OCCURRENCE AND PATHOGENICITY OF *PYTHIUM* SPP. IN THE DUST DEPOSITED ON THE GREENHOUSE ROOFS IN THE PONIENTE REGION OF ALMERIA (SOUTH-EAST SPAIN)

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SUMMARY

Dust accumulated on greenhouse roofs can be an important source of inoculum of *Pythium* spp. for the crops developing within. During the period March-May 1998, 20 greenhouses in the horticultural Poniente region of Almería (South-East Spain) were sampled monthly. *Pythium* isolates were cultured from the greenhouse dust. The density of the *Pythium* population was measured in the dust from 5 of these greenhouses, and the pathogenicity of 7 isolates was determined. Four species were identified and described: *Pythium aphanidermatum*, *P. catenulatum*, *P. mamillatum* and *P. spinosum*. This is the first report of *P. spinosum* for Spain. The mean total *Pythium* population densities ranged from 60 to 3450 propagules g^{-1} (d. wt) of greenhouse dust. All the isolates tested were pathogenic. The most pathogenic were the *P. aphanidermatum* isolates.

Key words: Pythium, pathogenicity, greenhouse, Alméria, dust.

INTRODUCTION

The species of the genus *Pythium* Pringsh. are major causative agents of damping-off diseases in nurseries throughout the world (Roberts and Boothroyd, 1972). *Pythium* spp. also produces root diseases in adult plants (Hendrix and Campbell, 1973). Almería province is an important European centre for the production of market garden crops (tomato, pepper, cucumber, melon, watermelon, etc.). With 15,000 ha of greenhouses under plastic cover (Garijo, 1994), Almería province represents 50% of the total greenhouse area in Spain. The Poniente region of Almería contains the majority of these greenhouses.

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The importance of these pathogenic *Pythium* species in greenhouse crops in this region of Almería has been highlighted (Cuadrado and Gómez, 1984). However, the capacity of the greenhouse covers to act as a reservoir for these pathogenic species of *Pythium* is unknown, as is the capacity of the dust to act as an inoculum source for these species.

Although various studies have measured the *Pythium* population densities in the irrigation water in Almería province (Sánchez and Gallego, 1996; Sánchez, 1998), little is known even now in terms of the dust deposited over the greenhouses (Gómez, 1993a) which, by means of the wind and rain, can penetrate into the interior of the greenhouses.

In the current article we describe the species that we have identified. Estimates of the population of *Pythium* spp. occurring in the dust on the plastic roofing material of these greenhouses are also given. Lastly, we present the results of a pathogenicity test, undertaken using cucumber seeds. It was designed to determine the pre-emergence damping-off mortality caused by the isolates.

MATERIALS AND METHODS

The study area was the Poniente region of Almería province (SE Spain). This is a coastal fringe about 10 km wide and 30 km long, located between 36°41'-36°48' N and 2°33'- 2°54' W. Absolute minimum temperatures lie above 0°C and the coolest months are January and February. The absolute maximum temperatures recorded exceed 30°C, during June to October. Rainfall is scarce, with an annual mean of less than 250 mm (López, 1993).

Twenty greenhouses were sampled, located along a north-south transect (Almerimar highway) through the middle of this region. A sample of the dust was collected from the plastic roofing of each greenhouse, on three occasions during the period March-May 1998.

The dust samples were desiccated at laboratory temperature over three days. They were ground and sieved to 0.2 mm. To obtain various isolates of *Pythium* spp.

immature carnation petals (*Dianthus caryophyllus* L.) were used, which were introduced during 2-5 days into various suspensions of the dust in sterilized distilled water (SDW) (Ponchet *et al.*, 1972; Ricci, 1974). Then the baits were placed on Ponchet's P medium (Ponchet *et al.*, 1972), though neither the antibiotics, nor the fungicides were autoclaved, and 5 ppm Rose Bengal was added to the medium (Sánchez, 1998). The suspensions were incubated for two days in darkness at 20ºC.

Pure cultures were made from selected isolates on potato-carrot agar (PCA) medium (Langeron, 1945) and maintained on PCA in darkness at 20ºC.

