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Isolation and Morphologic Characterization of Microsclerotia of *Verticillium dahliae* Isolate from Soil

F.J. López-Escudero and M. A. Blanco-López,
Department of Agronomy, ETSIAM, University of Córdoba, P.O. Box 3048, 14080 Córdoba, Spain

Abstract: The isolation of *Verticillium dahliae* from soil is necessary for studies of ecology and virulence and for designing preventative control strategies for Verticillium wilt diseases. In this study a methodology was described for improving the estimation of Verticillium inoculum density in soil and for extracting pure cultures of *V. dahliae* from such platings for studies of culture characteristics. The efficacy for recovery of *V. dahliae* colonies from soil platings was compared on a Modified Sodium Polipectate Agar Medium (MSPA) to that obtained on plates of MSPA covered by a 600 μm thick sterilized permeable cellophane sheet (MSPA+C). Soil samples from 15 olive orchards were processed by wet sieving. Then 1 mL of the suspensions containing the soil particles ranging from 35 to 150 μm in size in 100 mL of water were plated onto the two media, using 10 replications. Plates were incubated 14 days at 22°C. Then, the number of *V. dahliae* colonies were counted using a stereoscope after the soil residues were removed under running water for MSPA plates, or without removing the soil for MSPA+C plates. Colonies forming microsclerotia on the cellophane were recovered by cutting little squares of the sheet. The microsclerotia were removed from the cellophane by shaking in water and by treatment in an ultrasonic bath. The suspensions were then sieved through a filter connected to a pressure vacuum pump and the resulting residue was plated onto MSPA, PDA or acidified PDA media. Colonies which were reasonably free of contaminants were transferred again to obtain pure cultures. The morphology of the microsclerotia produced by these cultures were characterized by measuring length and width and values compared to those of two reference isolates, V117 (cotton defoliating) and V4 (cotton non-defoliating). Results showed a high variability in the morphological features of the microsclerotia produced by the isolated cultures. The MSPA+C medium was effective for estimating the inoculum density of the pathogen in soil and also for recovery of pure culture of *V. dahliae*. This technique provides an useful tool for studies of ecology, virulence of populations of the pathogen in soil and risk assessment.

Key words: Cellophane film, inoculum density, modified sodium polipectate agar

INTRODUCTION

Verticillium dahliae Kleb. is a soil-borne pathogen causing wilt diseases in a wide range of herbaceous and woody plants[1,2]. In Andalucía (Southern Spain) wilt disease incidence is increasing in hosts such as cotton, vegetables and olive trees, where causes significant crop losses[3-5]. Inoculum density of the pathogen in soils and the virulence of the resident strains are two factors that must be considered in the progress of Verticillium wilts[6,7].

Knowledge of these two aspects before planting is required for assessing risk of crop loss and for designing control strategies, especially for woody plants that remain in the field for many years.

Many methods utilizing semi-selective culture media have been developed for quantitative assessment of inoculum density of *V. dahliae* in soil[8-23]. These techniques involve plating a defined amount of soil onto plates of the selected medium and then counting colonies of *V. dahliae* which can be recognized by the production of microsclerotia (MS). The number of colonies formed is expressed as propagules per gram of soil (ppg). Almost all media are sufficiently selective to allow *V. dahliae* to form recognizable colonies but restrict the growth of other soil microorganisms. Nevertheless, none of them are completely satisfactory[12,17,24].

Other limitation in the quantification of *V. dahliae* is that it is very difficult to recover pure cultures of the fungus from soil. Then, the quantitative analyses of *V. dahliae* in a soil sample does not allow the simultaneous purification of the fungus, since the direct transference of individual or groups of MS from colonies produced over the selective medium is inconsistent or not success[11,14,17,19,20,25-27]. Isolation of such cultures however,

Corresponding Author: Dr. F. J. López-Escudero, Scientist on Plant Pathology, Department of Agronomy, ETSIAM, University of Córdoba, P.O. Box 3048, 14080, Córdoba, Spain
Tel: +0034957218530 Fax: +0034957218569.

is necessary for the characterization of the soil populations with regard to pathogenecity, Vegetative Compatibility Groups (VCG), morphology, population dynamics, etc.

