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Effect of soilless growing systems on the spread of *Verticillium dahliae* and the severity of the Verticillium wilt in strawberry

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Abstract

The dispersion of soilborne plant pathogens could be greater in closed soilless growing systems than in open ones. The effect of three soilless growing systems (open, closed and closed with slow sand filtration) on the dispersion of *Verticillium dahliae* propagules and the severity of the disease in strawberry *(Fragaria × ananassa* Duch.) has been analysed. *V. dahliae* dispersion in a closed system with slow sand filtration was studied by measuring propagules in the recirculating nutrient solution and in the growth medium. The growth medium used was coconut fiber. *V. dahliae* propagules were not removed by slow sand filtration. In the first crop cycle, an increase in the severity of Verticillium wilt was detected in the closed soilless growing system with slow sand filtration in comparison with the other two systems. This increase may be due to the non-elimination of *V. dahliae* propagules by filtration and to the lower microbial biomass in the filtered solution storage tank than in the drained solution storage tank. The decline in microbial biomass by filtration may improve the viability of the dispersed conidia, thus increasing the severity of the disease. This decline in microbial biomass by filtration may be compensated in the second crop cycle by the root debris from the first crop cycle. This debris may have provided nutrient sources to the microbes and increased the associated microbial biomass.

Additional key words: microbial biomass, slow sand filtration.

Resumen

Efecto de sistemas de cultivo sin suelo sobre la dispersión de *Verticillium dahliae* y de la verticilosis en el cultivo de la fresa

La dispersión de los patógenos de suelo podría ser mayor en los sistemas de cultivo sin suelo cerrados que en los sistemas abiertos. Se estudió el efecto de tres sistemas de cultivo (abierto, cerrado y cerrado con filtración lenta en lecho de arena) sobre la dispersión de los propágulos de *Verticillium dahliae* y sobre la severidad de la enfermedad en fresa *(Fragaria × ananassa* Duch.). Se analizó la dispersión de *V. dahliae* en el sistema cerrado con filtración lenta mediante la medida de los propágulos en la solución nutritiva recirculante y en el sustrato empleado, que fue fibra de coco. Los propágulos de *V. dahliae* no fueron eliminados por la filtración lenta. En el primer ciclo de cultivo, se detectó un incremento en la severidad de verticilosis en el sistema cerrado con filtración lenta en comparación con los otros dos sistemas. Este incremento puede ser debido a que el filtro no elimina los propágulos de *V. dahliae* y a que la biomasa microbiana en la solución filtrada es más baja que en la solución drenada. Este descenso que se produce en la biomasa microbiana debido a la filtración podría mejorar la viabilidad de las conidias dispersadas y por lo tanto incrementar la severidad de la enfermedad. El descenso de la biomasa microbiana por la filtración parece estar compensado en el segundo ciclo de cultivo por la activación de la misma debido a los nutrientes proveídos por los restos de raíces procedentes del primer ciclo de cultivo.

Palabras clave adicionales: biomasa microbiana, filtración lenta en lecho de arena.

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Abbreviations used: C (closed), CFU (colony-forming unit), CSSF (closed with slow sand filtration), O (open), SSF (slow sand filtration).

Introduction

Closed soilless growing systems offer several advantages over those that use soil. The use of the irrigation effluents in soilless growing systems may represent savings of up to 20% in water and fertilizers, in addition to avoiding subsoil contamination (Van Os *et al*., 2004). A disadvantage of these systems, however, is a higher risk of soil pathogens spreading through the recirculating nutrient solution. Certain methods of disinfection do not cause a microbial vacuum in the system (Postma *et al*., 1999). Furthermore, the surviving microflora collaborates with the disinfecting action in closed soilless growing systems with slow sand filtration (Van Os *et al*., 2004).

