efficiencies as $(E - 1) \times 100$ (Habib et al. 2017).

Traditional plating method. From each sample, the entire amount of dried residues was dissolved in 20 ml of sterile distilled water and the suspension was plated on pectate-based semiselective medium (Huisman and Ashworth 1974). Then, using a wide-orifice pipette, 2-ml aliquots withdrawn from the suspension maintained under continuous stirring were individually distributed on the surface of 10 Petri dishes per sample. After 15 days of incubation at 24°C in the dark, the residues on the medium were removed by gentle washing under running tap water. Plates were maintained for an additional 15 days at 24°C in the dark, and typical MS star-like colonies were recorded under a 20-fold magnification stereomicroscope. A representative number of star-like colonies were selected during the assessment and subcultured on potato dextrose agar (PDA) medium (Scharlau Chemie S.A., Barcelona, Spain) amended with streptomycin sulfate at 500 mg $liter^{-1}$ (Sigma-Aldrich, Milan, Italy) for at least three times until pure colonies were obtained. Their morphological characteristics on PDA were compared with reference strains of *V. dahliae* (CBS 807.97 and CBS 381.66) obtained from Westerdijk Fungal BIO Diversity Institute (Baarn, The Netherlands) to verify that recorded colonies were of *V. dahliae*. The ID of the pathogen was calculated as the number of MS per gram of dried soil.

Data analysis. The amount of *V. dahliae* DNA and the ID (MS per gram) of viable MS in samples of sediments from river and canal, as well as from residues on infested irrigation filters was compared by Pearson's correlation analysis by using Statistica (version 7.1; StatSoft Inc., Tusla, OK). A χ^2 test was applied on the frequencies of infested samples according to their origin using SPSS statistics V21.0 (IBM, Armonk, NY).

Results

Sampling from rivers and canals. The results of nested PCR, qnPCR, and plating methods carried out on sediment samples from Lebanese rivers and irrigation canals are summarized in Table 1. The species *V. dahliae* was detected in 18 of 29 sampled stretches of rivers or canals using the nested-PCR protocols, conventional or real-time, whereas only 12 stretches resulted that were infested with the pathogen propagules with the traditional plating method.

All rivers, except Arka and Hasbani, and all three sampled irrigation canals harbored the pathogen in at least one of the sampled stretches (Fig. 1; Table 2). The pathogen was detected with a higher frequency in stretches of irrigation canals (9 of 12) than rivers (9 of 17) and in samples gathered from Litani Basin (10 of 14), which includes the Bekaa and South Lebanon parts of the river and Canal 900, irrigating the West Bekaa area. However, these differences in the frequencies of infection according to the origin of the samples were not

Table 2. Infestation of *Verticillium dahliae* in samples of sediments collected from Lebanese rivers and irrigation canals in 2015 to 2016

				Nested real-time PCR		Plating method	
Region	Sampling site	Sampled stretches (*n*)	Nested PCR positive (*n*)	Positive stretches (*n*)	Starting DNA (ng)[a]	Positive stretches (*n*)	Inoculum density (MS/g)[b]
North	Al Kabir River	1	1	1	2.1318	1	0.2
	Ostwan River	1	1	1	0.040	1	0.02
	Ostwan Canal	2	1	1	0.435	1	0.08
	Arka River	2	0	0	0	0	0
	Al Bared River	2	1	1	3.503	1	0.1
	Al Bared Canal	5	3	3	0.024–12.893	3	0.06–0.1
Bekaa	Litani River	7	4	4	0.001–0.478	3	nd–0.02
	Litani Canal 900	5	5	5	0.0002–0.098	1	nd–0.08
South	Al Awali River	1	1	1	0.008	0	nd
	Hasbani River	1	0	0	0	0	0
	Litani River, South	2	1	1	1.917	1	0.04
Total[c]	…	29	18	18	0.0002–21.318 (2.283 ± 1.333)	12	nd–0.2 (0.05 ± 0.01)

[a] Range of starting DNA is reported for sites where more than one sample was positive.
[b] Range of inoculum density is reported for sites where more than one sample was positive. MS = microsclerotia and nd = nondetectable inoculum, designated for sites in which the pathogen was detected only with the molecular methods.
[c] Averages of starting DNA and inoculum density in infested sites are followed by standard error.

