

ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)



Bio Technology



ANSI*net*

Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Production of Homogeneous and Viable *Verticillium dahliae* Microsclerotia Effective for Verticillium Wilt Studies

F.J. López-Escudero, C. Mwanza and M.A. Blanco-López
Department of Agronomy, University of Córdoba, Campus Universitario de Rabanales,
Edificio Celestino Mutis, 14071, Córdoba, Spain

Abstract: Artificial production of *Verticillium dahliae* microsclerotia using potato dextrose agar plates covered by a cellophane sheet was optimized regarding thickness and sterilization method of the film. The objective was to standardize production per plate, homogeneity (size and propagules individuality) and viability (germinability and infective capability) of inoculum, for epidemiological studies. Autoclaved cellophane film, 600 µm in thickness, yielded the highest production of microsclerotia, after 7-10 days of incubation at 22°C, without causing its degradation. Amount of microsclerotia per plate was significantly higher for the defoliating (331.9×10^3) than the non-defoliating (202.1×10^3) isolate of the pathogen, maintaining the 93.0 and 89.6% of germinability, respectively, after 1 year. Disease developed on cotton plants of cultivars Tabladilla-16 and Tauro, grown on infested soils with 0.5 to 125 microsclerotia per gram of soil, demonstrated its capability of survival and infection. A high relationship between inoculum density and onset and severity symptoms was observed, that depended on the isolate virulence and cultivar susceptibility.

Key words: Cellophane film, cotton, defoliating and non-defoliating isolates, inoculum, inoculum potential

INTRODUCTION

Verticillium dahliae Kleb. is a widespread soil-borne pathogen that cause important losses in a broad range of herbaceous and woody host (Schnathorst, 1981; Hiemstra and Harris, 1998; Pegg and Brady, 2002). The pathogen survives in soil for a long period of time by its microsclerotia (MS), produced in senescent tissues of affected plants. Moreover, MS are the structures of infection and dispersion of the fungus in the soil. The studies of the relationships between Inoculum Density (ID) in the soil and virulence of *V. dahliae* on wilt diseases, require the use of naturally or artificially infested soils with these propagules. In the second case, major limitations are the availability of enough amount of inoculum to generate disease and the viability of the MS over time, especially for long term experiments. Microsclerotia of *V. dahliae* obtained from infested plant debris or produced on grains or plant straw (Lacy and Horner, 1966; Basu, 1987; Nagtzaam *et al.*, 1997) are usually associated to organic matter or tissues, grouped in cluster, or mixed with mycelium and conidia. Thus, attempts to produce standardized inoculum of this pathogen have been aimed on different culture media for studies of survival (Farley *et al.*, 1971; Hawke and Lazarovits, 1994), assessment of control measures

(Spink and Rowe, 1989; Blok *et al.*, 2000) or to determine the relationship between inoculum density and disease progress (DeVay *et al.*, 1974; Francl *et al.*, 1988; Xiao and Subbarao, 1998). In these researches, although enough amount of inoculum was obtained, accuracy of results strongly depended on viability, age and the variability in the size of propagules of the pathogen. Therefore, objective of this study was to produce artificially a significant amount of homogeneous and viable inoculum of *V. dahliae*, consisting of well-defined individual infective microsclerotia, for practical use in etiological, epidemiological or control studies. A preliminary report has been published by Mwanza and Blanco-López (2001).

MATERIALS AND METHODS

Fungal material: For the experiments, two single-spore isolates of *V. dahliae*, V4 and V117, from a collection of the Plant Pathology laboratory of Agronomy Department, University of Cordoba were used. V4 represents a mildly virulent Non-defoliating (ND) and V117 a highly virulent Defoliating (D) isolate of the pathogen (Blanco-López *et al.*, 1989). Isolates were maintained on Potato Dextrose Agar (PDA) slants at 4°C (Dhingra and Sinclair, 1995). Conidial suspensions of each isolate were prepared from plates of 6 day old cultures

incubated on PDA at 24°C in the dark, 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 adjusted to 10^7 conidia mL⁻¹.

Artificial production of microsclerotia: Microsclerotia of each isolate were produced by plating 0.5 mL/plate of the conidial suspension onto PDA plates covered with a sterilized permeable cellophane disc (9 cm in diameter) (UCB Film, La Cellophane Española S. A.). After several days of incubation at 22-23°C in the dark, both isolates produced on the sheets mycelium, conidia and, then, microsclerotia.

