

INCIDENCE OF VERTICILLIUM WILT ON OLIVE IN APULIA AND GENETIC DIVERSITY OF *VERTICILLIUM DAHLIAE* ISOLATES FROM INFECTED TREES

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

Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi di Bari,
Via G. Amendola 165/A, 70126 Bari, Italy

SUMMARY

To collect information on the incidence of Verticillium wilt of olive in Apulia (southern Italy), about 6,000 woody samples from 1,390 young and old olive orchards were analysed. Moreover, 565 soil samples from commercial orchards and nurseries located all over Apulia region were tested for the presence of *V. dahliae* microsclerotia. Verticillium wilt was found in the 18% of the surveyed orchards, mainly in seaside-located intensive plantations, which are usually intercropped with vegetables susceptible to *V. dahliae*. However, the disease was also found in young plantations established on soil which resulted free of the pathogen, thus indicating a role of the propagating material in the spread of the disease. On the whole, 16% of surveyed fields and 50% of nurseries were contaminated by the pathogen. Overall, the results suggest that preventive diagnostic tests of leafy cuttings, soil and soil mix are mandatory to obtain *V. dahliae*-free propagative material to limit the spread of the disease. The analysis of more than 60 *V. dahliae* isolates by RAPD-PCR technique indicated a low level of genetic diversity in the Apulian population of the pathogen from infected olive trees. A distinct cluster including few isolates was found, but no correlation could be established among the isolates and olive cultivars and location. Molecular tests to characterize the pathotype of *V. dahliae* from infected trees indicated that all the isolates belong to the non-defoliating pathotype, except one for which the results were not conclusive.

Key words: olive nursery, RAPD-PCR analysis, non-defoliating pathotype, defoliating pathotype.

INTRODUCTION

Verticillium wilt, caused by the soil-borne fungus *Verticillium dahliae* Kleb., is one of the most important

disease of olive worldwide. In the last two decades, the disease has occurred with increasing frequency and severity in most olive growing areas of the Mediterranean basin (Jiménez-Díaz *et al.*, 1998). It affects olive trees in the nursery, commercial orchards and landscape plantings. On young trees the disease can take the form of apoplexy, usually developing from late winter to early spring and consisting in a rapid and complete dieback of twigs and branches and, sometimes, the whole plant. On mature plants, the disease becomes chronic and develops slowly.

The pathogen is a soil inhabiting fungus and inoculum consists of microsclerotia, which form in the senescing tissues of the diseased plant, and may survive in the soil for many years. The species *V. dahliae* exhibits high variability of important traits (e.g. pathogenicity, vegetative compatibility, morphology, etc.) and isolates can be genetically diversified also according to their ecological niches in which many factors may intervene (Roberts *et al.*, 1995; Barbara *et al.*, 1998). During the last decades, molecular techniques have been largely used to characterize *Verticillium* species (Carder and Barbara, 1991; Robb *et al.*, 1993; Typas, 2000). Among the different techniques, Randomly Amplified Polymorphic DNA (RAPD) has been used for the characterization of *V. dahliae* isolates from different host species. The results demonstrate that molecular variation is diffused in *V. dahliae*, and different clusters, more or less discrete, correlate with host, geographical origin, virulence, and vegetative compatibility group (Messner *et al.*, 1996; Paplomatas and Lampropoulos, 2000; Cherrab *et al.*, 2000; Zeise and Tiedemann, 2002a). RAPD-PCR also enabled the characterization of two pathotypes, Defoliating (D) and Non-Defoliating (ND) possessing high and low virulence, respectively, and allowed the development of sequence characterized amplified regions (SCARs) to identify both D and ND isolates (Pérez-Artés *et al.*, 2000). Usually, infections caused by D pathotype lead to the plant death whereas infections by ND pathotype can result in a complete symptom remission, thus facilitating the spread of the pathogen in symptomless planting material (Mercado-Blanco *et al.*, 2002). Recently, it has been reported that D pathotype has spread from the area in Southern Spain where it was first found to dis-

Corresponding author: F. Nigro
Fax: +39.080.5442911
E-mail: nigrof@agr.uniba.it

tant olive-growing areas in the same region, thus causing severe *Verticillium* wilt in newly established olive orchards (Bejarano-Alcázar *et al.*, 2001). To date, no information are available about the occurrence of D pathotype on olive trees in Apulia.

Considering the severe wilting of olive trees observed in Apulia over the past several years, and the role that genetic variation can play in the biology of the pathogen, objectives of this research were: i) to collect data on the incidence of *Verticillium* wilt on olive, and on the occurrence of *V. dahliae* microsclerotia in the soil of the main olive-growing areas, ii) to analyse genetic variation by RAPD-PCR technique, and iii) to check the occurrence of the defoliating pathotype in a *V. dahliae* population from infected olive trees.

MATERIALS AND METHODS

Incidence of *Verticillium* wilt of olive and assessment of *V. dahliae* inoculum density in the soil. Surveys were carried out in young and old olive plantations both randomly chosen in the most representative olive-growing areas of the Apulian provinces [Bari (BA), Brindisi (BR), Lecce (LE), Foggia (FG), and Taranto (TA)] and suggested by the farmers since wilt symptoms occurred. Twigs, branches or stems were sampled from the late winter-early summer to early autumn from plants with or without wilting symptoms. About 6,000 woody samples from 1,390 orchards were collected (Table 1). Occurrence of *Verticillium* wilt in the nursery

Table 1. Occurrence of *Verticillium* wilt disease on olive trees growing in the different Apulian provinces.

Province	Surveyed fields (No.)	Fields with infected plants (%)
Bari	515	13.9
Brindisi	159	27.5
Foggia	154	8.4
Lecce	169	6.2
Taranto	393	35.8

was assessed on 150 olive-seedlings sampled from 29 nurseries spread all over the Apulia region (Table 2) from 1999 to 2001 growing seasons.

Inoculum density of *V. dahliae* microsclerotia was assessed on 224 soil samples collected from 82 of the surveyed orchards. Two to five soil samples (1-1.5 kg each) per orchard were taken, depending on the size of the field. Each sample consisted of four subsamples taken in a w- or double-w shaped pattern from the uppermost 20 cm of soil. Inoculum density was also determined on 341 samples from 29 nurseries located in the five Apulian provinces (Lecce, Taranto, Brindisi, Bari, and Foggia). Samples consisted of soil, soil mix used to fill pots or single components of the soil mix (sand, peat, and pumice), when available.

The presence of *V. dahliae* in the wood was assessed by isolating the fungus on 1.5% potato dextrose agar (PDA), according to the standard methods (Pegg and Brady, 2002). Fungal colonies isolated from the xylem were identified according to their morphological characteristics, and by comparison with type strains of *V. dahliae* (CBS 807.97 and CBS 381.66) obtained from Centraalbureau voor Schimmelcultures (Baarn, The Netherlands).