Table 3. Infestation of *Verticillium dahliae* in samples of soil and organic residues collected from farmers' irrigation filters during June 2016

				Nested real-time PCR		Plating method	
Region	Filter type	Sampled sites (*n*)	Nested PCR positive (*n*)	Positive stretches (*n*)	Starting DNA (ng)[a]	Positive stretches (*n*)	Inoculum density (MS/g)[b]
North	Mesh	8	4	4	0.114–0.390	4	0.3–1.0
Bekaa	Mesh	1	1	1	0.335	1	0.4
	Sand	5	0	0	0	0	0
Total[c]	…	14	5	5	0.114–0.390 (0.280 ± 0.047)	5	0.3–1.0 (0.5 ± 0.2)

[a] Range of starting DNA is reported for sites where more than one sample was positive.
[b] Range of inoculum density is reported for sites where more than one sample was positive. MS = microsclerotia.
[c] Averages of starting DNA and inoculum density in infested sites are followed by standard error.

significant ($P > 0.05$) according to the χ^2 test. In the infested stretches, the amount of starting DNA ranged from 0.2 pg to 21.318 ng in Litani Canal 900 and Al Kabir River, respectively (mean 2.283 ng), whereas the ID ranged from nondetectable to 0.2 MS/g in six stretches and in Al Kabir River, respectively (mean 0.05 MS/g). Remarkably, five of these six positive stretches, in which the inoculum was nondetectable by the traditional wet-sieving and plating method, are parts of Litani Basin in Bekaa.

Among the samples from rivers, the stretch of Al Kabir River located in Tal Bireh had the highest ID and starting DNA, followed by a stretch of Al Bared River at Oyoun Al Samak area (0.2 MS/g; 3.503 ng). Among samples from canals, the highest infestation was recovered in a stretch of Al Bared Canal (0.1 MS/g; 1.289 ng). Moreover, *V. dahliae* ID of some canal stretches of Litani (0.08 MS/g), Ostwan (0.08 MS/g), and Al Bared (0.1 MS/g) were greater than the highest ID detected in their corresponding river, indicating that more viable MS can accumulate in the canals. In addition, an increase of ID with the direction of water flow was noticed mainly in Al Bared Canal (data not shown).

Sampling from farmer's filters. Conventional PCR and qnPCR protocols together with plating method detected *V. dahliae* propagules in 5 of 14 sampled filters (Table 3), notably in 5 of 9 mesh-type filters but never in sand filters, with starting DNA quantities of 0.114 to 0.390 ng (mean 0.280 ng). Viable MS were found in all positive samples, and ID varied between 0.3 and 1.0 MS/g (mean 0.5 MS/g), more than 10-fold greater than *V. dahliae* ID in sediments from rivers and canals.

The Pearson's correlation analysis performed on data of rivers, canals, and filters obtained from traditional plating and qnPCR showed a significant ($P < 0.03621$) correlation coefficient ($r^2 = 0.84$) between quantities of *V. dahliae* DNA and ID (Fig. 2).

Discussion

V. dahliae is of increasing concern for the Lebanese agricultural sector. In this study, viable MS DNA were detected in several facilities of watering systems, starting from the natural perennial streams through irrigation canals and in irrigation filters implemented in commercial orchards. The findings from this study clearly explain the wide distribution of the fungus in the country, coupled with its high frequencies (Baroudy et al. 2016; Habib et al. 2017), particularly in riverside olive and potato orchards (Fig. 1).