To encourage the formation of the different structures of *Pythium* sp., pieces of the pure culture were placed in Petri dishes with SDW, baited with hempseed cotyledons (*Cannabis sativa* L.) (Paul, 1986). Production of sporangia was stimulated by subjecting the SDW Petri dishes to different temperatures of between 5 and 30ºC (Plaats-Niterink, 1981). Production of oogonia was stimulated by adding 5 ppm β-sitosterol in SDW to dual cultures on PCA.

Fungal structures on the colonies grown on the hemp cotyledon baits were observed with a light microscope (Olympus BH-2). Observations were made during the first 48 hours, after one week, two weeks, and over the following months (Sánchez, 1998).

Colony growth was measured on PCA in darkness at 25ºC. Colony patterns were described on PCA at 25ºC. They may consist of acute triangles (chrysanthemum pattern), obtuse lobes (rosette pattern) or may simply radiate with all possible intermediates (Plaats-Niterink, 1981).

Identification of the isolates was carried out with the aid of keys and descriptions by different authors (Middleton, 1943; Frezzi, 1956; Waterhouse, 1967; Plaats-Niterink, 1981; Dick, 1990).

In addition, on the first sampling run, the *Pythium* population densities (propagules g^{-1}) were estimated using McCrady's technique using baits of immature carnation petals (Ricci, 1974). This was done by taking a subsample of dust (10 g) and making serial dilutions (10^{-1} a 10^{-6}) in SDW. 15 ml of each dilution was placed in each of 5 Petri dishes. An immature carnation petal was placed in each dish. Finally, the number of plates with baits colonized by *Pythium* during the first 5 days was counted. Based on these data, and with the aid of the McCrady table, an estimate of the population was made.

A pathogenicity test was also undertaken to estimate pre-emergence damping-off mortality caused by the isolates. A disk of potato-dextrose agar (PDA) 9 cm in diam. with a *Pythium* sp. colony developed up to the edge of the disk was placed into a 10 cm diam. pot containing 4 cm depth of vermiculite; a 2 cm diam. central disc was removed from the Petri dish (Fig. 1A). Then 10 cucumber seeds (*Cucumis sativus*) cv. 'Ashley' were sown in a circle (Fig. 1B) and covered with 1 cm of vermiculite (Fig. 1C). These were watered to 70-90% humidity, and placed at 25ºC, 60% relative humidity, and 1000 lux illumination for 12 hours/day (Bouhot, 1975; Messiaen *et al.*, 1977; Sánchez, 1998). Three replicates were made per isolate, on two different occasions. A count was made of the number of seedlings emerging during the first 20 days after sowing. The number of seedlings which did not germinate was calculated and expressed as % mortality.

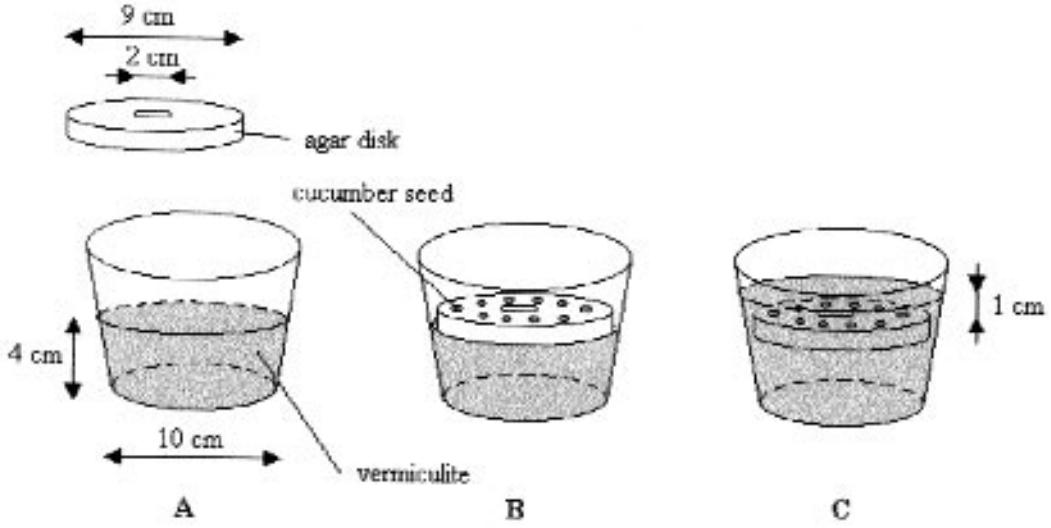

Fig. 1. Pathogenicity test method.