Low effectiveness of these techniques has been attributed to: 1) the difficulty to distinguish MS of *V. dahliae* from soil particles or other structures formed by other fungal species[19,20,25]; 2) the difficulty to discern MS embedded in microscopic plant tissue debris[8,11,14,19,20,25]; 3) the growth of other fungus from MS, due to contaminant attached to these propagules or propagules-like-microsclerotia[13,14,19,20,25,28]; 4) the low selectivity of culture media that allows contaminants growing (fungus, bacteria, hiperparasites of MS, etc.) more profusely and faster than *V. dahliae* and 5) the dormancy of MS produced onto the selective media[29].

In this study we developed a methodology that increases the frequency of isolation of *V. dahliae* into pure culture from a soil sample, that could be simultaneously used for quantitative estimation of *V. dahliae* from soil. A preliminary work has been published by Núñez-Santos *et al.*[28]. A second objective was to study the morphology variability of the MS produced by the recovered soil isolates onto the selective media with the aim of providing more exactitude in the detection of colonies of the pathogen from the plates.

MATERIALS AND METHODS

Experiments were conducted during years 2001 and 2002 in the Department of Agronomy of the University of Córdoba. Fifteen soil samples included were collected from established olive orchards affected by Verticillim wilt (Table 1 and 2). Soil samples were initially processed by the wet sieving technique as described by López-Escudero and Blanco-López[30] for determining the inoculum density of the pathogen. Soil samples were bulked and crumbled by hand, mixed and air-dried for 2 weeks at room conditions. Afterwards, the samples were sifted through a 0.8 mm sieve to remove organic debris and large particles. They were then mixed manually and air-dried for 2 additional weeks. Twenty-five gram of each sample were suspended in 100 mL of distilled water, shaken for 1 h at 270 rpm and the suspension filtered through two stacked sieves with 150 and 35 μm openings. The residue retained in the 35 μm sieve was resuspended in 100 mL of distilled water.

The use of cellophane paper placed over Potato Dextrose Agar plates (PDA)[31] had been successfully to induce the production of microsclerotia by *V. dahliae*[32,33]. An experiment was carried out to evaluate the influence of cellophane whether the cellophane, as additional carbon source, influenced the estimation of the number of propagules that *V. dahliae* forms onto a Modified Sodium Polypectate Agar Medium (MSPA)[12]. For samples 1 to 8 (Table 1), aliquots of 1 mL from the suspension obtained after wet sieving were sowed onto plates of MSPA and plates of MSPA covered with a 600 μm thick sterilized permeable cellophane sheet (UCB Film, La Cellophane Española S. A.)[33] laid on its surface (MSPA+C). Ten replications were used for each medium. Plates were incubated at 22°C in the dark for 14 days and then, the number of typical star-shaped colonies of *V. dahliae* were counted using a stereoscope (Nikon SMZ-2T). Colonies on MSPA plates were counted after the soil residues were removed under running water. For MSPA+C plates, observations were done without removing the soil. Inoculum density in each soil sample and medium was estimated by the number of colonies of *V. dahliae* and expressed as propagules or MS per gram of air-dried soil (ppg).

Recovery of soil isolates from soil samples: To obtain pure cultures of *V. dahliae* from soil a second experiment (Experiment II) was conducted using samples 9 to 15 (Table 2). Samples were initially processed by wet sieving, and suspensions of particles between 35-150 μm were used to determine the inoculum density of the pathogen on MSPA for each soil. Simultaneously, 1 mL aliquots of each sample were sowed onto plates of MSPA+C, with 10 replications and incubated at 22°C in the dark. Starting after 8 days of incubation, plates were observed daily using a stereoscope, without removing the soil on the plates. Colonies of *V. dahliae* could be detected 7-8 days after plating, well before the 14 days used for standard quantification. Sampled colonies earlier allowed to reduce contamination of microesclerotial colonies by excessive growth of other soil fungi that grew on the MSPA plates, particularly *Fusarium* spp.