The pathogen was detected in six rivers flowing in the main agricultural areas of the country. In general, the infested river stretches revealed in this study are surrounded by commercial fields intensively planted with *Verticillium*-susceptible hosts. These rivers are the sink of erosion events resulting from heavy rains or excessive irrigation, which might bring down soil particles and plant residues infested with the pathogen MS from nearby fields. For instance, a high number of potato fields where *V. dahliae* was previously detected (Baroudy et al. 2016) are located on the riversides of Al Kabir and Ostwan Rivers in North Lebanon and Litani River in Bekaa.

The highest ID values and starting DNA quantities were detected in North Lebanon; namely, in Al Kabir and Al Bared Rivers. In addition to the abundance of *Verticillium*-susceptible hosts planted on riversides, the possible reason is related to the fact that some farmers discard the pomace and untreated wastewater of olive mills, which might harbor contaminated plant residues (Jiménez-Díaz et al. 2012; Trapero et al. 2011), in Al Kabir River. Furthermore, the infested stretch of Al Bared was sampled from the touristic Oyoun Al Samak Lake, where the flow of water decreases, which probably leads to higher sedimentation rate of soil particles and, thus, accumulation of a higher amount of associated inoculum. Further samplings are needed to confirm the absence of the pathogen from Arka and Hasbani Rivers, the former flowing in a valley historically cultivated with table grape and now with greenhouse vegetables, and the latter passing through the economically important olive-growing areas of Hasbaya, Rachaya, and Marjayoun.

A general increase of ID in canals was observed compared with their corresponding river, probably because irrigation canals exclusively pass through agricultural lands, where erosion events and

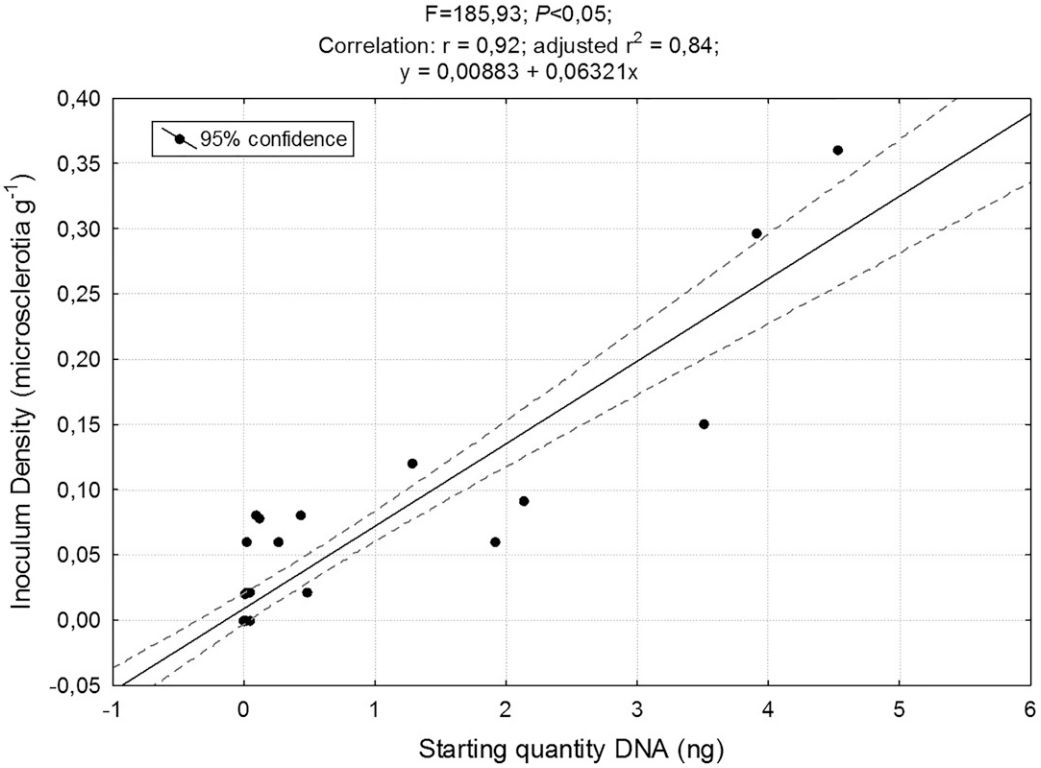

Fig. 2. Linear relationship between starting DNA quantity from real-time polymerase chain reaction quantitative assay for *Verticillium dahliae* and number of microsclerotia of *V. dahliae* per gram of soil from plating of the same samples.