The incubation period: A first experiment was carried out to find the optimum incubation period that allowed maximum production of MS after removing the cellophane disc from the PDA plates, before cellophane degradation due to fungal activities was caused. In preliminary studies it was observed that the degradation of the film could be influenced by the cellophane thickness and the sterilization method. Thus, three types of cellophane film (300, 400 and 600 µm in thickness) and two different sterilization methods (autoclaving, at 1 atm for 20 min; or dry oven, at 120°C for 2 h) were tested. Starting 6 days after conidial suspensions plating, a cellophane disc from each treatment was daily removed from PDA plates for 10 successive days or until cellophane degradation did not allow the removing. Optimum time for removing cellophane discs was based on visual observation of MS production and feasibility of removing the films without breaking. This experiment was repeated twice.

Selection of cellophane type and sterilization method for production of microsclerotia: A new experiment was conducted to compare production of MS using cellophane film of 300, 400 or 600 µm, sterilized by autoclave or dry oven as explained above for either isolate. After 7 days of incubation, for each treatment, MS from 4 cellophane sheets were pooled by removing the films from PDA plates and gently cleaning the surface with a paintbrush in 200 mL sterilized water. Suspensions were shaken at high speed 1 min. The process was repeated till confirm by microscopic observations that clusters of propagules were disintegrated in individual microsclerotia free of hyphal fragments. Then, the suspension was filtered in tandem through 150 and 35 µm sieves, that is the size range reported for microsclerotia present in soils and usually considered in the quantification methods of the inoculum density of this pathogen from the soil (Harris *et al.*, 1993; Hawke and Lazarovits, 1994; López-Escudero and Blanco-López,

2001, 2005). Microsclerotia retained in the 35 µm were recovered in a flask with 100 mL of sterilized water. For counting the total amount of MS, this suspension was diluted 100 times and MS in 0.1 mL drops (5 replications) of each dilution were counted and results expressed as number of MS per cellophane film. The experiment was repeated 3 times. Analyses of variance were performed by Statistic 7.0 for Windows (Analytical Software, Tallahase, USA) and mean values were compared by the Fisher's protected LSD test at $p = 0.05$ (Steel and Torrie, 1995).

Production of microsclerotia on 600 µm cellophane with the selected method: Experiments were conducted to determine MS production per each cellophane-PDA plate with the previous selected methodology. In these experiments, ND and D isolates of *V. dahliae* were plated onto PDA plates covered with autoclaved 600 µm cellophane sheets (4 replications). Films were removed from plates after 7 days incubation and processed as described above to obtain 100 mL suspensions of MS retained in the 35 µm sieve. Each suspension was decanted (settled for 30-45 min) in a separating funnel and the top layer with mycelium debris and conidia, was decanted off and discarded. The slurry containing decanted MS was transferred and uniformly distributed onto empty Petri plates forming a thin layer, excess water rejected and plates dried in an incubator at 28-29°C for 48 h. Separated and dried MS produced onto the 4 plates were collected by scrapping off using a scalpel and mixed in glass sterilized tubes with 4 g of sterile talc that was homogenized by shaking.

For counting MS in the mix with talc, samples from 0.05 to 0.5 g were taken and suspended in 10 mL of sterilized water. After shaking 30 sec, dilutions 1/10 and 1/100 were prepared and 0.5 mL aliquots of the suspensions were spread onto 3 plates (replications) of a Modified Sodium Polipectate Agar (MSPA) (Butterfield and DeVay, 1977). Plates were incubated at 22-23°C in the dark and at 24 and 48 h, the total amount of MS/plate was counted under a stereoscope. Mean number of MS produced per cellophane disc (N) was calculated according to following formula: $N = P \cdot N_1 / (0.1 \cdot d \cdot P_1 \cdot 0.5 \cdot n)$, where N_1 = mean number of MS on the MSPA plate; d = dilution factor; P = total weight of the mix MS+talc in the tube; P_1 = weight of a sample of MS+talc to obtain the suspension in 10 mL of water and n = total number of PDA cellophane plates used to produce the MS added to the talc. This experiment was repeated 3 times.