Colonies were isolated by cutting out with a scalpel a square piece of the cellophane and conserved at 5°C until these were processed. Colonies of *V. dahliae* were subjected to following process depending on the grade of contamination.

Cellophane squares containing colonies covered with soil particles, were suspended in tubes with 10 mL of distilled water, and then were shaken in an ultrasonic bath (J.P. Selecta S.A., Barcelona) for 30 sec while shaking at the same time with sterilized tweezers. This resulted in the release of both the MS and soil particles from the colony into the suspension. When necessary, the suspension was subjected to the same process for a second time to completely clean the cellophane. The resultant suspension was filtered through a filter (35 μm) in a glass

Table 1: Comparison of inoculum density of *Verticillium dahliae* from soil samples assessed on Modified Sodium Polipectate Agar (MSPA) with or without cellophane medium

Soil sample reference	Origin	Texture	ID in MSPA [a]	ID in MSPA+C [b]	Recovered soil isolates (nº)[c]
Corchuela 9	S.J. Rinconada (Sevilla)	Clay loam	7.6b	14.8a	3
Corchuela 15	S.J. Rinconada (Sevilla)	-	0.0	1.2	-
Corchuela 21	S.J. Rinconada (Sevilla)	Loam	4.4a	5.2a	-
Corchuela 22	S.J. Rinconada (Sevilla)	-	2.8a	4.8a	3
Piedras Altas	Marinaleda (Sevilla)	Clay loam	54.0a	55.6a	3
LaVega	Nac. Zambra (Córdoba)	Siltyclayloam	4.0a	3.6a	1
Batán	Écija (Sevilla)	-	1.2a	2.0a	2
Alburquerque	La Carlota (Córdoba)	-	0.0	0.8	1

[a,b]Inoculum density, expressed by microsclerotia per gram of dry soil was assessed by wet sieving technique using petri plates of Modified Sodium Polipectate Agar (MSPA) or MSPA with cellophane (MSPA+C). Values in rows followed by the same letter were not significantly different at P=0.05 according to Fisher's protected LSD test, [c]Soil isolates were recovered from MSPA + C plates 14 days after soil sowing

Table 2: Soil samples processed to purify soil isolates of *Verticillium dahliae* using Modified Sodium Polipectate Agar with cellophane medium

Soil sample reference	Origin	Texture	ID in MSPA[a]	Recovered soil isolates (n⁰)[b]
9 Corchuela 2	S.J. Rinconada (Sevilla)	Sandy clay loam	1.6	1
10 Corchuela 4	S.J. Rinconada (Sevilla)	-	10.2	2
11 Corchuela 6	S.J. Rinconada (Sevilla)	Sandy loam	1.2	1
12 Santana	Espeluy (Jaén)	Clay loam	12.0	6
13 Dehesa Algarín	Lora del Río (Sevilla)	Loam	13.6	1
14 M. Guillén	Jaén	-	13.6	1
15 Almodóvar	Almodóvar (Córdoba)	Loam	27.6	3

[a]Inoculum density, expressed by microsclerotia per gram of dry soil was assessed by wet sieving technique using petri plates of Modified Sodium Polipectate Agar (MSPA), [b]Soil isolates were recovered from MSPA + C plates from 8 to 14 days after soil sowing

funnel attached to a vacuum pressure pump (Model D-95, Dinko Instruments, Barcelona). The filter was washed with 500-1000 mL of sterilized water and the residue retained on the filter was resuspended in 10 mL of sterilized water by subjecting it to a 10 sec of ultrasonic bath. Aliquots of 0.5 mL of the final suspension were sowed onto MSPA plates (5 replications) and incubated at 22°C in the dark. From 6 to 14 days of incubation, plates were observed under a stereoscope to detect colonies of *V. dahliae*. Then, individual, clean and uncontaminated colonies were transferred to acidified PDA plates to obtain pure cultures.