accumulation of contaminated sediments are mostly higher than in rivers. Remarkably, farmers benefiting from the Ostwan irrigation canal do not use water pumps but create closable apertures in the canal, a practice that allows water to flow to their fields for furrow irrigation. In some cases, these apertures are broken or are not tightly closed, which could easily transport soil particles and plant debris to the canal.

The maximum ID of *V. dahliae* in rivers and canals from Lebanon recorded in this study was 0.21 MS/g, a density that is comparable with the lowest value (0.28 MS/g) detected in a main irrigation canal in Andalucía, southern Spain (García-Cabello et al. 2012). The higher ID can be linked to a more intensive and historical production of *V. dahliae*-susceptible hosts in the Guadalquivir Valley of Andalucía under irrigation regimes; namely, cotton (Blanco-López and DeVay 1987) and olive (Blanco-López et al. 1984). Nonetheless, even MS densities lower than 1 MS/g in soil are enough to cause severe infection, depending on the virulence of isolates and the susceptibility of the olive cultivar (López-Escudero and Blanco-López 2007; Trapero et al. 2013). Moreover, the different stretches of rivers or canals did not show constant ID values, similarly to the results of pellet samples from the main canal in Andalucía, Spain (García-Cabello et al. 2012). This might be related either to the different flow of water passing by each stretch that affects the sedimentation rate or the abundance of commercial sites around rivers or canals.

In the sampled area of Bekaa region, water is delivered to farmers' plots from Canal 900 through an underground water pipe network whereas, in the Akkar area of North Lebanon, water from Al Bared Canal flows by gravity to narrower canals to reach commercial fields or is extracted by farmers using irrigation water pumps placed directly in the canal. Two different kinds of filters are used in the farmers' plots to retain suspended soil particles and plant debris. However, some farmers producing potato under sprinkler irrigation and receiving water from Canal 900 do not use any kind of filters to prevent the decrease in water pressure. In addition, farmers benefiting from Ostwan Canal were not considered in the sampling because they generally do not use irrigation filters and practice furrow irrigation by allowing water to flow through artificial closable apertures. This study clearly highlights the need and the urgency to abandon these two practices because water from canals can harbor the pathogen MS.

Furthermore, through analyses of samples from irrigation filters, we demonstrated that MS of *V. dahliae* are efficiently transferred in association with soil particles and plant debris from irrigation canals, thus reaching commercial fields in North Lebanon and Bekaa. This is due, in part, to the resuspension of MS from sediments in canals and water pipes as a result of activating the irrigation water pumps or heavy rains and wind that cause turbidity in water and, therefore, increase the ID in water supplied to the crops (García-Cabello et al. 2012). In the present study, *V. dahliae* could not be detected in sand filters from Lebanon; nevertheless, five of nine mesh-type filters were infested by the pathogen propagules, with ID ranging from 0.3 to 1.0 MS/g and starting DNA ranging from 0.114 to 0.390 ng, suggesting that these filters can retain more pathogen propagules than sand filters. Even though irrigation filters can trap the inoculum, this would not prevent the local spread of MS once they reach the field. Indeed, farmers commonly clean the filters on site and this would wash the MS onto the soil, thus allowing their spread in the field with the machinery and cultural practices.