RESULTS

Species encountered. Four species were encountered in the isolates obtained from dust collected from greenhouse covers in the Poniente region of Almería (SE Spain). These are described below, using an isolate of each species. First reports for Spain are noted.

P. aphanidermatum *(Edson) Fitzp. (Figs 3A-G and 5B-C).* Isolate described: I8B, obtained from greenhouse no. 8. Other isolates of the same species: I8A, I8C, I8D, I8E, I8F, I8G and I8H. *Colony* on PCA with radiate pattern, cottony mycelium. Radial growth rate 32 mm day^{-1} on PCA at 25ºC. *Hyphae* 2-8 µm thick. *Appressoria* club-shaped or in the form of bagpipes. *Sporangia* inflated (Figs 3E-G and 5C), with a digitate aspect and tangled nature, 4-20 µm thick, with varying length, more frequently 25-120 µm in size. *Oogonia* smooth-walled (Figs 3A-D and 5B), (18-)22(-26) µm. *Oospores* aplerotic (Figs 3A-D and 5B), 16-20 µm diam, wall thickness 2-6 µm. *Antheridia* 1(-2) (Figs 3A-D and 5B) per oogonium, terminal or intercalary, 8-10 x 11-15 in size.

P. catenulatum *Matthews (Figs 3H-O and 4B, D).* Isolate described: M3A, from greenhouse no. 3. Also isolate M1E. *Colony* on PCA with a radiate pattern, completely submerged mycelium. Radial growth rate 15 mm day^{-1} on PCA at 25ºC. *Hyphal swellings* globose (Figs 3N-O and 4B), 9-15 µm diam. in extensive chains of variable length. *Sporangia* (Fig. 3 L-M) filamentous slightly inflated. *Oogonia* (Figs 3H-K and 4D) terminal or intercalary, smooth-walled, (20-)25(-27) µm diam. *Oospores* (Figs 3H-K and 4D) plerotic, 18-26.5 µm diam., wall thickness 1.5-2 µm. *Antheridia* (Figs 3H-K and 4D) monoclinous and diclinous, 1-3 per oogonium, 3-5 x 9.5-12 µm in size.

P. mamillatum *Meurs (Figs 2K-P and 5A).* Isolate described: C2.2, obtained from greenhouse no. 2. *Colony* lost, not described. *Sporangia* globose (Fig. 2O), 14-21(27) µm diam., terminal or intercalary. *Oogonia* (Figs. 2 K-N and Fig. 5A) mamillate ornamented-walled, 2-6 projections (1-7 µm long) around the perimeter, 16-21 µm diam. (excluding the ornament), terminal or intercalary. *Oospores* (Figs 2K-N and 5A) 12-18 µm diam., wall thickness 0.8-1.6 µm. *Antheridia* (Figs 2K-N and 5A) monoclinous, with 1-2(3) per oogonium, 4-10 x 9-14 µm in size.

P. spinosum *Sawada (Figs 2A-J and 4A, C).* Isolate described R2A, from greenhouse no. 2. Other isolates of the same species: R2B. *Colony* on PCA with a rosette pattern, aerial mycelium. Radial growth rate 7 mm day^{-1} on PCA at 25ºC. *Hyphae* 5-10 µm thick. *Appressoria* chained (Fig. 2I-J). *Sporangia* globose (Figs 2G-H and 4A). *Oogonia* (Figs 2A-F and 4A, C) (25-)31(-35) µm diam., with digitate ornamentations, 6-10 µm long and 0.3-0.5 µm wide at the base. *Oospores* aplerotic (Figs 2B-C, E-F and 4A), 25-30 µm diam., wall thickness 1.5-2 µm. *Antheridia* (Figs 2A-C, E-F and 4C), 1-3 per oogonium, 8-14 µm wide and 15-20 µm long, monoclinous or sometimes diclinous, characteristically borne on hyphae that are wrinkled or septate. This is the first report of this species in Spain.