Viability of microsclerotia: The viability of MS of D and ND isolates of *V. dahliae* artificially produced was assessed in two experiments. For each isolate, aliquots of

0.5 mL of a MS suspension in sterilized water or a mix of MS and talc were spread onto MSPA plates. Suspensions were previously adjusted to obtain densities between 50 to 100 MS/plate with 8 replications (plates). Germination of MS was assessed at 6, 12, 48, 72 and 96 h of incubation at 22-23°C. In a second experiment survival of MS, produced by the current method, stored in sterilized glass tubes at 4°C, was tested periodically every 3 months during one year, using the same procedure and replications as describe above. For the second experiment, ordinary least squares regression analyzes were performed with transformed data of germination, $y = \arcsin(\text{percentage of germination})^{0.5}$, to describe the evolution over the time of this parameter by Statistix 7.0 for Windows (Analytical Software, Tallahassee, USA).

Capability of infection of microsclerotia: Infectivity of *V. dahliae* MS of the two pathotypes of the pathogen on cotton was investigated under controlled conditions in a growth chamber, using an autoclaved and artificially infested soil (1:1:1, peat:sand:lime) in three experiments: In Experiments 1 and 2 0, 5, 25 and 125 MS/g of soil were used as inoculum and «Tabladilla-16» (very susceptible) and «Tauro» (moderately susceptible) *Gossypium hirsutum* L. cultivars were tested, respectively; in Experiment 30, 0.5, 1 and 5 MS/g of soil were used and cultivar Tauro was infected. A sterile soil (1:1:1, peat:sand:lime) was infested by mixing with the corresponding amount of MS using 1 L clay pots.

In all experiments cotton seeds were pre-germinated at 22°C for 48 h. Plants grew in controlled conditions at 22-23°C, with a photoperiod of 14 h (216 $\mu\text{Em}^{-2} \text{s}^{-1}$ fluorescent light) and 80% HR, for 10, 17 and 23 weeks for Experiments 1, 2 and 3, respectively. Plants were arranged according to a split-plot completely randomized block design, where the main-plot was the *V. dahliae* pathotype and ID were assigned to sub-plots.

Foliar symptoms of wilt were weekly assessed starting 6 weeks after sowing until the end of experiments, according to a 0-4 scale (0 = no symptoms; 1 = 1 to 33% foliage affected, 2 = 34 to 66%; 3 = 67 to 100%; 4 = dead plant) for chlorosis, necrosis, flaccidity, distortion of the shoot tips and defoliation. The percentage of dead plants was also assessed. In Experiment 3, height and fresh weight of plants were assessed at the end of evaluation. At the end of experiments, all plants showing severity of symptoms <3.0 were processed for isolating the pathogen. Petioles of the third and fourth leaves, with or without symptoms, were washed under tap water for 45 min and disinfected in 0.5% sodium hypochlorite for 1 min. Little pieces of petioles were cut and placed onto PDA plates and incubated at 24°C in the dark for 7-8 days.

From severity of symptom values, the area under disease progress curve (AUDPC) for each combination cultivar/ID was calculated, considering its percentage with regard to the maximum possible value that could be reached in the period of assessment, based on Campbell and Madden (1990): $\text{AUDPC} = [(\Delta t/2*(S_2+2*S_3+\dots+2*S_{i-1}+S_i))/4*n]*100$ (t = interval in days between observations; S_i = final mean severity; 4 = maximum value of severity; n = number of observations). Values of mean severity, AUDPC, height and fresh weight of plants and percentage of dead plants were analyzed by ANOVA and mean values were compared by the Fisher's protected LSD test at $p = 0.05$.

RESULTS

Influence of cellophane type and sterilization method on the incubation period: Microsclerotia formation started from 4-5 days after plating. Degradation of cellophane sheets was influenced by the isolate, the cellophane thickness and sterilization method. Disintegration was faster for the ND isolate than the D one for the thinnest films sterilized in dry oven. Optimum time for removing cellophane discs depended on the isolate, since the discs plated with the D isolate, that grew faster and formed higher amount of MS over time, became degraded sooner and its removing was recommended before those plated with the ND one. Optimum time ranged from 7 to 10 days of incubation. Before this time, the amount of MS was not optimum and after 10 days of incubation, the degradation of the sheets caused breakings during the film extractions and the formation of aerial mycelium made it more difficult the process.

Selection of cellophane type and sterilization method for production of microsclerotia: For both isolates and the three types of films, MS started to form at 5 days of incubation and covered the full film surface at the seventh day. The D isolate yielded a significantly higher amount of MS than the ND one (Table 1). The amount of MS produced onto the 600 μm film was significantly higher than that produced on the others (Table 1). Sterilization methods applied to the cellophane had a significant influence only on the production of MS of the ND isolate.

Cellophane film of 600 μm was finally selected due to higher production of MS, resistance to degradation and feasibility of management. Similarly, autoclaving was selected since affected less the resistance of the film and prolonged the time for degradation.