The molecular diagnostic method optimized for detection of *V. dahliae* in soil (Habib et al. 2017) was successfully applied in the present study on samples of sediments and organic residues from rivers, canals, and filters. The specific qnPCR showed that average *V. dahliae* DNA quantities were 2.283 ng in the 18 infested samples from rivers and irrigation canals and 0.280 ng in the 5 infested filters implemented on irrigation systems, values that are comparable with DNA quantities detected in soil samples from different regions of Lebanon (Habib et al. 2017). In addition, the molecular method was more sensitive than the traditional plating method, as observed in previous studies using the same intergenic spacer primers (Bilodeau et al. 2012; Gramaje et al. 2013; Habib et al. 2017). In the present study, the method was also modified into a conventional nested-PCR and applied for screening the presence in samples of sediments and plant residues, and was as sensitive as the qnPCR. The conventional nested-PCR is cheap and rapid and can be used as a preliminary step to reduce the number of samples processed with the traditional time-consuming method used to quantify the viable inoculum of the pathogen, when needed.

The traditional wet-sieving and plating technique did not detect the pathogen in 6 of 18 PCR-positive samples. Molecular diagnosis of natural samples can, in fact, give different results from those obtained with traditional techniques (Bridge and Spooner 2001), because studies report that DNA of dead organisms persists in soil for a long time by forming complexes with soil components (England et al. 1997). On the other hand, the higher sensitivity and specificity of molecular techniques compared with traditional methods used for *V. dahliae* in soils or plants are well documented (Aljawasim and Vincelli 2015; Bilodeau et al. 2012; Gramaje et al. 2013). Consequently, the difference in the results obtained by the traditional and molecular methods in the present study could be due to either the very low amount of MS, undetectable by the traditional plating method, or the presence of dead MS, which could be perceived only by qnPCR or conventional nested PCR.

Our study revealed the actual risk of MS spread by irrigation water in North Lebanon and Bekaa but imminent risks should be projected in the South Lebanon region, in case *V. dahliae*-susceptible crops are introduced. In fact, the prevalent crops in that region are citrus and banana, which are not hosts of *V. dahliae* but are intensively irrigated using water from the MS-infested rivers Litani South and Al Awali. Thus, the pathogen inoculum could be increasing in soil of those sites without expressing symptoms of wilt in the crop. Therefore, soil analysis for the detection of *V. dahliae* is recommended before establishing new fields in South Lebanon.

In order to limit the risk of MS dispersal with water, many approaches could be considered, starting from reducing ID in the riverside field plots by using an integrated management strategy involving soil solarization (López-Escudero and Blanco-López 2001) and crop rotation (López-Escudero and Mercado-Blanco 2011) with drought-tolerant or nonirrigated crops. The improvement of the structure and correct management of canals could reduce the amount of inoculum transported for long distances. This could be done, for instance, by building physical barriers to prevent the flow of eroded soil from reaching the canals and periodically removing the accumulating sediments through more frequent cleaning. Moreover, structures used to reduce sediment intake such as on-farm sedimentation ponds, wetlands, and some components of irrigation infrastructure such as weirs, U-shaped pipes, and storage tanks in irrigation schemes were shown to significantly improve the microbiological quality of water delivered through natural streams or canals to commercial fields (Ensink et al. 2010; Keraita et al. 2008). The role of mesh-filters as a physical control measure for the management of Verticillium wilts together with other filtration systems should be further investigated.

This study clearly confirms the effective role of irrigation water in the spread of *V. dahliae* (Easton et al. 1969; García-Cabello et al. 2012; Rodríguez-Jurado and Bejarano-Alcázar 2007). Despite the high prevalence of the pathogen in Bekaa and the high number of *V. dahliae*-positive samples detected by molecular techniques, there was a general low ID (nondetectable to 0.08 MS/g) in samples from Litani Basin. Therefore, more periodic sampling and a higher number of samples are needed in order to monitor the ID during different seasons.

It has been suggested that irrigation together with other agronomic and cultural factors can favor the spread of the most aggressive strains of the pathogen in different geographical areas within the Mediterranean basin (Dervis et al. 2010; López-Escudero et al. 2010). Thus, it is necessary to assess the pathogenicity of the isolates transported in the different rivers and canals in Lebanon through biological assays and determine their pathotype, race, and vegetative compatibility group. Research studies aiming at characterizing the population of *V. dahliae* occurring in the different areas of Lebanon are currently in progress in order to better evaluate the epidemiological impact of the spread through the rivers and irrigation systems.