Population density of *Pythium* spp. Table 1 shows the estimate using McCrady's technique of the *Pythium* population densities contained in the dust on the roofs of various greenhouses sampled during March 1998. The mean total *Pythium* population densities ranged from 60 to 3450 propagules g^{-1} (d. wt) of greenhouse dust. This shows that the presence of *Pythium* is highly variable on this substrate, although in all the greenhouses surveyed some propagule of the *Pythium* species was found.

Table 1. Population density of *Pythium* in the dust found over greenhouse roofs.

Propagule density	Sampled greenhouse				
g^{-1} (d. wt)	no. 1	no. 6	no. 9	no. 13	no. 20
mean	3450	60	390	140	270
CI (95%)	1170-9990	5-150	130-1060	40-340	90-800

Pathogenicity of *Pythium* isolates. The results of the pathogenicity test (Table 2) demonstrate that the isolates obtained from the dust on the greenhouse roofs are fairly pathogenic, in all cases differing from the test control and resulting in values of more than 85%. The most pathogenic isolates (I8A to I8H), affecting 100% of the seeds, belong to *P. aphanidermatum*.

The tests were carried out under optimum conditions for the pathogens: the temperature was adequate for the isolates being tested (Table 2), the amount of inoculum was more than sufficient and the host plant was susceptible. It is therefore assumed that such high pre-emergence mortality would not always be achieved under field conditions.

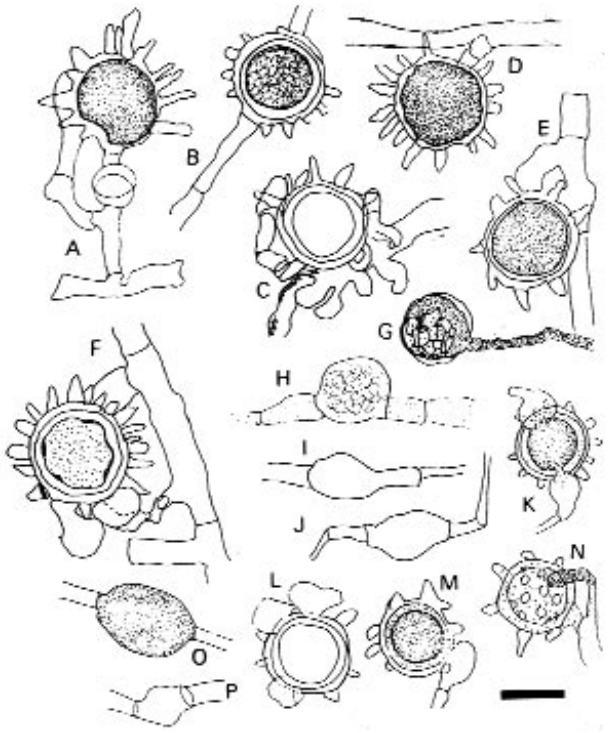

Fig. 2. *P. spinosum*. **A**-**F**: oogonia and antheridia; **G**-**H**: sporangia, **I**-**J**: appressoria. *P. mamillatum*. **K**-**N**: oogonia and antheridia; **O**: globose sporangium; **P**: appressorium. (bar = 20 mm).

Table 2. Pre-emergence damping-off (% mortality[1]) caused by *Pythium* isolates on cucumber seedlings (*Cucumis sativus* cv. 'Ashley') at 25ºC.