Production of microsclerotia on 600 μm cellophane with the selected method. The experiments were not different statistically and mean production expressed as MS per plate of cellophane-covered PDA were 331.9×10^3

Table 1: Production of microsclerotia by the non-defoliating and defoliating isolates of *Verticillium dahliae* on cellophane-covered PDA using different sterilization processes

Cellophane type	Non defoliating			Defoliating		
	Dry oven	Autoclave	Mean	Dry oven	Autoclave	Mean
	300 ^x	86.2 ^y	121.2	103.7c	231.2	242.5
400	112.5	176.2	144.3b	301.2	281.2	291.2b
600	192.5	251.2	221.8a	355.0	358.7	357.5a
Mean	130.4c	182.9b		295.8a	294.1a	

^xThickness (in µm), ^yValues are the mean of three experiments of the number of microsclerotia obtained from 9 cm diameter cellophane film. Mean values in rows or columns followed by different letter(s) were significantly different at p = 0.05 according to Fisher's protected LSD test

Table 2: Final disease parameters of cotton plants affected by *Verticillium* wilt grown on an artificially infested soil by microsclerotia of the non-defoliating or defoliating isolates of *Verticillium dahliae*^a

Experiment ^b	ID (MS g ⁻¹)	AUDPC		Mean severity		Dead plants (%)	
		ND	D	ND	D	ND	D
1	5	40.8	10.5	2.8	1.5	42.9	28.6
	25	33.9	55.2	2.8	3.9	42.9	100.0
	125	41.2	69.9	3.1	3.9	57.1	100.0
	Mean	38.7a	45.2a	2.9m	3.1m	47.6	76.2
2	5	47.4	32.9	2.5	2.3	66.7	66.7
	25	33.8	32.1	1.2	2.6	33.3	33.3
	125	60.3	92.3	2.5	4.0	66.7	100.0
	Mean	47.2a	52.4a	2.0m	2.9m	55.6	55.6
3	0.5	1.1	31.8	0.5	3.1	0.0	71.4
	1	17.0	43.1	2.4	3.2	28.6	71.4
	5	12.3	72.5	2.7	4.0	14.3	100.0
	Mean	10.1b	49.1a	1.9n	3.4m	14.3	81.0

^aDisease parameters were evaluated at 10, 17 and 23 weeks after sowing plants in the infested soil for Experiments 1, 2 and 3, respectively. For each disease parameter, mean values in rows followed by different letters were significantly different at p = 0.05 according to Fisher's protected LSD test. ^bFor Experiment 1 was used the susceptible cultivar Tabladilla-16 and for Experiments 2 and 3, the moderately susceptible cultivar Tauro

and 202.1×10³ for the D and the ND isolate, respectively. These values were similar than those obtained in previous experiments, during the selection the cellophane type and the sterilization method, when the 600 µm autoclaved film was used, that accounted for 294.1×10³ and 182.9×10³ for the D and ND isolate, respectively (Table 1).

Viability of microsclerotia: Talc did not affect MS germination. No differences in germinability over time were found between isolates. Microsclerotia germination started at 24 h of incubation and more than the 90% of them had already germinated at 48 h. Final percentages at 96 h of incubation were 96.5 and 98.0% for the D and the ND isolate, respectively. Microsclerotia of both isolates remained with high viability after 1 year of storing at 4 °C, although the percentage of germination slightly decreased from 95.2 to 89.6 and 98.0 to 93.0 for the D and ND isolates of *V. dahliae*, respectively (Fig. 1). Transformed values of the percentage of germination of MS showed negative and significant linear relationships with time, that were y = -0.01066x+1.34 (at p = 0.04 and determination coefficient R² = 0.79) and y = -0.00347x+0.99 (at p = 0.01 and determination coefficient R² = 0.89) for the D and ND isolate, respectively.

Capability of infection of microsclerotia: Symptoms of wilt in cotton plants were similar in Experiments 1 to 3 and consisted of wilt, leaf necrosis and flaccidity. Leaves remained attached in plants infected by the ND. In addition, the D isolate caused defoliation, apical necrosis of twigs and dwarfing, causing earlier dead of plants. Isolation from petioles of affected plants consistently yielded cultures of *V. dahliae*.