Literature Cited

Aljawasim, B., and Vincelli, P. 2015. Evaluation of polymerase chain reaction (PCR)-based methods for rapid, accurate detection and monitoring of *Verticillium dahliae* in woody hosts by real-time PCR. Plant Dis. 99:866-873.

Baroudy, F., Habib, W., Puri, K., Al Sabbagh, C., Subbarao, K., and Nigro, F. 2016. *Verticillium dahliae*: The main cause of potato wilt in Lebanon. Page 48 in: (Abstr.) 12th Int. Verticillium Symp. Ljubljana, Slovenia.

Bilodeau, G. J., Koike, S. T., Uribe, P., and Martin, F. N. 2012. Development of an assay for rapid detection and quantification of *Verticillium dahliae* in soil. Phytopathology 102:331-343.

Blanco-López, M. A., and DeVay, J. E. 1987. Effect of different irrigation regimes on Verticillium wilt, proline content and leaf water potential in cotton. In: Proc. VII Congr. Mediterr. Phytopathol. Union, Granada, Spain.

Blanco-López, M. A., Jiménez-Díaz, R. M., and Caballero, J. M. 1984. Symptomatology, incidence and distribution of Verticillium wilt of olive trees in Andalucía. Phytopathol. Mediterr. 23:1-8.

Bridge, P., and Spooner, B. 2001. Soil fungi diversity and detection. Plant Soil 232: 147-154.

Butterfield, E. J., and DeVay, J. E. 1977. Reassessment of soil assays for *Verticillium dahliae*. Phytopathology 67:1073-1078.

Cappaert, M. R., Powelson, M. L., Christensen, N. W., and Crowe, F. J. 1992. Influence of irrigation on severity of potato early dying and tuber yield. Phytopathology 82:1448-1453.

Cappaert, M. R., Powelson, M. L., Christensen, N. W., Stevenson, W. R., and Rouse, D. I. 1994. Assessment of irrigation as a method of managing potato early dying. Phytopathology 84:792-800.

Cullen, D. W., Lees, A. K., Toth, I. K., and Duncan, J. M. 2001. Conventional PCR and quantitative real-time PCR detection of *Helminthosporium solani* in soil and on potato tubers. Eur. J. Plant Pathol. 107:387-398.

Davis, J. R., and Everson, D. O. 1986. Relation of *Verticillium dahliae* in soil and potato tissue, irrigation method, and N-fertility to Verticillium wilt of potato. Phytopathology 76:730-736.

Dervis, S., Mercado-Blanco, J., Erten, L., Valverde-Corredor, A., and Pérez-Artés, E. 2010. Verticillium wilt of olive in Turkey: A survey on disease importance, pathogen diversity and susceptibility of relevant olive cultivars. Eur. J. Plant Pathol. 127:287-301.

Easton, G. D., Nagle, M. E., and Bailey, D. L. 1969. A method of estimating *Verticillium albo-atrum* propagules in field soil and irrigation waste water. Phytopathology 59:1171-1172.

El-Zik, K. M. 1985. Integrated control of Verticillium wilt of cotton. Plant Dis. 69: 1025-1032.

England, L. S., Lee, H., and Trevors, J. T. 1997. Persistence of *Pseudomonas aureofaciens* strains and DNA in soil. Soil Biol. Biochem. 29:1521-1527.

Ensink, J. H. J., Scott, C. A., Brooker, S., and Cairncross, S. 2010. Sewage disposal in the Musi-River, India: Water quality remediation through irrigation infrastructure. Irrig. Drain. Syst. 24:65-77.

García-Cabello, S., Pérez-Rodríguez, M., Blanco-López, M. A., and López-Escudero, F. J. 2012. Distribution of *Verticillium dahliae* through watering systems in widely irrigated olive growing areas in Andalucía (southern Spain). Eur. J. Plant Pathol. 133:877-885.