The inoculum density of the pathogen in the soil was determined according to the protocol of Harris *et al.* (1993), with some modifications. Briefly, soil samples were thoroughly mixed, air dried at room temperature for 30-40 days, carefully crumbled, and sieved with a 2-mm openings. Twenty-five g soil were weighed into a 250 ml Erlenmeyer flasks and made up to 100 ml volume with sterile distilled water. Flasks were vigorously agitated for 1 h on a gyratory shaker operating at 250 rev per min. The suspension was washed under running tap water through nested sieves with 150- μ m and 20- μ m openings. The residue collected on the 20- μ m sieve was washed into a 50-ml beaker to obtain a final volume of 20 ml. Aliquots of 2-ml soil suspension under continuous stirring were individually distributed, by using a wide orifice pipette, on the surface of 10 Petri dishes containing a semiselective medium (Huisman and Ashworth, 1974). The Petri dishes were incubated in the

Table 2. Occurrence of *Verticillium dahliae* microsclerotia in soil samples from olive nurseries located in the different Apulian provinces.

Province	Surveyed nurseries (No.)	Soil samples (No.)	Infested nurseries (%)	Infested samples (%)	Inoculum density (mscl g ⁻¹) ^a
Bari	7	22	57.5	13.7	1.5
Brindisi	2	20	50.0	5.0	1.0
Foggia	2	18	50.0	5.1	7.0
Lecce	3	15	33.3	6.7	1.0
Taranto	15	145	60.0	7.6	1.7

^a For each province, the mean inoculum density calculated over the infested nurseries is reported.

dark at room temperature for 10-14 days, after which soil was removed from the surface with a gentle stream of running water; the plates were then drained and incubated again at room temperature for 15-20 days. The inoculum density of *V. dahliae*, determined by recording the colonies arising from microsclerotia in each plate under a 20x magnification stereomicroscope, was calculated as number of microsclerotia per gram of dried soil (mscl g⁻¹).

Genetic diversity and pathotype analysis. The isolates of *V. dahliae* examined are listed in Table 3. They were collected mainly in Apulia (south-eastern Italy) from infected olive trees (58 isolates) showing or not wilting symptoms. Additional isolates, made available by the collection of the Department of Plant Protection and Applied Microbiology of the University of Bari (DPPMA), were previously obtained from various herbaceous (7 isolates) and trees (1 isolate) hosts. All fungal isolates were maintained on 2% PDA slants at 4°C. For each isolate a conidial suspension, prepared from 7-days-old cultures, was triple-streaked on PDA plates and incubated at 22°C until small single colonies grew on the medium. From each isolate one single conidial strain was obtained by transferring onto new PDA plate a portion of a single-growing colony.

For DNA extraction, isolates were cultured on 2% Potato Dextrose Broth (PDB) in 100 ml Erlenmeyer flasks, agitated using an orbital shaker (120 rpm) for 8-10 days at 21±2°C. Mycelial mat was collected by vacuum filtration, washed with sterile distilled water, air dried in a laminar flow hood, and frozen at -80°C for future use.

Genomic DNA was obtained from fungal mycelium (20-30 mg) which was suspended in 400 µl of lysis buffer (2% Triton X-100; 1% SDS; 100 mM NaCl; 10 mM Tris-HCl, pH 8; 1 mM EDTA pH 8), and extracted with 400 µl of phenol/chloroform/isoamyl alcohol (25:24:1) in presence of acid-washed glass beads (425-600 µm diameter) and 2 steel beads (5 mm diameter). This mixture was vortexed at 3,000 rpm for 10 min and centrifuged at 13,000 g for 15 min. The supernatant was extracted again with an equal volume of chloroform/isoamyl alcohol (24:1) and DNA was precipitated with absolute ethanol, then washed with 70% ethanol, dried, and re-suspended in "nuclease free" water. DNA was quantified by measuring optical density at 260 nm, diluted to 50 ng µl⁻¹ and stored at -20°C. At least three independent DNA extractions were made from each isolates to verify the repeatability of PCR products among different extractions. DNA from defoliating and non defoliating cotton's pathotypes of *V. dahliae* was kindly provided by R.M. Jiménez-Díaz (Instituto de Agricultura Sostenible - CSIC, Cordoba, Spain).

Arbitrary random amplification of DNA sequences was carried out with 5 primers, of which 4 (OPH9,

OPH13, OPH19, OPH20) were 10-mer oligonucleotide primers corresponding to the OPH primer set (Operon Technology, Alameda, CA); the last primer was a 17-mer oligonucleotide, named KS (Kelly *et al.*, 1994) and was derived from the multiple cloning site of the phagemid pBluescript (Stratagene, Cambridge, UK). These primers were chosen because they produce polymorphic amplicons in *V. dahliae* (Pérez-Artés *et al.*, 2000).

PCRs were performed in a total volume of 25 ml, containing 100 ng of genomic DNA, 50 mM KCl, 10 mM Tris-HCl (pH 9), 50 mM each of dNTPs, 2 mM MgCl₂, 1 unit of Taq polymerase (Red-Taq, Sigma-Aldrich Co., St Louis, MO, USA), and 5 pmols of a single primer. DNA was amplified in a programmable thermal cycler (PCR express, Hybaid Limited, Ashford, UK) starting with 5 min of denaturation at 94°C, followed by 45 cycles of 1 min at 94°C, 1 min of annealing at 36°C (for 10-mer primers) or 40°C (for the KS primer), and 2 min of extension at 72°C. The final step consisted of 7 min at 72°C. Negative controls (no template DNA) were used in each amplification. Amplicons were separated by electrophoresis in 1.5% agarose gels in TAE buffer at 60V for 2 h, and stained for 10 min with ethidium bromide (2 µg ml⁻¹).

V. dahliae population was screened for D or ND pathotypes using the primers Df/Dr and NDf/NDr, as described by Pérez-Artés *et al.* (2000). Primers were synthesized by MWG-Biotech Spa (Florence, Italy) and the amplification products were analysed as reported above.

Patterns from the analysis of the isolates tested were compared for reproducibility of RAPD markers. Positions of reproducible RAPD bands, in at least two independent experiments, were scored as present (1) or absent (0) directly from the photographs of gels, and recorded as a binary character matrix. Only the clearest and most reproducible bands were scored; faint bands, which could not be systematically visualized, were not taken into account. Relationships among isolates were determined by calculation of Jaccard's similarity coefficients (S_j), which calculates the proportion of positive bands shared by each pair of isolates. S_j was calculated according to the formula $S_j = (N_{ab}) / (N_a + N_b - N_{ab})$, where N_{ab} is number of shared bands; N_a and N_b are number of bands in isolates a and b, respectively. The resulting similarity matrix was subjected to the unweighted paired group method with arithmetic averages (UPGMA) clustering algorithm and a dendrogram was constructed (Sneath and Sokal, 1973). Significance of the clusters generated was assessed by a bootstrapping procedure (Felsenstein, 1985). Bootstrapping was accomplished by using "Resampling Methods" option in Freetree program to bootstrap the presence/absence data sets 1,000 times (Pavlicek *et al.*, 1999). Only bootstrap values higher than 70% were considered significant and supported with 95% probability (Hillis and Bull, 1993).