For semi-clean colonies, cellophane pieces containing them were suspended in 10 mL of sterilized water and subjected to an ultrasonic bath 15 sec. This caused the release of large soil particles from the colony to the suspension, while the mycelium and the microsclerotia remained attached to the cellophane. The thin layer of micelium and MS were scraped off the cellophane using a scalpel and placed into an empty glass sterilized petri plate. Under the stereoscope, individual or small groups of MS were picked up using a needle, washed in a sterilized water drop in a petri plate and finally sowed on acidified PDA or MSPA plates and incubated at 22°C in the dark. Colonies free of contaminants were transferred to new PDA plates as pure cultures.

Cellophane square containing clean colonies, such as those formed in the border or folds of the cellophane film, were laid on a filter paper, that was placed onto a sieve and washed by pouring sterilized water. Under the stereoscope, individual or groups of 2-3 MS were transferred to MSPA plates and incubated at 22°C in the dark. Colonies free of contaminants were transferred to new PDA plates as pure cultures and maintained at 4°C.

Additionally, they were also processed by this method several microesclerotial colonies of *V. dahliae* recovered from MSPA+C plates used in Experiment I after 14 days of incubation, from soil samples 1 to 8. The aim was to evaluate the possibility of counting and isolating the pathogen from a soil sample simultaneously in the same process. For samples 9-15 inoculum density onto MSPA+C was not assessed since only a limited number of colonies were selected for being processed.

Morphological characterization of soil isolates on MSPA: *V. dahliae* soil isolates obtained from MSPA+C plates were morphologically characterized by measuring the length and the width of microsclerotia formed on MSPA. Pure cultures of each soil isolate were incubated on PDA at 24°C in the dark and from these, a conidial suspension was prepared by adding sterile distilled water to plates and gently rubbing the surface of the colony with a sterile bent glass rod. Conidial suspensions were filtered through a double layer of sterile cheesecloth and diluted with sterile water to obtain 20 to 50 conidia/mL.

Three plates of MSPA per isolate were sowed with 1 mL aliquot of the suspension and incubated at 22°C in the dark during 14 days. Three colonies, about 1 to 1.5 cm in diameter, for each isolate were selected from MSPA plates. Two microscope slides per colony were prepared with

acidified fucshsin A in lactophenol by taking pieces of agar from the center and border of colonies, to avoid morphologic differences among MS related to maturity and coalescence among propagules. Length and width of 200 MS from each isolate were measured (65-70 MS per colony) by means the analytical program Analysis (Soft Imaging System Gmbh 2000, Münster, Germany), connected to a microscope Nikon Optiphot-200 trough a video camera Kappa (CF 20/4 DX, K-Vision BV, Huisen). For all cases the relation length/wide (L/W) was also calculated.

Two soil isolates from the collection of Laboratorio de Patología Vegetal (Universidad de Córdoba), corresponding to a cotton defoliating (V117) and cotton non-defoliating (V4) isolates of *V. dahliae*, were used as control for comparing their morphological parameters with those of the rest of soil isolates. Microsclerotia of these two isolates were recovered from an loam soil, artificially infested two years ago[32] using the same methodology described above for the rest of soil samples.

Values of length, width and L/W of MS were analyzed by Statistics 7.0 for Windows (Analytical Software, Tallahase, USA) and mean values were compared by the Fisher`s protected LSD at $p = 0.05$[34].

RESULTS

The quantitative analyses by wet sieving using MSPA medium showed considerable differences in the inoculum density of *V. dahliae* of the processed soil samples, ranging from 54.0 to 1.2 ppg (Table 1 and 2). Analyses of texture available for some of the soil samples sowed that clay loam (samples 1 and 5) or loam soils (samples 3, 13 and 15) presented higher inoculum densities of the pathogen than those with a sandy component (samples 9 and 11) (Table 1 and 2). Inoculum density in almost all soil samples processed in Experiment I was nearly identical using MSPA or MSPA+C media. Nevertheless, MSPA plates produced a number of microesclerocial colonies significantly lower than MSPA+C in sample 1 and failed in the detection of the pathogen in samples 2 and 8 (Table 1).