Isolate	SOGT (ºC)[2]	Species	Mean[1]	2·SD[1]	[3]
C1		control	3.33	0.47	a
C2		control	5.00	0.05	a
R2B	25ºC	*P. spinosum*	85.00	0.13	b
R2A	25ºC	*P. spinosum*	86.67	0.09	b
M3A	30-35ºC	*P. catenulatum*	88.33	0.11	b
M1E	30-35ºC	*P. catenulatum*	93.33	0.07	bc
I8A	35-40ºC	*P. aphanidermatum*	100.00	0.00	c
I8B	35-40ºC	*P. aphanidermatum*	100.00	0.00	c
I8C	35-40ºC	*P. aphanidermatum*	100.00	0.00	c
I8D	35-40ºC	*P. aphanidermatum*	100.00	0.00	c
I8E	35-40ºC	*P. aphanidermatum*	100.00	0.00	c
I8F	35-40ºC	*P. aphanidermatum*	100.00	0.00	c
I8G	35-40ºC	*P. aphanidermatum*	100.00	0.00	c

[1] % mortality: 100 (1 – seedlings emerging/seeds sown).

[2] SOGT: species optimum growing temperature (Plaats-Niterink, 1981).

[3] Letters denote a significant difference at 95% level (test LSD, $P \leq 0.05$) calculated for the trigonometric transformation (arcsin $\sqrt{\%}$).

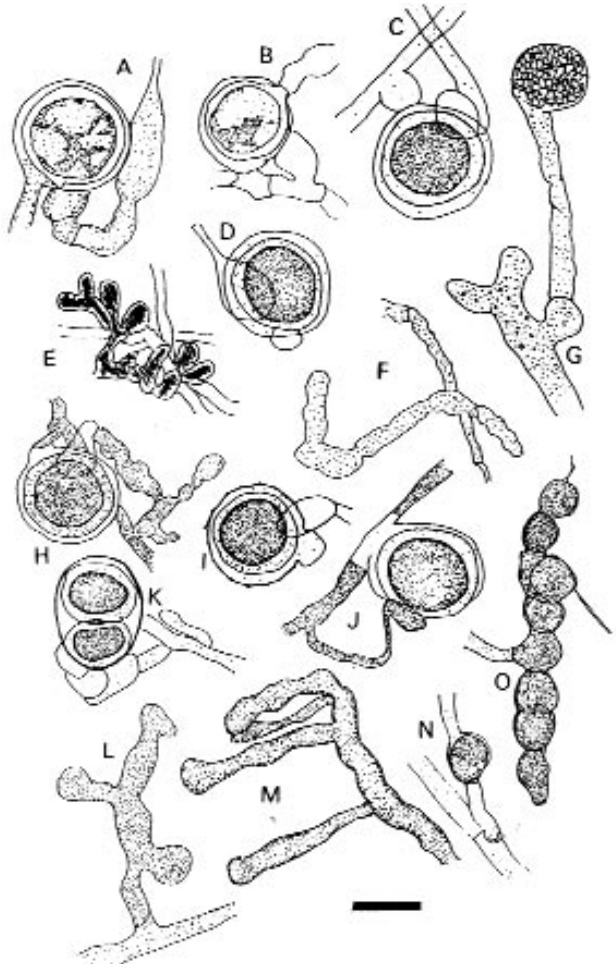

Fig. 3. *P. aphanidermatum*. **A**-**D**: oogonia and antheridia; **E**-**G**: inflated sporangia. *P. catenulatum*. **H**-**K**: oogonia and antheridia; **L**, **M**: inflated sporangia; **N**, **O**: hyphal swellings. (**A**-**D**, **F**-**O**: bar = 16 mm, **E**: bar = 40 mm).

DISCUSSION

Firstly, the *Pythium* species described are not considered unusual, if we take into account that *P. aphanidermatum* and *P. catenulatum* have been previously cited in the same region (Gómez, 1993b; Sánchez, 1998), that *P. mamillatum* has also been cited in another region in Extremadura (South-West of Spain) (Rodríguez, 1996) and that *P. spinosum* is a fairly frequent species on a world-wide scale (Plaats-Niterink, 1981).