Table 3. *Verticillium dahliae* isolates analyzed by RAPD-PCR.

Isolate code	Host origin	Cultivar ^a	Location (Province)	Source ^b	Year of isolation ^a
V1	Olive	NA	Bari (BA)	DPPMA	1996
V2	Olive	NA	Polignano (BA)	DPPMA	1996
V3	Olive	Nociara	Palagiano (TA)	DPPMA	1996
V4	Olive	Cellina di Nardò	Ceglie Messapica (BR)	DPPMA	1992
V5 ^c	Olive	NA	Serra Capriola (FG)	DPPMA	1992
V6	Olive	S. Agostino	Terlizzi (BA)	DPPMA	1997
V7	Olive	Cellina di Nardò	Francavilla Fontana (BR)	DPPMA	1997
V8	Olive	CNR I/77 Clone	Francavilla Fontana (BR)	DPPMA	1997
V9	Olive	Coratina	Valenzano (BA)	DPPMA	1997
V10 ^c	Olive	Carolea	Palagiano (TA)	DPPMA	1999
V11 ^c	Olive	NA	Sava (TA)	DPPMA	1999
V12	Eggplant	NA	Palagiano (TA)	DPPMA	1999
V13	Olive	Bella di Cerignola	Cerignola (FG)	DPPMA	1999
V14	Olive	NA	Sava (TA)	DPPMA	1999
V15	Olive	Cellina di Nardò	S. Marzano (TA)	DPPMA	1999
V16	Olive	NA	Sava (TA)	DPPMA	1999
V17	Olive	NA	Sava (TA)	DPPMA	1999
V18	Olive	Ogliarola	Copertino (LE)	DPPMA	2000
V19 ^c	Olive	Ogliarola	Copertino (LE)	DPPMA	2000
V20 ^c	Olive	Leccino	Ostuni (BR)	DPPMA	2000
V21	Olive	Picholine	Torre S. Susanna (BR)	DPPMA	2000
V22	Olive	NA	Capurso (BA)	DPPMA	2000
V23	Olive	NA	Massafra (TA)	DPPMA	2000
V24	Olive	NA	Campobasso (CB)	DPPMA	2000
V25	Olive	NA	S. Pancrazio (TA)	DPPMA	2000
V26 ^c	Olive	Cellina di Nardò	Ceglie Messapica (BR)	DPPMA	2000
V27	Olive	Cima di Melfi	Taranto (TA)	DPPMA	2000
V28 ^c	Olive	Leccino	Montalbano Jonico (MT)	DPPMA	2000
V29	Olive	Cellina di Nardò	Carpignano Salentino (LE)	DPPMA	2000
V30 ^c	Olive	Mele	Cerignola (FG)	DPPMA	2000
V31	Olive	NA	Ostuni (BR)	DPPMA	2000
V32	Olive	Picholine	Taranto (TA)	DPPMA	2000
V34	Olive	Cellina di Nardò	Carovigno (BR)	DPPMA	2001
V35	Olive	NA	Grottaglie (TA)	DPPMA	2001
V36 ^c	Olive	Nociara	Ostuni (BR)	DPPMA	2001
V37	Olive	Frantoio	Oria (BR)	DPPMA	2001
V39	Olive	Cima di Mola	Canosa di Puglia (BA)	DPPMA	1992
V40	Olive	Cima di Melfi	Mottola (TA)	DPPMA	1999
V41	Olive	Cima di Melfi	Mottola (TA)	DPPMA	1999
V42	Olive	NA	Monopoli (BA)	DPPMA	1999
V43	Almond	NA	Monopoli (BA)	DPPMA	2000
V46	Tomato	NA	Canada	CBS 381.66	1993
V52	Guar	NA	Monopoli (BA)	DPPMA	1990
V53 ^c	Olive	NA	Matera (MT)	DPPMA	1991
V54	Olive	Cellina di Nardò	Ceglie Messapica (BR)	DPPMA	1992
V56	Pepper	NA	Torre S. Susanna (BR)	DPPMA	1994
V57	Pepper	NA	Stornara (FG)	DPPMA	1994
V58	Pepper	NA	Monopoli (BA)	DPPMA	1998
V59	Eggplant	NA	Palagiano (TA)	DPPMA	2000
V64 ^c	Olive	NA	Foggia (FG)	DPPMA	2000
V71	Artichoke	NA	Brindisi (BR)	DPPMA	1997
V75	Olive	NA	Polignano (BA)	DISACD	NA
V76	Olive	NA	Maglie (LE)	DISACD	NA
V77	Olive	NA	Cerignola (FG)	DISACD	NA
V78	Olive	NA	Carapelle (FG)	DISACD	NA

V79	Olive	NA	Carmiano (LE)	DISACD	NA
V80	Olive	NA	Ruffano (LE)	DISACD	NA
V81	Olive	NA	Scorrano (LE)	DISACD	NA
V82	Olive	NA	Massafra (TA)	DISACD	NA
V83	Olive	NA	Manfredonia (FG)	DISACD	NA
V84	Olive	NA	Bari (BA)	DISACD	NA
V85	Olive	NA	Putignano (BA)	DISACD	NA
V86	Olive	NA	Monopoli (BA)	DISACD	NA
V88	Olive	NA	Molfetta (BA)	DISACD	NA
V90	Olive	NA	Cerignola (FG)	DISACD	NA
V91	Olive	NA	Ortanova (FG)	DISACD	NA
V92	Olive	NA	Cerignola (FG)	DISACD	NA

^a NA = Not Available.

^b DPPMA = isolates from the collection of Dipartimento di Protezione delle Piante e Microbiologia Applicata, University of Bari, Italy; CBS = Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; DISACD = isolates from the collection of the Dipartimento di Scienze Agroambientali, Chimica e Difesa Vegetale, University of Foggia, Italy.

^c Isolate from symptomless plant.

RESULTS

Field surveys on the incidence of verticillium wilt of olive and assessment of *V. dahliae* inoculum density in the soil. Isolation from wood tissues showed that Verticillium wilt occurs more frequently in the provinces of Taranto and Bari than in those of Lecce, Brindisi and Foggia (Table 1). The highest disease incidence was found in the province of Taranto, in which the mean percentage of olive orchards with infected trees reached 35.8% level. At municipality level, the maximum disease incidence (about 83% of the surveyed fields) was recorded in Mola di Bari (south-eastern of Bari province), where intercropping with *V. dahliae*-susceptible hosts, such as potato, tomato, and artichoke, is a common practice (data not shown).