Gramaje, D., Pérez-Serrano, V., Montes-Borrego, M., Navas-Cortés, J. A., Jiménez-Díaz, R. M., and Landa, B. B. 2013. A comparison of real-time PCR protocols for the quantitative monitoring of asymptomatic olive infections by *Verticillium dahliae* pathotypes. Phytopathology 103:1058-1068.

Habib, W., Choueiri, E., Baroudy, F., Tabet, D., Gerges, E., Saab, C., and Nigro, F. 2017. Soil inoculum density of *Verticillium dahliae* and Verticillium wilt of olive in Lebanon. Ann. Appl. Biol. 170:150-159.

Harris, D. C., Yang, Y. R., and Ridout, M. S. 1993. The detection and estimation of *Verticillium dahliae* in naturally infested soil. Plant Pathol. 42:238-250.

Huisman, O. C., and Ashworth, L. J. 1974. Quantitative assessment of *Verticillium albo-atrum* in field soil: Procedural and substrate improvements. Phytopathology 64:1043-1044.

Jiménez-Díaz, R. M., Cirulli, M., Bubici, G., Jiménez-Gasco, M. D., Antoniou, P. P., and Tjamos, E. C. 2012. Verticillium wilt, a major threat to olive production: Current status and future prospects for its management. Plant Dis. 96:304-329.

Keraita, B., Drechsel, P., Konradsen, F., and Vreugdenhil, R. C. 2008. Potential of simple filters to improve microbial quality of irrigation water used in urban vegetable farming in Ghana. J. Environ. Sci. Health Part A: Toxic/Hazard Subst. Environ. Eng. 43:749-755.

Klosterman, S. J., Atallah, Z. K., Vallad, G. E., and Subbarao, K. V. 2009. Diversity, pathogenicity and management of *Verticillium* species. Annu. Rev. Phytopathol. 47:39-62.

López-Escudero, F. J., and Blanco-López, M. A. 1999. First report of transmission of *Verticillium dahliae* by infested manure in olive orchards in Andalucía (Southern Spain). Plant Dis. 83:1178.

López-Escudero, F. J., and Blanco-López, M. A. 2001. Effect of a single or double soil solarization to control Verticillium wilt in established olive orchards in Spain. Plant Dis. 85:489-496.

López-Escudero, F. J., and Blanco-López, M. A. 2005. Effects of drip irrigation on population of *Verticillium dahliae* in olive orchards. J. Phytopathol. 153: 238-239.

López-Escudero, F. J., and Blanco-López, M. A. 2007. The relationship between the inoculum density of *Verticillium dahliae* and the progress of Verticillium wilt of olive. Plant Dis. 91:1372-1378.

López-Escudero, F. J., and 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.

López-Escudero, F. J., Roca, J. M., Mercado-Blanco, J., Valverde-Corredor, A., and Blanco-López, M. A. 2010. Verticillium wilt of olive in the Guadalquivir Valley (southern Spain): Relations with some agronomical factors and spread of *Verticillium dahliae*. Phytopathol. Mediterr. 49:370-380.

Navas-Cortés, J. A., Landa, B. B., Mercado-Blanco, J., Trapero-Casas, J. L., Rodríguez-Jurado, D., and Jiménez-Díaz, R. M. 2008. Spatiotemporal analysis of spreads of infections by *Verticillium dahliae* pathotypes within a high tree-density olive orchard in Southern Spain. Phytopathology 98:167-180.

Nigro, F., Gallone, P., Romanazzi, G., Schena, L., Ippolito, A., and Salerno, M. G. 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.

Nigro, F., Schena, L., and Gallone, P. 2002. Pages 454-461 in: Diagnosi in tempo reale della verticilliosi dell'olivo mediante Scorpion-PCR. Convegno Internazionale di Olivicoltura, Spoleto, Italy.