The onset and severity of the symptoms depended clearly on the ID, isolate and cultivar used. Experiment 1, finished at 10 weeks after sowing cotton plants since high ID and a susceptible cultivar Tabladilla-16 were used; while, Experiments 2 and 3, finished later, 17 and 23 weeks after inoculation, respectively, because cultivar Tauro was less susceptible and ID was either high (Experiment 2) or low (Experiment 3). There were not statistical differences between some of the treatments in Experiments 1 and 2, although mean values of AUDPC and final mean severity of symptoms in both experiments were higher in plants infected by the D isolate (Table 2).

The beginning of symptoms was influenced by the susceptibility of the cultivar and the ID of the pathogen. Symptoms in cultivar Tabladilla-16 in Experiment 1 appeared 6 weeks after sowing and the plants were

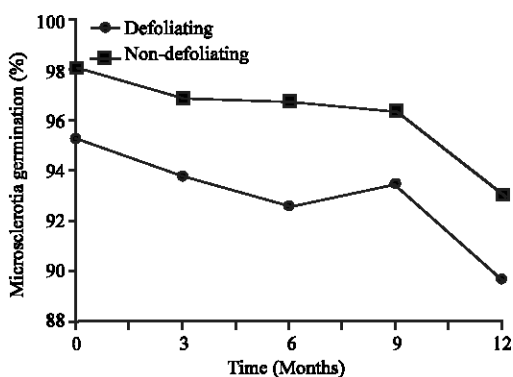


Fig. 1: Fluctuation of the viability of microscerotia of *Verticillium dahliae* artificially produced on cellophane-covered PDA

severely affected by both isolates. The D isolate caused faster the death of plants than the ND, particularly in higher ID treatments, where a 100% of mortality at the end of experiment was reached, with the exception of the lowest ID (5 MS/g) (Fig. 2A and Table 2). The lower susceptibility of cultivar Tauro was expressed as a delay in symptom expression in Experiment 2. It was especially marked in plants infested with 5 and 25 MS/g of soil of the D isolate, in which symptoms did not consistently started until 14 weeks after sowing (Fig. 2B). Thus, final mortality in these treatments (33.3%) was significantly lower than that reached by plants infested with 125 MS/g (100%), that started to show earlier symptoms at 8 weeks after sowing and developed fast reaching high values of AUDPC (90%) from the twelfth week (Fig. 2B and Table 2). In Experiment III, final values of AUDPC and mean severity of symptoms were significantly different between isolates and ID used. Plants of cultivar Tauro were severely affected by the D isolate at all ID and wilt symptoms started from 10 weeks after sowing in the 5 MS/g treatment (Fig. 2C). The D isolate caused severe symptoms even at the lowest ID (0.5 MS/g), that reached a final mortality of 71.4% (Table 2). Nevertheless, the ND isolate only induced mild symptoms on the plants at higher ID (5 or 1 MS/g), that started from 14 to 16 weeks after sowing, or very scarce symptoms at 0.5 MS/g, that were delayed to 22 weeks after sowing (Fig. 2C and Table 2). Plant high and fresh weight were diminished by the infection, with the exception of the lowest ID of the ND isolate. Highest reductions in height and fresh weight of plants were reached in the treatment of 5 MS/g, ranging from 75 and 98% and 21 and 31% for the D and ND isolate of *V. dahliae*, respectively.