In the provinces of Taranto, Brindisi, and Foggia, *V. dahliae* was also isolated from young (less than 10 years old) and old (more than 60 years old) symptomless plants; in particular, 3 samples out of 147 young plants tested resulted positive, whereas there were 11 positive samples out of 153 old plants tested.

The highest percentage of fields infested with *V. dahliae* microsclerotia (Table 4) was found in the province of Taranto (38.2%), whereas in the provinces

of Bari and Brindisi it reached values of 22-25%. No microsclerotia were found in soil samples from fields located in the provinces of Foggia and Lecce.

The percentage of nurseries contaminated with *V. dahliae* microsclerotia was higher in the province of Taranto (60.0%) than in the remaining provinces, in which the infestation level ranged from 33.3 to 57.5%. The highest mean inoculum density (7 mscl g⁻¹) was found in nurseries located in the province of Foggia (Table 2).

Data on the presence of *V. dahliae* microsclerotia in soil mix or in its components are reported in Table 5. In the province of Taranto 22.6% of soil mix samples from pots containing 1-2 year old plants resulted infested, with a mean inoculum density around 12.3 mscl g⁻¹; a lower percentage of infestation and inoculum density was found among the soil mix samples from the provinces of Bari and Lecce, whereas samples from Brindisi and Foggia were free of the pathogen. Sand from nurseries located in the provinces of Taranto and Foggia contained *V. dahliae* microsclerotia in the 11% and 25% of the analysed samples, respectively; the mean inoculum density was 41.5 mscl g⁻¹ in the samples from Taranto and 3.78 in those from Foggia. Peat and pumice resulted free of *V. dahliae*.

Table 4. Occurrence of *Verticillium dahliae* microsclerotia in soil samples collected from olive-tree orchards located in the different Apulian provinces.

Province	Surveyed fields (No.)	Soil samples (No.)	Infested fields (%)	Inoculum density (mscl g ⁻¹) ^a
Bari	18	54	22.4	5.7
Brindisi	16	49	25.2	4.6
Foggia	13	39	0	0
Lecce	14	42	0	0
Taranto	21	40	38.2	4.3

^a For each province, the mean inoculum density calculated over the infested fields is reported.

Table 5. Occurrence of *Verticillium dahliae* microsclerotia in samples of soil mix or its components, collected from olive-trees nurseries located in the different Apulian provinces.

Province ^a	Soil mix			Sand			Peat			Pomice		
	No.	%	ID	No.	%	ID	No.	%	ID	No.	%	ID
Bari	10	10.0	4.5	-	-	-	5	0	0	-	-	-
Brindisi	3	0	0	1	0	0	-	-	-	-	-	-
Foggia	5	0	0	4	25.0	3.8	1	0	0	1	0	0
Lecce	15	6.7	2.7	-	-	-	6	0	0	-	-	-
Taranto	40	22.6	12.3	26	11.6	41.5	3	0	0	1	0	0

^a For each province, the number of surveyed nurseries is the same reported in Table 2; No. = number of samples analysed; % = percentage of infested samples; ID = mean inoculum density of the infested samples, expressed as microsclerotia per gram of dry soil (mscl g⁻¹); - = data not available.

In 29 nurseries surveyed all over the Apulian provinces olive seedlings did never show *Verticillium* wilt symptoms nor was the pathogen isolated from 152 tested plants.

Genetic diversity and pathotype analysis. The five random primers used in this study (KS, OPH9, OPH13, OPH19, and OPH20) produced reproducible amplicons for all 66 *V. dahliae* isolates (Table 3). Thirty-eight bands were obtained, and 16 were polymorphic.

The amplicons ranged from 1.0 to 0.2 kb; representative banding patterns from primers OPH13 and OPH20 are illustrated in Fig. 1. The resulting UPGMA dendrogram showed 4 distinct clusters (Fig. 2). Fifty-eight isolates from olive trees plus one from almond grouped into two significantly distinct clusters (I and II) with a similarity coefficient of 0.85. Isolates from various herbaceous hosts (sweet pepper, eggplant, artichoke, and guar) formed two distinct clusters (III and IV) showing a lower value of genetic similarity coeffi-

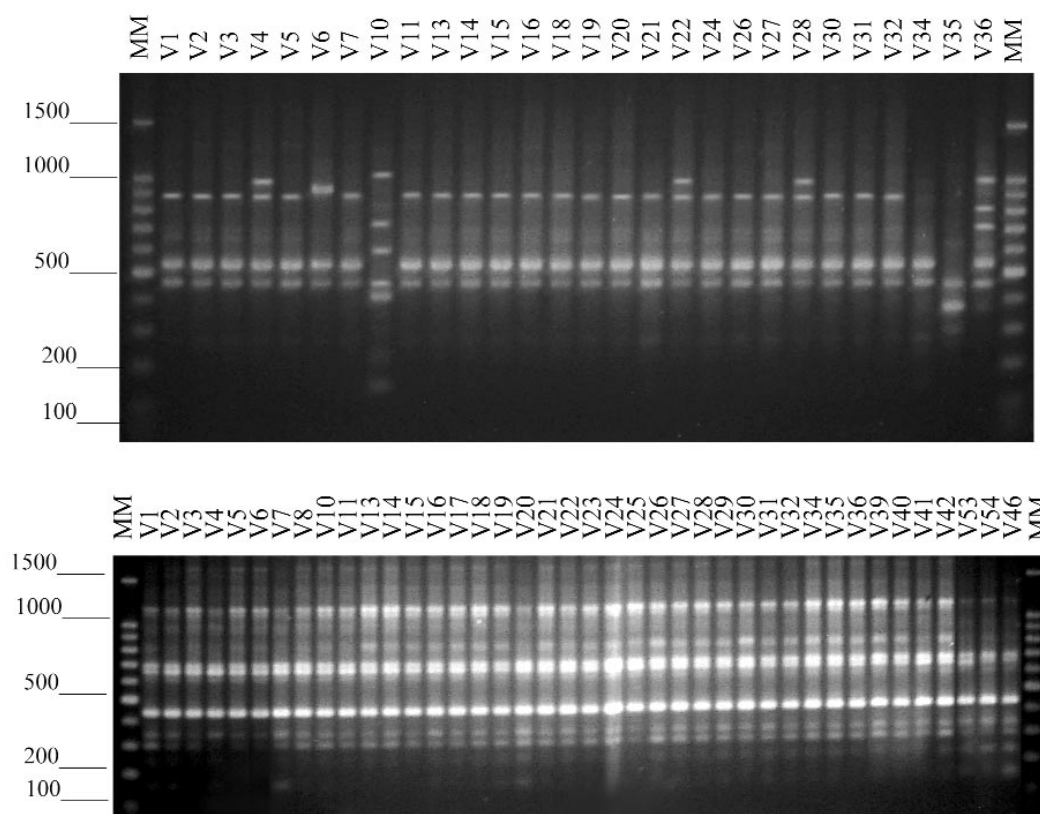


Fig. 1. Banding patterns of random amplified polymorphic DNA (RAPD) of *V. dahliae* isolates obtained with the primer OPH-13 (top) and OPH-20 (bottom). The list of isolates is reported in Table 3. MM = Molecular marker (100 bp DNA Ladder, Promega Corporation, WI, USA).

cient (0.80) in comparison with isolates from olive (Fig. 2). None of the four clusters showed relationship neither with symptoms expression nor with the locality of isolation.