Recovery of soil isolates from soil samples: The method for isolating pure cultures of *V. dahliae* from soil samples has been effective in a wide range of soils that presented different characteristics and inoculum densities of the pathogen (Table 1 and 2). It has been obtained a collection of 28 isolates from 13 of the 15 processed samples from colonies on MSPA+C: 13 isolates from samples 1 and 4 to 8, in Experiment I and 15 from samples 9 to 15, in Experiment II (Table 1 and 2). No isolates of the pathogen were recovered from samples 2 and 3 due to contamination during the transference to PDA plates.

Although colonies with different rates of dirtiness and size were processed in both experiments, in general cellophane film improved the formation of well-developed and typical star-shaped colonies with high number of ME. Therefore, the methodology allowed to select microsclerotial colonies of *V. dahliae* free of contaminants as much as possible. Little colonies, with low number of propagules, or those that could induce to doubt due to similarities with propagules or miscellaneous of other fungus, were discarded and not processed. These aspects elevated the efficacy of the cleaning process and the success of obtaining a pure culture without contamination during the transference to general culture media. The general frequency of purification was high, since the cleanest colonies could be previously chosen. As an example, for sample 12, 6 purified isolates were finally obtained when 7 colonies were selected and processed from plates on MSPA + C (Table 2).

Every *V. dahliae* isolate transferred to PDA as pure culture at the end of the process represented a monosclerotial isolate, since these came from an individual colony formed onto MSPA+C and it is assumed that each colony formed in this medium is produced from the germination of an individual microsclerotium from the soil sample.

Morphological characterization of soil isolates on MSPA: Results showed significant differences in morphologic values of MS produced on MSPA between the defoliating (V117) and non-defoliating (V4) isolates of *V. dahliae* from the collection of the Laboratorio of Patología Vegetal, used as reference isolates (Table 3). The average of the relation L/W were 4.06 and 2.92 for the D and ND isolate, respectively. Values of length, wide and the ratio L/W demonstrated that the D isolate produced on APSM much more elongated and bigger in size MS than those formed by the ND one, that were more rounded and smaller. Variation coefficients of values of length and wide were similar among colonies selected for each isolate and between the two isolates (Table 3).

Morphology of MS of *V. dahliae* isolates recovered from soil samples varied considerably on MSPA (Fig. 1 and Table 4), ranging from values of L/W of 5.15, corresponding to the most elongated one, to 1.61 to the most spherical and smallest one. Isolates were classified according to the intervals of the relation L/W in four morphologic types: I) elongated, with L/W relation higher

Table 3: Morphological parameters of microsclerotia of colonies developed on Modified Sodium Polipectate Agar of the defoliating (V117) and non-defoliating (V4) cotton isolates of *Verticillium dahliae*

		Length (μm)				Width (μm)				Length/Wide		
Isolate	Colony	Mean	Min.	Max.	V.C.[b]	Mean	Min.	Max.	V.C.	Mean	Min.	Max.
V117	1	62.16	26.04	130.29	35.4	21.48	13.15	36.17	23.2	3.07	1.09	7.21
	2	66.69	32.68	188.12	23.2	24.15	9.43	43.92	38.0	3.39	1.03	9.92
	3	65.14	30.14	171.51	45.2	12.96	7.60	26.38	32.7	5.72	1.35	19.16
	Mean[a]	64.67a				19.53a					4.06a	
V4	1	42.11	20.02	102.76	31.6	20.23	9.27	37.78	32.7	2.46	1.03	5.92
	2	48.61	22.00	100.81	33.3	16.31	8.70	35.50	34.9	3.42	1.06	8.23
	3	43.24	21.72	95.51	31.3	16.88	9.29	31.46	30.4	2.90	0.92	8.09
	Mean[a]	44.66b				17.81b					2.92 b	

[a]Figures are mean of values corresponding to 200 microsclerotia from the 3 colonies (among 65 and 70 per colony). Values in columns followed by the same letter were not significantly different at p=0.05 according to Fisher's protected LSD test, [b]Variation coefficient