A number of techniques have been developed for the disinfection of recirculating nutrient solutions. These can be active, such as UV treatment (Runia, 1994a), heat treatment (Runia *et al*., 1997) and ozonification (Runia, 1994b), or passive, such as slow sand filtration (SSF) (Wohanka, 1995). In the latter, mechanical, chemical and biological properties may interact. Passive methods do not require (or require a lower) energy supply, and chemical compounds than active ones. The objective of SSF is not to remove resident microflora but to prevent the dispersion of phytopathogenic soilborne pests and diseases (Postma *et al*., 1999; Wohanka, 1995; Wohanka *et al.,* 1999). Thus, SSF can contribute to suppress certain diseases (Postma *et al*., 1999, Van Os *et al.,* 1999). The type of soilless growing system has a significant effect on the microbiologic characteristics around the root (Martínez *et al*., 2005). Closed soilless growing systems, with disinfection of the recirculating nutrient solution by SSF, exhibit a lower density of copiotrophic bacteria and fungi than open soilless growing systems (Martínez *et al*., 2005). Several studies have shown that SSF removes *Pythium* spp. and *Phytophthora* spp. propagules (Wohanka, 1995; Wohanka *et al.,* 1999). In this regard, other authors have reported that the SSF and UV treatments are able to significantly reduce root rots caused by *Phytophthora cryptogea* (Garibaldi *et al.,* 2003).

Verticillium wilt in strawberry *(Fragaria × ananassa* Duch.) is caused by the pathogen *Verticillium dahliae* (Thomas, 1932), which invades the plant's vascular system and prevents the transport of water and nutrients (Kiraly *et al*., 1970). This microorganism has been described as phytopathogen in strawberry plantations in Huelva (Spain) (Tello *et al*., 1996) and could be introduced into soilless growing systems by infected cold storage plants, irrigated water and air dispersion.

Soilborne pathogens can be very virulent under certain conditions. Nevertheless, there are reports of instances where little or no disease is expressed in hydroponic systems, despite the presence of major pathogens, such as *Pythium aphanidermatum* or *Phytophthora cryptogea* (McPherson, 1998; Postma *et al*., 2000a,b). McPherson (1998) found that the suppressive effect against *P. cryptogea* in closed soilless growing systems was not always present, that it took time to develop when it did occur, and that it was lost or reduced when the system was switched to run-to-waste (open soilless growing systems).

The main purpose of this study was determine the effect of three soilless growing systems (open, closed, and closed with slow sand filtration) on the spread of *V. dahliae* and the severity of the disease in strawberry plants. In addition, the effect of filtering in closed soilless growing systems with SSF was evaluated.

Material and methods

Experimental design

The assays were performed in Huelva (SW Spain) in a metacrilate tunnel-type greenhouse using a randomized complete block design with three replicates. The assay was repeated twice, and each covered a two-year period (two crop cycles) in the same growth medium. The plants were transplanted to the three growing systems at the beginning of each crop cycle.

The typical crop cycle in SW Spain begins in October and finishes in May. The soilless growing systems used were: open (O), closed (C) and closed with slow sand filtration (CSSF). Non-inoculated rows of plants were grown as controls for all the systems. The total number of rows was: 3 replicates × 3 systems × 2 rows (inoculated and non-inoculated). Each replicate consisted of one independent, continuous, 6 m-long crop row, with 65 hanging plants of the Camarosa variety. Plant density was 11 plants m^{-2}. The plants were watered with drip irrigation (1 drip/plant, flow of 2.3 L h^{-1}) and grown in "Hanging Bed-Pack" (Polygal Plastic Industries, Ramat Hashofet, Israel) troughs containing a coconut fiber growing medium (Bas Van Buuren B.V., Maasland, Netherlands). The CSSF system was constructed as described by Wohanka (1995) (Figure 1). The drained water was pumped into the sand filter to maintain a water layer of 35 cm, thereby obtaining a final flow rate of 100 to 300 L m^{-2} h^{-1}. The three replicates for the inoc-

ulated and non-inoculated crop rows of the three systems were fed separately. Each treatment had an independent storage tank for collecting drain water (drained solution) from the crop, a storage tank for collecting the effluents from the sand filter, and a storage tank for mixing the nutrient solutions. The content of the drained solution storage tank was pumped into the nutrient solution storage tank. The nutrient solution was then passed through the sand filter and stored in the filtered solution storage tank. The filtered solution was applied to the strawberry crop (Figure 1). Sand filter activation was done one month before transplanting.

The twelve plants at the end of each row were artificially inoculated before nutrient solution run-off. The drained solution was in contact with inoculated and non-inoculated plants in the same crop row. Inoculation and transplanting were performed simultaneously and only during year one. The artificially infected plants had the possibility to infect other plants via the nutrient solution and plant-to-plant contact. The *V. dahliae* strains were previously isolated from a naturally-infected strawberry plant grown in soil. The roots of the inoculated plants were dipped for 30 min in the inoculum suspension containing approximately 1×10^8 *V. dahliae* conidia per mL. This suspension was grown in potato dextrose agar plates at 25°C for seven days, after which conidia were removed by flooding the plates with sterile distilled water and scraping the mycelium with a sterile glass rod. Water containing the conidia was strained through four layers of cheesecloth to filter out mycelial fragments.