PCR analysis with specific primers to identify the

pathotype D or ND indicated that all the isolates belonged to the ND group, showing the specific 1.5 kb band independently from the hosts of origin (Fig. 3). However, isolate V77 from olive was not assigned to D or ND pathotype because gave inconsistent results.

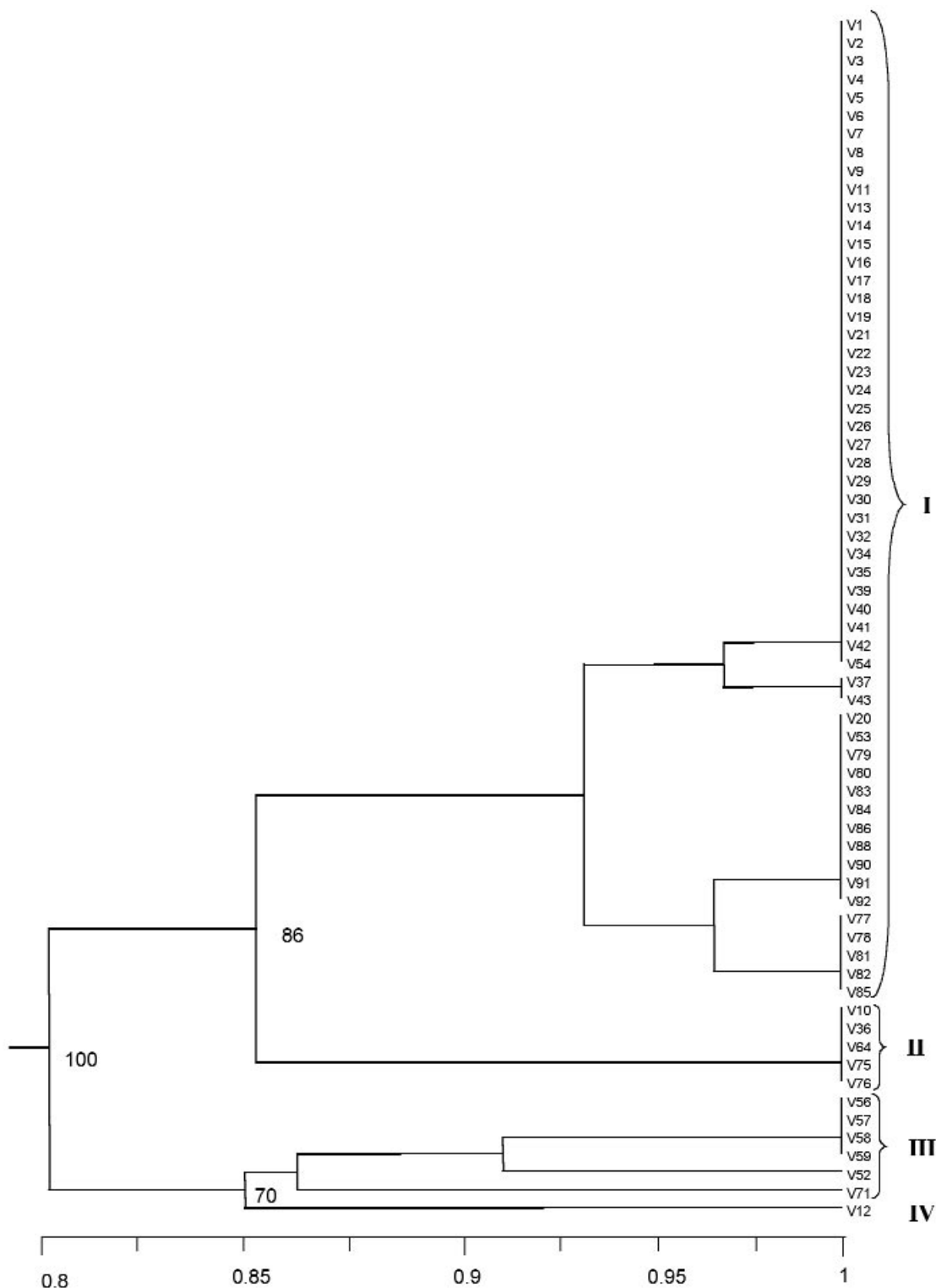


Fig. 2. Dendrogram derived from cluster analysis (UPGMA) of 66 isolates of *V. dahliae* based on data from RAPD-PCR analysis. The scale along the bottom represents Jaccard's similarity coefficient as metric distance. The numbers at each node are the bootstrap values (bootstrap performed using 1,000 replications) significant at 95% probability level.