Table 4: Morphology parameters of microsclerotia of colonies developed on Modified Sodium Polipectate Agar of isolates of *Verticillium dahliae* recovery directly from soil samples

			Morphology parameters of microsclerotia [a]		
Soil sample and reference	Origin	Isolate	Length /Width[b]	Type	Shape
12 Santana	Espeluy	29	5.15a	E	H
5 Piedras Altas	Marinaleda	2	5.06a	E	H
14 M. Guillén	Jaén	12	4.73b	E	H
12 Santana	Espeluy	30	4.17c	E	H
- Colección	Córdoba	V117	4.06cd	E	H
12 Santana	Espeluy	26	3.82de	O	H
7 Batán	Écija	8	3.69e	O	H
10 Corchuela 4	S.J. Rinconada	18	3.31f	O	H
- Colección	Córdoba	V4	2.92g	R	H
1 Corchuela 9	S.J. Rinconada	17	2.91g	R	H
8 Alburquerque	La Carlota	6	2.84g	R	H
12 Santana	Espeluy	27	2.76gh	R	H
4 Corchuela 22	S.J. Rinconada	20	2.50hi	R	H
9 Corchuela 2	S.J. Rinconada	14	2.35ij	R	H
6 La Vega	Nac. Zambra	4	2.22ijk	R	H
1 Corchuela 9	S.J. Rinconada	16	2.22ijk	R	I
4 Corchuela 22	S.J. Rinconada	22	2.16jkl	R	I
12 Santana	Espeluy	28	2.16jkl	R	H
12 Santana	Espeluy	31	2.14jkl	R	H
5 Piedras Altas	Marinaleda	3	2.10jkl	R	H
1 Corchuela 9	S.J. Rinconada	15	2.09jkl	R	H
5 Piedras Altas	Marinaleda	1	2.08jkl	R	I
4 Corchuela 22	S.J. Rinconada	21	2.01kl	R	H
15 Almodóvar	Almodóvar	10	1.99kl	S	I
11 Corchuela 6	S.J. Rinconada	13	1.98kl	S	H
10 Corchuela 4	S.J. Rinconada	19	1.93lm	S	I
13 Dehesa Algarín	Lora del Río	5	1.77m	S	H
7 Batán	Écija	7	1.69mn	S	I
15 Almodóvar	Almodóvar	9	1.66mn	S	H
15 Almodóvar	Almodóvar	11	1.61n	S	I

[a]Figures are the mean of 200 microsclerotia of soil isolates recovery from artificially (isolates V117 and V4) or naturally (rest of isolates) infested soil by *V. dahliae*. Soil isolates were classified according to intervals of the relation Length/Wide in elongated (E), >4; oval (O), 3-4; round (R), 2-3; spherical (S), 1-2 and to the presence or absence of cell projections from microsclerotia, in homogeneous (H) or irregular (I)

[b]Values in columns followed by the same letter were not significantly different at p=0.05 according to Fisher's protected LSD test

[c]Soil isolate cultures are preserved in Dpto. Agronomía, University of Cordoba, Cordoba, Spain

than 4 where the D isolate (V117) was included (Table 3 and 4); oval, with L/W ranging from 3 to 4; rounded, from 2 to 3, that was the largest group and included the ND isolate (V4) and spherical, from 1 to 2, that included also the isolates producing the smallest MS (Table 4). This classification was only tentative, with a comparative aim, since a determinate group included isolates showing significant statistical differences with regard to L/W values (Table 4).It is has been also considered the shape of MS related to the presence or absence of short melanized cell clusters or projections, observed in several isolates (Fig. 1). Then, isolates were additionally classified as homogeneous (H) or irregular (I) in shape (Table 4).

Results showed important morphologic differences among MS formed by isolates recovered from the same sample. Therefore, the 6 isolates from sample 12 presented among them high morphologic variability, ranging in values of L/W from 2.14 (isolate 31) to 5.15 (isolate 29) and they were classified as rounded or elongated. Equally, within each of the samples 5, 7 or 10, recovered isolates were included in different morphologic types (Table 4).