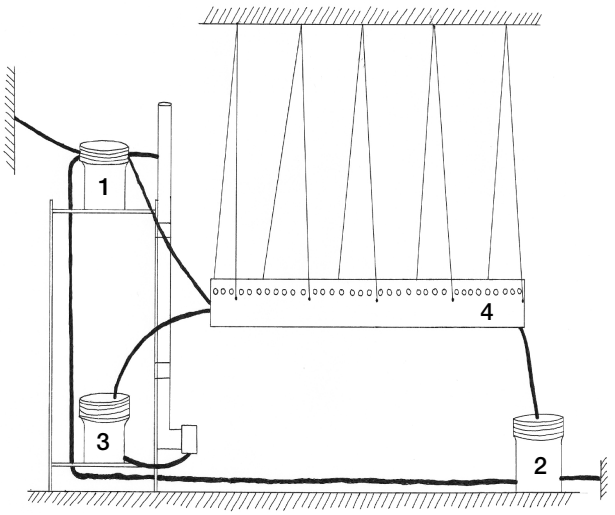

Figure 1. Diagram of closed soilless growing system with slow sand filtration (CSSF). 1: Nutrient solution storage tank (mixing of the nutrient solution and the drained solution from the strawberry crop before passing to filter). 2: Drained solution storage tank (storage tank of drainwater from the strawberry crop). 3: Filtered solution storage tank (storage tank of effluent from sand filter). 4: The last twelve plants of each row were artificially inoculated.

Dispersion

The concentration of propagules was determined by filtering the nutrient solution through cellulose membrane filters (2.5 µm pore diameter). Conidia (2.5–8 × 1.5–3 µm) and microsclerotia (15–100 µm diameter) can be detained by the cellulose membrane filters. The filter paper was transferred to a culture plate and covered with 2 mm of *Verticillium* selective medium (Dhingra and Sinclair, 1995). After three days, colonies were counted and confirmed for *V. dahliae* under a dissection microscope. Each estimate consisted of filtering ten 20-mL samples from the drained solution and filtered solution storage tanks of the CSSF system, and from the drained solution storage tank of the O and C systems (for each crop row of plants). *V. dahliae* propagules were evaluated every 15 days. Analyses were repeated on five sampling dates during the first crop cycle and on nine during the second crop cycle.

Results were expressed as colony-forming units per liter (CFU L^{-1}) of solution. For multifactorial ANOVA, the different sampling dates were considered as independent factors.

The density of *V. dahliae* propagules in growth medium samples was estimated each year at the end of the crop cycle by the wet-sieving technique (Ashworth *et al.,* 1972) and plate counts on semi-selective media. The medium used was modified soil extract agar based on that of Isaac *et al.* (1971), with modifications suggested by Huisman and Ashworth (1974). Six samples of 25 g were taken from each inoculated crop row (replicate) of the three systems.

Severity

In the artificially-inoculated crop rows, disease severity was measured at the end of the crop cycle in all the non-inoculated plants. The severity of Verticillium wilt in the crown was measured by a symptom scale, where 0 = plant with no symptoms, 1 = lightly colored vascular tissue, 2 = small brown speckles, and 3 = abundant necrotic speckles. Very few *V. dahliae* from the crown rot of affected plants at the end of the cycle could be detected by plating in a *Verticillium* selective medium; detection

by means of PCR was used to improve the sensitivity.

For PCR analysis, sections of the crown (8–18 mm long) were separated from the rest of the tissues, cut with a scalpel, washed with distilled water and soaked in bleach for 2 min. The crowns were frozen in Eppendorf tubes and stored at −80°C until use. The total genomic DNA was extracted using the commercially available EZNA Plant DNA Miniprep Kit (Omega Bio-Tek, Doraville, GA, USA). In the first PCR reaction, the specific primers were NMS1 and NMS2 (Li *et al*., 1994). Samples were processed as follows: 1 cycle (95°C for 2 min), 35 cycles (94°C for 1 min, 60°C for 1 min, 72°C for 1 min) and 1 cycle (72°C for 5 min). In the second PCR reaction, 2.5 µL of the product obtained in the previous reaction were used and the specific primers were VMSP1 and VMSP2 (Li *et al*., 1994). Samples were processed as follows: 1 cycle (95°C for 5 min) and 40 cycles (94°C for 1 min, 60°C for 1 min, 72°C for 2 min, and 72°C for 5 min).