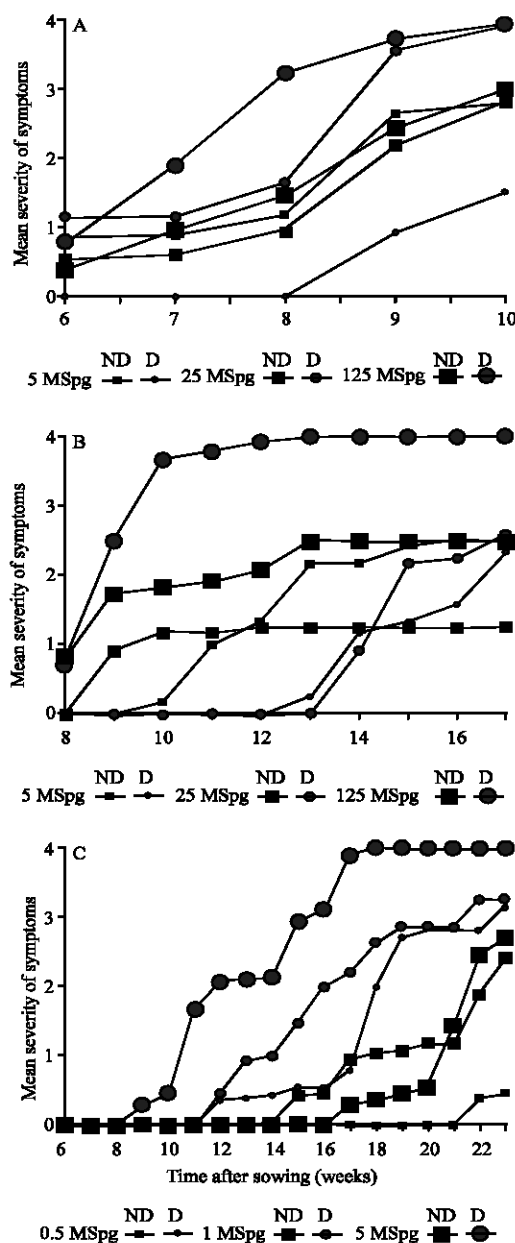


Fig. 2: Evolution of the severity of foliar symptoms in cotton plants affected by *Verticillium* wilt in an artificially infested soil with different inoculum densities (MS/g of soil) of a Non-defoliation (ND) or defoliation (D) isolate of *Verticillium dahliae*. A, Experiment 1 with cultivar Tabladilla and B and C, Experiments 2 and 3, respectively, with cultivar Tauro

DISCUSSION

The use of cellophane for producing MS of *V. dahliae* has been used by several authors (Schnathorst

and Mathre, 1966; Davis *et al.*, 1979; Al-Rawahi and Hancock, 1998). In our studies, parameters involved in massive production of these structures have been optimized: kind of cellophane film, incubation time for recovering MS from the sheets and sterilization method. Therefore, enough amount of MS can be produced for *Verticillium* studies. Microsclerotia produced are very homogeneous in age, size and are highly purified, without conidia or mycelium that could have an extra and non-controlled influence on the research. For purifying, decanting method, as used by Tjamos and Fravel (1995), resulted effective in removing conidia and mycelium from the inoculum produced. Moreover, the air-drying process of MS during 48 h at 28-29°C reduced debris of mycelium and conidia.

Verticillium sp. have cellulositic activity, using the cellophane as an energy source due to the high availability of carbohydrates (Talboys, 1958; Wyllie and DeVay, 1970; Ashworth *et al.*, 1972; Huisman and Ashworth, 1974). This probably explains that production of MS was significantly higher using the thicker film of 600 µm than other ones with less thickness. Both isolates responded positively to extra nutrients provided by the cellophane, but the fungus capability for growing and producing MS caused cellophane biodegradation. The D isolate produced higher amount of MS than the ND one, in agreement with Wyllie and DeVay (1970). Nevertheless, in our experiments, MS production by the ND isolate of *V. dahliae* started before that of the D one and it degraded the cellophane film after the D one. Irrespectively the kind of film and isolate, cellophane sheets were more resistant to degradation when these were autoclave sterilized instead of using a dry oven.

Studies of viability and capability of infection were carried out to check if there are real problems on this parameters when MS are artificially produced as suggested by Hawke and Lazarovits (1994), who reported that type of inoculum might not be effective for research on epidemiology or disease control in experiments taking place during several weeks or months. However, these authors pointed out that these parameters should depend on the method of production. Thus, Tjamos and Fravel (1995) attained to produce laboratory MS with high viability for studies of biological control and solarization. By this method of production we have also achieved to obtain MS of *V. dahliae* with viability higher than 90% one year after their production.

Talcum powder did not interfere on viability of MS after 30 days of incubation and it was an appropriate inert substrate to avoid regrouping of propagules during conservation period. Thereafter, MS of both isolates maintained high levels of germinability during one year when they were conserved at 4°C.

Inoculum obtained by this technique is very useful for epidemiological studies, since it provides homogeneous infective units formed by individual microsclerotia, avoiding clusters of propagules that could mask the relationship between the inoculum density added to the soil and the disease progress in the tested host. Disease developed on cotton plants growing on infested soil with artificially produced MS demonstrates the capability of survival and infection of our inoculum. Symptoms on wilt in cotton plants were similar to those described by other authors in this crop (Schnathorst, 1981; El-Zik, 1985; Blanco-López *et al.*, 1989). Furthermore, the beginning of the symptom expression and severity depended on the isolate, ID and susceptibility of the cultivar used, as reported by Ashworth *et al.* (1979) and El-Zik (1985). Thus, in the first experiment, cultivar Tabladilla-16 was severely infected by both isolates in all ID treatments. For both isolates first symptoms appeared 6 weeks after sowing, with the exception of the ID of 5 MS/g of the D isolate which were delayed to 9 weeks. The high ID used could explain the high and early severity of foliar symptoms caused by the ND pathotype, that usually induces less-severe symptoms than the D one in cotton (Schnathorst, 1981). Similarly, results reported by Ashworth *et al.* (1979) showed that densities of 1.7 or 20.9 MS/g could induce 70-90% of vascular infections in tolerant cultivars to *V. dahliae* of cotton such as Acala SJ-4 and SJ-5.