On the contrary, isolates recovered from each one of other samples did not presented morphologic differences according to described morphologic types (Table 4). Therefore, the 3 isolates from sample 15 formed spherical ME, homogeneous or irregular, although significant differences in the relation L/W were detected among them. Similarly, the 3 isolates coming from sample 1 and the 3 isolates from sample 4 were classified as rounded, although with significant differences among isolates of the two sample and those belonging to the same sample (Table 4).

DISCUSSION

The method for obtaining soil isolates of *V. dahliae* has provided results that were effective for a wide range of soils. This technique overcomes difficulties reported by DeVay *et al.*[13] in their approach to recover the fungus from soil and by an other author who described the difficulty of purifying the pathogen in an assay for

quantification[25]. Cellophane paper overlaid onto PDA plates for induction of MS production of *V. dahliae*[32,33], provides an additional carbon source for its growth[13,35]. In this study, cellophane allowed the fungus to grow on the plates for at least 14 days of incubation without becoming degraded[33]. Therefore, this technique can be used for the quantitative assessment of *V. dahliae* in the soil sample, since results on MSPA+C medium do not differ from those obtained with MSPA, or even these are improved. After or during the process of quantification, MS colonies can be selected and recovered free of agar, that is one of the main sources of contamination in the transference of the pathogen to PDA for purification. It is also possible to choose the best formed colonies, free of contaminants and soil particles, so the purification efficacy increased considerably. Moreover, since the reported technique starts from a colony formed MSPA-cellophane with high amount of MS that represent the same isolate, results are improved related to other works where individual propagules are selected as source to attempt the purification of each isolate.

It is recommended to start the detection of microesclerotial colonies on MSPA+C plates from 8-10 days after sowing, before the normal incubation period of 14 days reported for quantitative analyses. This will reduce the contamination of colonies by other microorganisms, with micelial growth rates much higher than *V. dahliae*.

Inoculum density, texture and microbiologic composition of the soil samples might be major factors influencing on the efficacy of the method for obtaining soil isolates. In cases of soils with low inoculum densities, probabilities of detecting and purified MS colonies are strongly reduced, since there are not enough amount of colonies to select the most appropriate ones to process. For high inoculum density soils the success increments considerably and selected colonies will be also more representative of those isolates that are in high amount in the sample. Soil texture has an influence on the sieving process, in the detection effectiveness of the quantitative method, and during the washing process of the colony suspension for recovering isolates. Finally, the microbiologic composition of the soil has a direct influence on the competitiveness that occurs during the growth of *V. dahliae* onto the cellophane sheet, since this specie has not a growth rate comparable with other fungus like *Fusarium* sp. or *Aspergillus* sp. that grow habitually on MSPA.

Several studies indicate that morphology of MS that *V. dahliae* produces on different culture media might be connected to the virulence of the isolate. Therefore, on water agar, defoliating isolates produce colonies with mixtures of elongated and rounded ME and non-defoliating ones, only rounded[25,36]. The culture medium composition or substrate where the fungus grows seems to have an influence on MS morphology and this feature has been used to established, at least preliminarily, the pathotype of the isolate[25]. However, for our morphological studies on MSPA, such relations should be confirmed by means biological test of pathogenicity or molecular analyses. Particularly, in the Agronomy Department of University of Cordoba, the wet sieving technique and the MSPA medium have been habitually used on research programs of epidemiology and control of Verticillium wilt of olive carried out for several years[30,32,37]. Consequently, morphologic differences on MSPA observed in the present work between MS formed by our reference isolates V117 (defoliating pathotype) and V4 (non-defoliating pathotype) are very interesting. Additionally, in precedents works it has been observed that our D reference isolate used to form on this medium bigger and more complete typical star-shape colonies, with MS elongated, than those formed by the ND, that also usually present a higher proportion of rounded ME. However, it has been also observed frequently colonies with a high proportion of irregular-oval propagules[28].

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