Amplification was performed in a thermocycler and DNA was detected by electrophoresis in 2% agar gels.

Microbial biomass in drained solution storage tank and filtered solution storage tank from the CSSF system

Three samples from the drained solution and filtered solution storage tanks of the CSSF system, respectively, were taken from each inoculated crop row (replicate).

Two and a half months after transplanting, microbial biomass was measured with the acridine orange direct count technique. Each sample was repeated twice. The volume of each analyzed sample was 1.8 mL. For this purpose, cells from the recirculating nutrient solution were counted using an epifluorescence microscope (Leica Microsystems D-35578; Wetzlar, Germany) (Kepner and Pratt, 1994). The recirculating nutrient solution sample was filtered through polycarbonate membranes with a pore size of 0.2 µm (Isopore, Millipore Iberica S.A, Madrid, Spain) (Kepner and Pratt, 1994). Counting was done with a magnification of 1.250× and a minimum of 400 cells/filter. The control samples were made with sterile water.

Data analysis

Data from two assays for each crop cycle were pooled for final analysis after finding no significant system × assay interaction in preliminary analysis of variance. Data were analyzed with Statgraphics Plus (version Plus 5.1; Statistical Graphics Corp., Rockville, MD). The variables analyzed by multifactorial ANOVA were: *V. dahliae* propagule density in the drained solution storage tank and the filtered solution storage tank, *V. dahliae* microsclerotia in growth medium, severity of Verticillium wilt in the three systems, and microbial biomass of the drained solution and filtered solution storage tanks of the CSSF system. Significant means were compared by Tukey's honestly significant difference test ($P < 0.05$).

Results

The density of *V. dahliae* propagules in the drained solution storage tank of the three soilless growing systems revealed no effect of the system used in either the first or second crop cycles (Table 1). The density of *V. dahliae* propagules in the drained solution storage tank and the filtered solution storage tank of the CSSF system did not differ significantly (Table 2).

No significant differences were observed in the density of *V. dahliae* microsclerotia in the growth medium of the three systems during the first crop cycle. Nevertheless, the density of microsclerotia at the end of the second crop cycle was lower in the C system than in the O and CSSF systems (Table 3). With regard to the severity of Verticillium wilt, significant differences were detected at the end of the first crop cycle between CSSF and the other two systems (Table 4). In the second crop cycle, however, no significant differences in severity were observed between the systems (Table 4). Symptoms of Verticillium wilt were not detected in the non-inoculated rows of plants of the three systems at any time in the crop cycle.

Table 1. *Verticillium dahliae* propagule density[1] (CFU L^{-1}) in drained solution storage tank of three soilless growing systems during each crop cycle

	First crop cycle[2]	Second crop cycle[2]
Closed	45	101
Open	15	22
Closed with SSF[3]	25	34

[1] Average of the five sample dates for first crop cycle and nine for second crop cycle. [2] Not significant; analysis of variance was performed with transformed data: Ln (CFU mL^{-1} + 0.1). [3] SSF: slow sand filtration.

Table 2. *Verticillium dahliae* propagule density[1] (CFU L^{-1}) in drained solution storage tank and filtered solution storage tank of closed soilless growing system with slow sand filtration (CSSF) during each crop cycle

	First crop cycle[2]	Second crop cycle[2]
Drained solution storage tank	25	34
Filtered solution storage tank	12	8

[1] Average of the five sample dates for first crop cycle and nine for second crop cycle. [2] Not significant, analysis of variance was performed with transformed data: Ln (CFU mL^{-1} + 0.1).

There were significant differences in the microbial biomass of the drained solution and the filtered solution storage tanks in both crop cycles of the CSSF system (Table 5). Overall, microbial biomass was higher in the second crop cycle than in the first crop cycle, and the microbial biomass of the filtered solution in second crop cycle was higher than that of the drained solution in the first crop cycle.