Inoculum densities of 25 or 125 MS/g of the D isolate produced 100% of mortality in plants 10 weeks after sowing, while values for the ND one were much lower. Isolations of the pathogen from aerial parts of asymptomatic plants did not yield positive cultures, confirming there was not aerial tissue colonization by *V. dahliae*. Probably plants escaped from the pathogen infection as described by Tjamos *et al.* (1991), due to the reduction of the probability of contact between inoculum and plant roots.

When the less-susceptible cultivar Tauro was used, first symptoms of the disease delayed, starting around 2 weeks after sowing for the highest ID. In Experiment 3, differences on disease development (symptoms onset, severity and percentage of dead plants) were more pronounced between the two isolates. For the ND isolate, using the lowest dosages of inoculum, mortality was 0%, according to results from Blanco-López *et al.* (1992).

These studies confirm the high viability and capability of infection of microsclerotia of *V. dahliae* produced by this technique, that present the uniformity necessary for research on studies of the relationship between the inoculum density and the disease development.

ACKNOWLEDGMENTS

The present study was supported by Comisión Interministerial de Ciencia y Tecnología (Proyecto AGF 97-0546) and by the European Commission (Project reference number QLRT-1999-1523).

REFERENCES

- Al-Rawahi, A.K. and J.G. Hancock, 1998. Parasitism and biological control of *Verticillium dahliae* by *Pythium oligandrum*. Plant Dis., 82: 1100-1106.
- Ashworth, L.J., J.E. Waters, A.G. George and O.D. McCutcheon, 1972. Assessment of microsclerotia of *Verticillium albo-atrum* in field soils. Phytopathology, 62: 715-719.
- Ashworth, L.J. Jr., O.C. Huisman, D.M. Harper, L.K. Stromberg and D.M. Bassett, 1979. Verticillium wilt disease of cotton: Influence of inoculum density in the field. Phytopathology, 69: 483-489.
- Basu, P.K., 1987. Longevity of *Verticillium albo-atrum* within alfalfa stems buried in soil or maintained without soil at various temperatures. Can. Plant Dis. Survey, 67: 15-16.
- Blanco-López, M.A., J. Bejarano-Alcázar, J.M. Melero-Vara and R.M. Jiménez-Díaz, 1989. Current Status of Verticillium Wilt of Cotton in Southern Spain. Pathogen Variation and Population in Soil. In: Vascular Wilt Disease of Plants. Tjamos, E.C. and C.H. Beckman (Eds.). NATO-ASI series, Berlin: Springer-Verlag, H28: 123-132.
- Blanco-López, M.A., R.M. Jiménez-Díaz, J.M. Melero-Vara and J. Bejarano-Alcázar, 1992. Integrated Control of Verticillium Wilt of Cotton by Soil Solarization and Tolerant Cultivars. In: Biological Control of Plant Diseases. Tjamos, E.C., G.C. Papavizas and R.J. Cook, (Eds.), Plenum Press, New York, pp: 63-67.
- Blok, W.J., J.G. Lamers, A.J. Termorshuizen and G.J. Bollen, 2000. Control of soilborne plant pathogens by incorporating fresh organic amendments followed by tarping. Phytopathology, 90: 253-259.
- Butterfield, E.J. and J.E. DeVay, 1977. Reassessment of soil assays for *V. dahliae*. Phytopathology, 67: 1073-1078.
- Campbell, C.L. and L.V. Madden, 1990. Introduction to Plant Disease Epidemiology. John Wiley and Sons, New York.
- Davis, R.M., J.A. Menge and D.C. Erwin, 1979. Influence of *Glomus fasciculatus* and soil phosphorus on Verticillium wilt of cotton. Phytopathology, 69: 453-456.
- DeVay, J.E., L. Forrester Linda, R.H. Garber and E.J. Butterfield, 1974. Characteristics and concentration of propagules of *Verticillium dahliae* in air-dried field soils in relation to the prevalence of Verticillium wilt in cotton. Phytopathology, 64: 22-29.
- Dhingra, O.D. and Sinclair, J.B. 1995. Basic Plant Pathology Methods. 2nd Edn., CRC Press, Inc. Boca Raton, pp: 359.
- El-Zik, K.M., 1985. Integrated control of Verticillium wilt of cotton. Plant Dis., 69: 1025-1032.
- Farley, J.D., S. Wilhelm and W.C. Snyder, 1971. Repeated germination and sporulation of microsclerotia of *Verticillium albo-atrum* in soil. Phytopathology, 61: 260-264.
- Franel, L.J., R.C. Rowe, R.M. Riedel and L.V. Madden, 1988. Effects of three soil types on potato early dying disease and associated yield reduction. Phytopathology, 78: 159-166.
- Harris, D.C., J.R. Yang and M.S. Ridout, 1993. Estimating *Verticillium dahliae* in soil. The detection and estimation of *Verticillium dahliae* in naturally infested soil. Plant Pathol., 42: 238-250.
- Hawke, M.A. and G. Lazarovits, 1994. Production and manipulation of individual microsclerotia of *Verticillium dahliae* for use in studies of survival. Phytopathology, 84: 883-890.
- Hiemstra, J.A. and D.C. Harris, 1998. A Compendium of Verticillium Wilt in Tree Species. Ponsen and Looijen, Wageningen, The Netherlands.
- Huisman, O.C. and L.J. Ashworth, 1974. Quantitative assessment of *Verticillium albo-atrum* in field soils: Procedural and substrate improvements. Phytopathology, 64: 1043-1044.
- Lacy, M.L. and C.E. Horner, 1966. Behavior of *Verticillium dahliae* in the rizosphere and on roots of plants susceptible, resistant and immune to wilt. Phytopathology, 56: 427-430.
- López-Escudero, F.J. and M.A. Blanco-López, 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 M.A. Blanco-López, 2005. Isolation and morphologic characterization of microsclerotia of *Verticillium dahliae* isolate from soil. Biotechnology, 4: 296-304.
- Mwanza, C. and M.A. Blanco-López, 2001. The viability an infective capacity of *Verticillium dahliae* microsclerotia produced on cellophane paper. Abstracts of the 8th Inter. Verticillium Symposium. Córdoba, Spain.