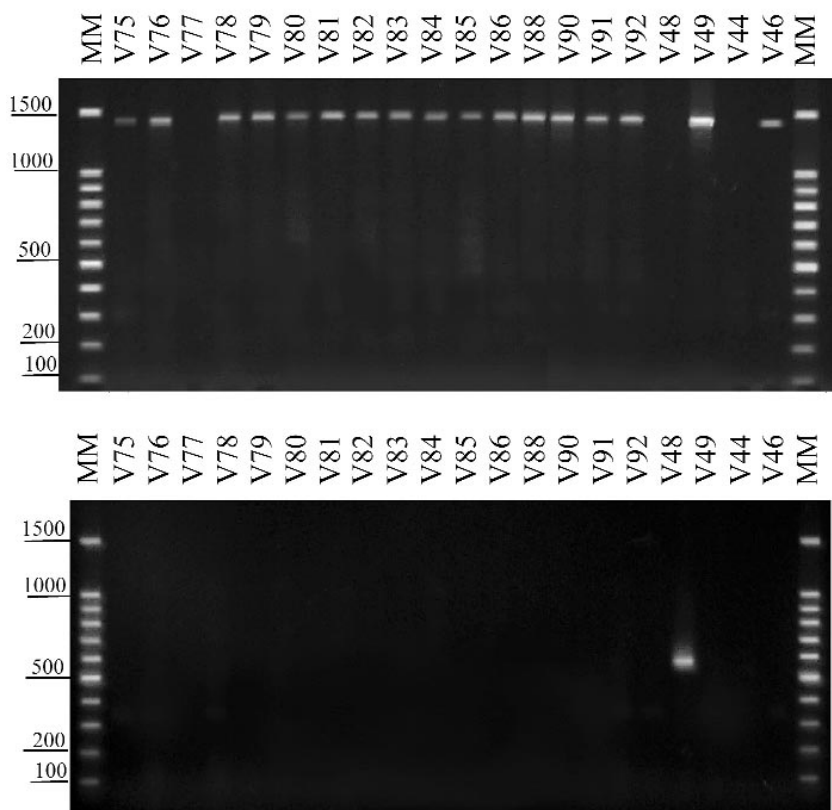


Fig. 3. Agarose gel electrophoresis of PCR products with NDf/NDr (top) and Df/Dr (bottom) primers for the detection of defoliating and non-defoliating pathotypes of *V. dahliae*. The list of isolates is reported in Table 3; V48 and V49 = DNA from defoliating and non-defoliating pathotype, respectively; MM = Molecular marker (100 bp DNA Ladder, Promega Corporation, WI, USA).

DISCUSSION

Since the 1980's, *Verticillium* wilt is expanding in the Mediterranean olive-growing areas (Jiménez-Díaz *et al.*, 1998). Recent field surveys in Southern Spain (Sánchez Hernández *et al.*, 1998) and Israel (Levin and Tsror, 2002) showed that *Verticillium* wilt has become the most important fungal disease affecting olive trees. In Italy, the disease was initially reported from Sicily (Ruggieri, 1946); afterwards, infections of varying severity have been reported from other regions, such as Tuscany (Bonifacio and Parrini, 1975), Umbria (Marte and Zizzerini, 1977), Calabria, and Sicily (Iannotta *et al.*, 1998). In Apulia, the severity of *Verticillium* wilt of olive was evidenced since 1975 (Cirulli, 1975), and the results obtained in this research update the little comforting picture about the disease incidence in Apulia. The high disease incidence, especially in young and intensive orchards, is probably due to both planting in contaminated soil and the use of infected propagative material. In particular, in some locations of the Bari, Brindisi and Taranto provinces (e.g. Mola di Bari, Polignano a Mare, Brindisi, and Palagianò) potato, sweet pepper, artichoke, eggplant, and tomato are usually intercropped with olive; probably, the use of infected propagative material

and crop residues may enrich the soils of *V. dahliae* microsclerotia. Although specificity has been reported in isolates of *V. dahliae* from herbaceous hosts (Bhat and Subbarao, 1999; Ciccarese *et al.*, 2000), it has long been recognized that cross-pathogenicity between *V. dahliae* isolates from olive and from other hosts exists (Cirulli and Montemurro, 1976; Tsror and Levin, 2003). A high disease incidence on olive-trees planted in soils previously cropped with hosts susceptible to the pathogen has been largely demonstrated (Cirulli, 1981; Tjamos, 1993; Serrhini and Zeroual, 1995; Blanco-López and Jiménez-Díaz, 1995; Naser and Al-Raddab, 1998).

In all of the surveyed provinces the pathogen was also isolated from old and young olive-trees planted in soils which had no microsclerotia. In this case the disease spread could be mainly attributed to the use of apparently healthy plantlets carrying *V. dahliae* inoculum in the soil mix used to fill pots in which olive seedlings are usually grown and commercialised. In fact, results from soil, sand, and the resulting mix samples collected in nurseries indicate a widespread occurrence of *V. dahliae* microsclerotia.

On the whole, the results obtained from field and nursery surveys indicate that appropriate prophylaxis and diagnostic tests are mandatory to limit the spread of

the Verticillium wilt of olive trees. According to the Italian and European specifications on the quality of nursery productions (EU Dir. 93/48 of June 23, 1993 "Conformitas Agraria Communitatis", CAC), a protocol for the certification of *V. dahliae*-free planting material has recently been developed in Italy (Cariddi *et al.*, 2001) and several critical control points have been identified (Saponari *et al.*, 2001). The laboriousness and length of the current methods to assess the inoculum density of *V. dahliae* in the soil is still a limiting factor for large-scale diagnosis. However, preliminary results on the detection of *V. dahliae* microsclerotia in the soil by real-time Scorpion PCR seem encouraging (Nigro *et al.*, 2001) and a reduction of the time for the diagnosis can be foreseen in the next future.

Symptomless plants were found to harbor the pathogen indicating the active role they could play in the spread of the pathogen and in the build up of inoculum in the soil (Tjamos and Tsougriani, 1990). The occurrence of symptomless plants is not new for Verticillium wilt of olive. The remission of symptoms has been reported by several Authors (Cirulli, 1981; Blanco-Lopez *et al.*, 1984) who observed a disease reduction with the increase of plant age. It is believed that recovery is probably due to the compartmentalization of the fungus in the xylem due to the apposition of new annual ring (Wilhelm and Taylor, 1965). However, in this research wood samples from both young (less than 10-year old) and old (more than 60-year old) symptomless plants resulted positive to *V. dahliae* isolation. This result could be explained considering the occurrence of low virulence isolates on olive trees. In fact, molecular characterization of the pathotype indicated that all the *V. dahliae* isolates belong to the Non-Defoliating group. This result, although comforting as it excludes the occurrence of highly virulent pathotypes on olives in Apulia, suggests that diagnostic tests are mandatory both on olive mother blocks, from which the leafy cuttings are taken, and on planting material imported from Mediterranean countries, where the D pathotype already occurs (Saponari *et al.*, 2001). Plants infected by ND pathotypes can recover with a complete symptoms remission (Jiménez-Díaz *et al.*, 1998), thus making more difficult the identification of healthy plants on the basis of symptom expression. Molecular analysis also indicated the presence of one isolate (V77) not referable to any of the two pathotypes. The significance of this difference, related to the virulence and other characteristics, is currently under investigation.

Variations in RAPD banding patterns detected for *V. dahliae* isolates revealed that there is low genetic diversity in the Apulian population of this pathogen from infected olive trees. Cluster analysis of RAPD bands resulted in the classification of 2 clusters with a coefficient similarity of 0.85. Moreover, the dendrogram indicated that 53 isolates from olive cluster at the level of 93%

similarity and no correlation could be established among the isolates included into the two clusters, neither based on the presence of symptoms, nor on the olive cultivars and locality of fungal isolation. These results agree with other works demonstrating high genetic homogeneity among populations of *V. dahliae* wilting olive trees from some Mediterranean countries (Bellahcene *et al.*, 2001; Tsror and Levin, 2003); in contrast, evidences that populations of the fungus from infected olive-trees are genetically variable have also been reported, thus allowing clustering based on their geographical origin (Cherrab *et al.*, 2000, 2002).