Discussion

V. dahliae propagules were not removed by SSF. To our knowledge, no study has reported the elimination of propagules of this pathogen by this method of filtration. Other studies have shown that oomycete propagules (*Pythium* spp. and *Phytophthora* spp.) are eliminated by SSF (Wohanka, 1995; Runia *et al.,* 1997; Van Os *et al.,* 1999; Wohanka *et al.,* 1999) and that this filtering technique shows high efficacy against other phytopathogenic fungi and bacteria (Waechter-Kristensen *et al.,* 1997). Slow filtration is a living system in which a number of factors (pathogen, filtration rate, grain size, filter medium, specific surface, temperature, microbial life, and preferential channeling) determine efficacy. The SSF did not affect the concentration of *V. dahliae* propagules in the drained solution storage tanks of the systems tested, as expected. This finding is consistent with the observation that SSF did not remove *V. dahliae* propagules.

With regard to the density of *V. dahliae* microsclerotia in the growth medium, no significant differences were observed in any of the three growing systems during the first crop cycle. However, the decline in the density of microsclerotia in the C system during the second crop cycle could be related with the high accumulation of plant exudates and plant decomposition residues–and the associated microbial biomass and activity–in comparison with the other two systems.

Table 3. Number of *Verticillium dahliae* microsclerotia per gram dry weight growth medium measured in three soilless growing systems at the end of each crop cycle

Growth system	First crop cycle[1]	Second crop cycle[1]
Closed	0.313	0.033 b
Open	0.169	0.158 a
Closed with SSF	0.142	0.284 a

[1] Analysis of variance was performed with transformed data = = Ln[(nº microsclerotia g^{-1} dry weight growth medium) + $(2n)^{-1}$]. Values followed by different letters within each column are significantly different based on Tukey's test at $P \leq 0.05$

Table 4. Verticillium wilt severity[1] in the crown rot of strawberry plants in three soilless growing systems at the end of each crop cycle in all non-inoculated plants of artificially-inoculated crop rows

	First crop cycle[2]	Second crop cycle[2]
Closed	0.8 b	0.6
Open	0.8 b	0.5
Closed with SSF	1.3 a	0.4

[1] Disease severity was measured in the crown rot of non-inoculated plants. Verticillium wilt severity in plant with infection was confirmed by selective medium isolation and/or PCR and was scored with the following symptom severity scale: 0 = plant with no symptoms, 1 = lightly colored vascular tissue, 2 = small brown speckles, and 3 = abundant necrotic speckles. [2] Values followed by different letters within each column are significantly different based on Tukey's test at $P \leq 0.05$.

Table 5. Microbial biomass determined by acridine orange direct count technique in drained solution storage tank and filtered solution storage tank of closed soilless growing system with slow sand filtration (CSFF) during each crop cycle

Filtering treatment	Number of cells mL^{-1} solution	
	First crop cycle[1]	Second crop cycle[1]
Drained solution storage tank	0.5×10^5 a	1.8×10^5 a
Filtered solution storage tank	0.3×10^5 b	0.7×10^5 b

[1] Analysis of variance was performed with transformed data: (number of cells $mL^{-1})^{0.4}$. Values followed by different letters within each column are significantly different based on Tukey's test at $P \leq 0.05$.

In the first crop cycle, an increase in the severity of Verticillium wilt in the CSSF system with regard to the other two systems was detected. This increase may be due to the non-elimination of *V. dahliae* propagules by filtration and the lower microbial biomass in the filtered solution storage tank than in the drained solution storage tank. The decline in microbial biomass by filtration may improve conidial viability, thus increasing the severity of the disease. For other soilborne pathogens, the activity of the surrounding microorganisms can increase endogenous carbon loss from propagules, thereby reducing pathogen viability (Arora, 1998). A direct relationship has been reported between the loss of endogenous carbon and a decrease in germinability and virulence in chlamydospores of *Fusarium solani* f.sp. *phaseoli* and in oospores of *P. aphanidermatum* (Mondal *et al.,* 1995; Mondal *et al.,* 1996). In addition, the same relationship with regard to the viability of the sclerotia of *Sclorotium rolfsii* has been described (Hyakumachi and Lockwood, 1989).

In the second crop cycle, the severity of Verticillium wilt in the three systems was similar. Despite the reduction in microbial biomass due to filtration, the biomass in the filtered solution storage tank remains very high. The roots debris from the first crop cycle may have provided nutrient sources to the microbes, thus increasing the microbial biomass (Table 5).

Results show that growing strawberries in a closed system does not imply a threat of Verticillium wilt for the crop, because no differences were observed in the severity and density of microsclerotia between C and O systems.

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