Pegg, G. F., and Brady, B. L., eds. 2002. Verticillium Wilts. CABI Publishing, New York.

Pérez-Rodríguez, M., Serrano, N., Arquero, O., Orgaz, F., Moral, J., and López-Escudero, F. J. 2016. The effect of short irrigation frequencies on the development of Verticillium wilt in the susceptible olive cultivar 'Picual' under field conditions. Plant Dis. 100:1880-1888.

Powelson, M. L., and Rowe, R. C. 1993. Biology and management of early dying of potatoes. Annu. Rev. Phytopathol. 31:111-126.

Rodríguez-Jurado, D., and Bejarano-Alcázar, J. 2007. Dispersión de *Verticillium dahliae* en el agua utilizada para el riego de olivares en Andalucía. Bol. Sanid. Veg. Plagas 33:547-562.

Schena, L., Nigro, F., and Ippolito, A. 2004a. Real-time PCR detection and quantification of soilborne fungal pathogens: The case of *Rosellinia necatrix, Phytophthora nicotianae, P. citrophthora*, and *Verticillium dahliae*. Phytopathol. Mediterr. 43:273-280.

Schena, L., Nigro, F., Ippolito, A., and Gallitelli, D. 2004b. Real-time quantitative PCR: A new technology to detect and study phytopathogenic and antagonistic fungi. Eur. J. Plant Pathol. 110:893-908.

Schnathorst, W. C. 1981. Life cycle and epidemiology of *Verticillium*. Pages 81-111 in: Fungal Wilt Diseases of Plants. M. E. Mace, A. A. Bell, and C. H. Beckman, eds. Academic Press, New York.

Schnathorst, W. C., and Sibbett, G. S. 1971. *Verticillium* strain: Major factor in cotton and olive wilt. Calif. Agric. 25:3-5.

Schneider, H. 1948. Susceptibility of guayule to Verticillium wilt and influence of soil temperature and moisture on development of infection. J. Agric. Res. 76: 129-143.

Serrhini, M. N., and Zeroual, A. 1995. Verticillium wilt in Morocco. Olivae 58: 58-61.

Thanassoulopoulos, C. C., Biris, D. A., and Tjamos, E. C. 1981. Dissemination of *Verticillium* propagules in olive orchards by irrigation water. Pages 52-53 in: (Abstr.) Proc. 5th Congr. Mediterr. Phytopathol. Union, Patras, Greece.

Trapero, C., Roca, L. S., Alcántara, E., and López-Escudero, F. J. 2011. Colonization of olive inflorescences by *Verticillium dahliae* and its significance for pathogen spread. J. Phytopathol. 159:638-640.

Trapero, C., Serrano, N., Arquero, O., del Rio, C., Trapero, A., and López-Escudero, F. J. 2013. Field resistance to Verticillium wilt in selected olive cultivars grown in two naturally infested soils. Plant Dis. 97:668-674.

Vallad, G. E., Bhat, R. G., Koike, S. T., Ryder, E. J., and Subbarao, K. V. 2005. Weed-borne reservoirs and seed transmission of *Verticillium dahliae* in lettuce. Plant Dis. 89:317-324.

Wang, Y. L., Wang, Y., and Tian, C. 2013. Quantitative detection of pathogen DNA of Verticillium wilt on smoke tree *Cotinus coggygria*. Plant Dis. 97: 1645-1651.

Wilhelm, S. 1955. Longevity of the Verticillium wilt fungus in the laboratory and field. Phytopathology 45:180-181.

Xiao, C. L., and Subbarao, K. V. 2000. Effects of irrigation and *Verticillium dahliae* on cauliflower root and shoot growth dynamics. Phytopathology 90:995-1004.

Xiao, C. L., Subbarao, K. V., Schulbach, K. F., and Koike, S. T. 1998. Effects of crop rotation and irrigation on *Verticillium dahliae* microsclerotia in soil and wilt in cauliflower. Phytopathology 88:1046-1055.