- Nagtzaam, M.P.M., A.J. Termorshuizen and G.J. Bollen, 1997. The relationship between soil inoculum density and plant infection as a basis for a quantitative bioassay of *Verticillium dahliae*. *Eur. J. Plant Pathol.*, 103: 597-605.
- Pegg, G.F. and B.L. Brady, 2002. *Verticillium* Wilts. Cromwell Press, Trowbridge, pp: 552.
- Schnathorst, W.C., 1981. *Verticillium* wilt. In: *Compendium of Cotton Diseases*. Watkins, G.M. (Ed). The American Phytopathological Society, St. Paul, MN, pp: 41-44.
- Schnathorst, W.C. and D.E. Mathre, 1966. Host range and differentiation of a severe form of *Verticillium albo-atrum* in cotton. *Phytopathology*, 56: 1155-1161.
- Spink, D.S. and R.C. Rowe, 1989. Evaluation of *Talaromyces flavus* as a biological control agent against *Verticillium dahliae* in potato. *Plant Dis.*, 73: 230-236.
- Steel, G.D. and J.H. Torrie, 1995. *Bioestadística: Principios y procedimientos*. McGraw Hill, Bogotá.
- Talboys, P.W., 1958. Degradation of cellulose by *Verticillium albo-atrum*. *Trans. Br. Mycol. Soc.*, 41: 242-248.
- Tjamos, E.C, D.A. Biris and E.J. Paplomatas, 1991. Recovery of olive trees with *Verticillium* wilt after individual application of soil solarization in established olive orchards. *Plant Dis.*, 75: 557-562.
- Tjamos, C.E. and D.R. Fravel, 1995. Detrimental effects of sub-lethal heating and *Talaromyces flavus* on microsclerotia of *Verticillium dahliae*. *Phytopathology*, 85: 388-392.
- Wyllie, T.D. and J.E. DeVay, 1970. Growth characteristics of several isolates of *Verticillium albo-atrum* and *Verticillium nigrescens* from cotton. *Phytopathology*, 60: 907-910.
- Xiao, C.L. and K.V. Subbarao, 1998. Relationships between *Verticillium dahliae* inoculum density and wilt incidence severity and growth of cauliflower. *Phytopathology*, 88: 1108-1115.