RAPD markers clearly differentiated between isolates from woody (cluster I and II) and herbaceous hosts (cluster II and IV), thus allowing the identification of isolates from eggplant, sweet pepper, artichoke, and guar (*Cyamopsis tetragonolobus* L. Taub.). Previous report demonstrates that pepper and eggplant isolates exhibit host specificity and differential pathogenicity, whereas isolates from artichoke and other vegetables do not; however, these characteristics were not associated to a specific RAPD patterns, suggesting that RAPD fragments are independent on host specificity (Bhat and Subbarao, 1999). Recently, it has been demonstrated that a limited diversity exist among *V. dahliae* isolates from olive in Israel; most of the isolates were classified as Vegetative Compatibility Group (VCG) 4B and the remaining as VCG 2A. No differences in aggressiveness to olive twigs were observed between isolates from these two VCGs (Tsror and Levin, 2003). In the present work only five isolates from olive constituted a distinct cluster but the reason of this grouping is not clear. Researches are in progress to further characterize these strains, evaluating their pathogenicity on young olive plantlets and assessing the VCG. In *V. dahliae* isolates from herbaceous hosts the VCG and genetic variation are related to host specialization and virulence characteristics (Zeise and Tiedemann, 2002a,b). Whether or not the grouping observed in this work between isolates from herbaceous hosts and olive is related to host specificity, differential virulence or VCG need to be ascertained.

ACKNOWLEDGMENTS

The work was funded by Programma Operativo Multiregionale (P.O.M.) "Attività di sostegno ai servizi di sviluppo per l'agricoltura", Misura 2, Progetto A32, and by CEGBA (Centro di Eccellenza di Genomica in campo Biomedico e Agrario), Research Line n. 6. Thanks are expressed to Prof. S. Frisullo, University of Foggia, for providing additional isolates of *V. dahliae*. We are also grateful to Prof. R.M. Jiménez-Díaz (Instituto de Agricultura Sostenible - CSIC, Cordoba, Spain) for providing DNA from defoliating and non-defoliating reference strains of *V. dahliae*.

REFERENCES

- Barbara D.J., Paplomatas E.J., Jiménez-Díaz R.M., 1998. Variability in *V. dahliae*. In: Hiemstra J.A., Harris D.C. (eds.). A compendium of *Verticillium* wilts in tree species, pp. 43-45. Ponsen and Looijen, Wageningen, The Netherlands.
- Bejarano-Alcázar J., Pérez-Artés E., Jiménez-Díaz R.M., 2001. Spread of the defoliating pathotype of *Verticillium dahliae* to new cotton-and olive-growing areas in Southern Spain. In: *Proceedings of the 8th International Verticillium Symposium, Córdoba 2001*, 57.
- Bellahcene M., Fortas Z., Matallah A., Geiger J.P., Nicole M., Vigouroux A., Assigbétsé K., 2001. Genetic diversity within *Verticillium dahliae* isolates from olive trees in Algeria. Comparison with some strains from France and Syria. In: *Proceedings 12th Congress of the Mediterranean Phytopathological Union, E'vora 2001*, 193-195.
- Bhat R.G., Subbarao K.V., 1999. Host range specificity in *Verticillium dahliae*. *Phytopathology* **89**: 1218-1225.
- Blanco-López M.A., Jiménez-Díaz R.M., 1995. Una propuesta de lucha integrada contra la verticilosis del olivo. *Fruticultura Profesion* **70**, 52-58.
- Blanco-Lopez M.A., Jiménez-Díaz R.M., Caballero J.M., 1984. Symptomatology incidence and distribution of *Verticillium* wilt of olive tree in Andalucía. *Phytopathologia Mediterranea* **23**: 1-8.
- Bonifacio A., Parrini C., 1975. Presenza di *Verticillium dahliae* Kleb. in oliveti della Toscana. *Informatore Fitopatologico* **25** (12): 21-25.
- Carder J.H., Barbara D.J., 1991. Molecular variation and restriction fragment length polymorphism (RFLP) within and between six species of *Verticillium*. *Mycological Research* **95**: 935-942.
- Cariddi C., Gallone P., Grieco F., Loconsole G., Nigro F., Romanazzi G., Saponari M., Trisciuzzi N., Vovlas N., 2001. Protocolli per gli accertamenti sanitari degli organismi patogeni di qualità dell'olivo. In: Savino V., Amenduni T., Bazzoni A., Boscia D., Pollastro S., Saponari M. (eds.). *Proceedings Convegno Nazionale Norme fitosanitarie e commercializzazione delle produzioni vivaistiche, Locorotondo (Bari) 2001*, 815-839.
- Cherrab M., Serrhini M.N., Charest P.M., 2000. Characterization of Moroccan isolates of *Verticillium dahliae* Kleb using RAPD markers. *Journal of Phytopathology* **148**: 243-249.
- Cherrab M., Bennani A., Charest P.M., Serrhini M.N., 2002. Pathogenicity and vegetative compatibility of *Verticillium dahliae* Kleb. isolates from olive in Morocco. *Journal of Phytopathology* **150**: 703-709.
- Ciccarese F., Schiavone D., Botalico A., 2000. Survey on *Verticillium*-wilt of artichoke in Apulia and pathogenic variations among isolates of *Verticillium dahliae*. In: *IV International Congress on Artichoke, Valenzano (Bari) 2000*, 103.
- Cirulli M., 1975. Il deperimento dell'olivo da *Verticillium dahliae* Kleb. *Italia Agricola* **112**: 119-124.
- Cirulli M., 1981. Attuali cognizioni sulla verticilliosi dell'olivo. *Informatore fitopatologico* **31** (1-2): 101-105.
- Cirulli M., Montemurro G., 1976. A comparison of pathogenic isolates of *Verticillium dahliae* and sources of resistance in olive. *Agriculturae Conspectus Scientificus* **39**: 469-476.
- Felsenstein J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783-791.
- Harris D.C., Yang J.R., Ridout M.S., 1993. The detection and estimation of *Verticillium dahliae* in naturally infested soil. *Plant Pathology* **42**: 238-250.
- Hillis, D.M., Bull J.J., 1993. An empirical test of bootstrapping as a method assessing confidence in phylogenetic analysis. *Systematic Biology* **42**: 182-192.
- Huisman O.C., Ashworth L.J., 1974. Quantitative assessment of *Verticillium albo-atrum* in field soils: procedural and substrate improvements. *Phytopathology* **64**: 1043-1044.
- Iannotta N., Fodale A.S., Tocci C., 1998. La Verticilliosi dell'olivo: grave malattia in forte espansione in Calabria e Sicilia. *Notiziario Protezione Piante* **9**: 139-146.
- Jiménez-Díaz R.M., Tjamos E.C., Cirulli M., 1998. *Verticillium* wilt of major tree hosts. Olive. In: Hiemstra J.A., Harris D.C. (eds.). A compendium of *Verticillium* wilts in tree species, pp. 13-16. Ponsen and Looijen, Wageningen, The Netherlands.
- Kelly A.G., Alcalá-Jiménez A.R., Bainbridge B.W., Heale J.B., Pérez-Artés E., Jiménez-Díaz R.M., 1994. Use of genetic fingerprinting and random amplified polymorphic DNA to characterize pathotypes of *Fusarium oxysporum* f.sp. *ciceris* infecting chickpea. *Phytopathology* **84**: 1293-1298.
- Levin A., Tsrur L., 2002. Epidemiology of *Verticillium dahliae* on olive. *Phytoparasitica* **30** (3): 21.
- Marte M., Zizzerini A., 1977. Principali malattie diagnosticate in Umbria. *Informatore Fitopatologico* **27** (11): 19-24.
- Mercado-Blanco J., Rodríguez-Jurado D., Pérez-Artés E., Jiménez-Díaz R.M., 2002. Detection of the defoliating pathotype of *Verticillium dahliae* in infected olive plants by nested PCR. *European Journal of Plant Pathology* **108**: 1-13.
- Messner R., Schweigkofler W., Ibl M., Berg G., Prillinger H., 1996. Molecular characterization of the plant pathogen *Verticillium dahliae* Kleb. using RAPD-PCR and sequencing of the 18S rRNA-gene. *Journal of Phytopathology* **144**: 347-354.
- Naser Z.W., Al-Raddab Al-Homany A., 1998. Dissemination factors of *Verticillium* wilt of olive in Jordan. *Dirasat. Agricultural Sciences* **25** (1): 16-21.
- Nigro F., Schena L., Gallone P., Romanazzi G., Ippolito A., 2001. Diagnosi nel terreno di *Verticillium dahliae*, agente di tracheomicosi nell'olivo, con l'uso della PCR in tempo reale. In: Savino V., Amenduni T., Bazzoni A., Boscia D., Pollastro S., Saponari M., (eds.). *Proceedings Convegno Nazionale Norme fitosanitarie e commercializzazione delle produzioni vivaistiche, Locorotondo (Bari) 2001*, 785-791.
- Paplomatas E.J., Lampropoulos C.J., 2000. Molecular characterization of *Verticillium* spp. by Random Amplified Polymorphic DNA analysis. In: Tjamos E.C., Rowe R.C., Heale J.B., Fravel D.R. (eds.). *Advances in Verticillium: Research and Disease Management*, pp. 48-52. APS Press, St Paul, Minnesota (USA).

- Pavlicek A., Hrda S., Flegr J., 1999. FreeTree - Freeware program for construction of phylogenetic trees on the basis of distance data and bootstrap/jackknife analysis of the tree robustness. Application in the RAPD analysis of the genus *Frenkelia*. *Folia Biologica Praha* **45**: 97-99.
- Pegg G.F., Brady B.L., 2002. Verticillium wilts. CABI Publishing International, Wallingford, Oxon, UK.
- Pérez-Artés E., García-Pedrajas M.D., Bejarano-Alcázar J., Jiménez-Díaz R.M., 2000. Differentiation of cotton-defoliating and nondefoliating pathotypes of *Verticillium dahliae* by RAPD and specific PCR analyses. *European Journal of Plant Pathology* **106**: 507-517.
- Robb J., Moukhamedov R., Hu X., Platt H., Nazar R.N., 1993. Putative subgroups of *Verticillium albo-atrum* distinguishable by PCR-based assay. *Physiological and Molecular Plant Pathology* **43**: 423-436.
- Roberts D., Bainbridge B.W., Evans H., Heale J.B., 1995. Genome analysis in isolates of *Verticillium*: *V. albo-atrum*, *V. dahliae*, and *V. lecanii*. *Phytoparasitica* **23**: 40.
- Ruggieri G., 1946. Una nuova malattia dell'olivo. *L'Italia Agricola* **83**: 369-372.
- Sánchez Hernández M.E., Ruiz Dávila A., Pérez de Algaba A., Blanco Lopéz M.A., Trapero Casas A., 1998. Occurrence and etiology of death of young olive trees in southern Spain. *European Journal of Plant Pathology* **104**: 347-357.
- Saponari M., Nigro F., Vovlas N., Cariddi C., Grieco F., Trisciuzzi N., Savino V., Martelli G.P., 2001. Punti critici dell'olivo. In: Savino V., Amenduni T., Bazzoni A., Boscia D., Pollastro S., Saponari M., (eds.), *Proceedings Incontro Divulgativo POM A32 - Risultati di due anni di attività, Termoli (Campobasso) 2000*, 311-324.
- Serrhini M.N., Zeroual A., 1995. Verticillium wilt of olive trees in Morocco. *Olivae* **58**: 58-61.
- Sneath P.A., Sokal R.R., 1973. Numerical Taxonomy. Freeman W.H., San Francisco (CA), USA.
- Tjamos E.C., 1993. Prospects and strategies in controlling Verticillium wilt of olive. *Bulletin OEPP/EPPO Bulletin* **23**: 505-512.
- Tjamos E.C., Tsougriani H., 1990. Formation of *Verticillium dahliae* microsclerotia in partially disintegrated leaves of *Verticillium* affected olive trees. In: *Proceedings 5th International Verticillium Symposium, Leningrad 1990*, 20.
- Tsrer L., Levin A.G., 2003. Vegetative compatibility and pathogenicity of *Verticillium dahliae* Kleb. isolates from olive in Israel. *Journal of Phytopathology* **151**: 451-455.
- Typas J., 2000. Molecular characterization of *Verticillium* species. In: Tjamos, E. C., Rowe R. C., Heale J. B., Fravel D. R. (eds.), *Advances in Verticillium: research and disease management*, pp. 109-111. APS Press, St. Paul, Minnesota, USA.
- Wilhelm S., Taylor J.B., 1965. Control of Verticillium wilt of olive through natural recovery and resistance. *Phytopathology* **55**: 310-316.
- Zeise K., von Tiedemann A., 2002a. Application of RAPD-PCR for virulence type analysis within *Verticillium dahliae* and *V. longisporum*. *Journal of Phytopathology* **150**: 557-563.
- Zeise K., von Tiedemann A., 2002b. Host specialization among vegetative compatibility groups of *Verticillium dahliae* in relation to *Verticillium longisporum*. *Journal of Phytopathology* **150**: 112-119.

Received 28 February 2004

Accepted 20 January 2005