P. aphanidermatum has been cited in hydroponic crops in Almería (Gómez, 1993b) and *P. catenulatum* has been reported in the water of reservoirs within the region (Sánchez, 1998). Therefore its detection in the dust, in addition to predicting its possible presence in

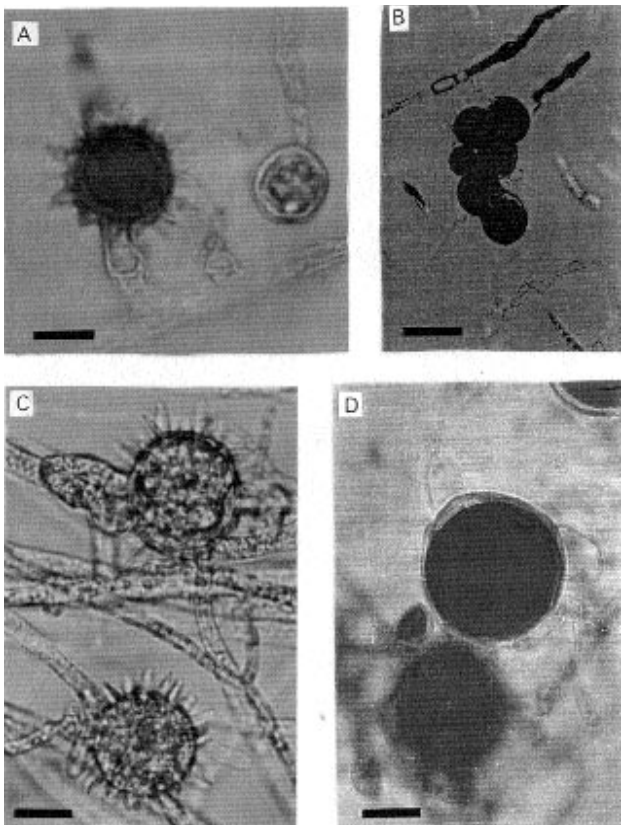

Fig. 4. *P. spinosum*. **A**, **C**: oogonia and antheridia. *P. catenulatum*. **B**: hyphal swellings; **D**: oogonium and antheridium. (**A**-**C**: bar = 20 mm, **D**: bar = 8 mm).

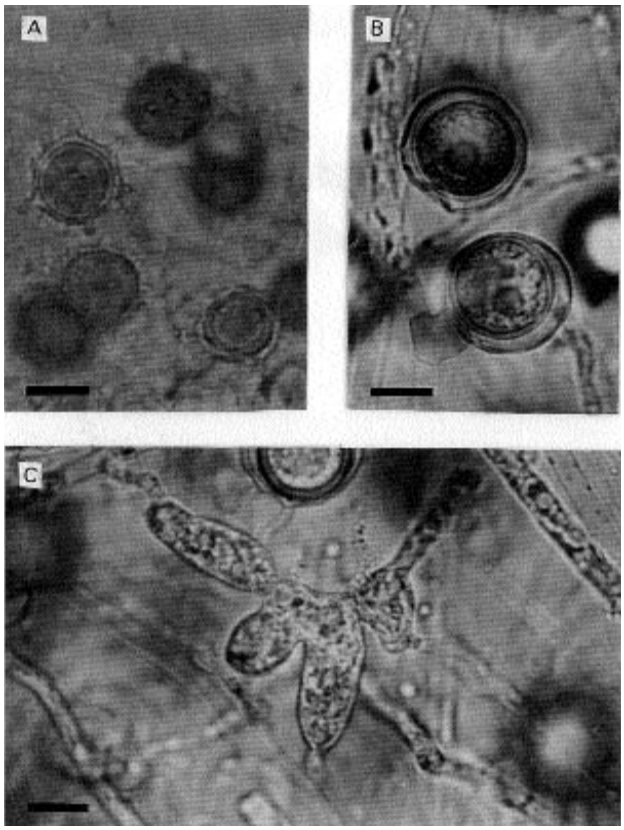

Fig. 5. *P. mamillatum*. **A**: oogonia and antheridia. *P. aphanidermatum*. **B**: oogonia and antheridia; **C**: inflated sporangium. (bar = 20 mm).

the soils of the region, indicates the more than probable soilborne origin of the propagules of these two species detected in aquatic environments, an environment that has already been listed for *P. catenulatum* (Sánchez, 1998).

The presence of *P. spinosum* as a new report for Spain is probably due to the relative lack of knowledge about the *Pythium* flora from Spanish soils.

In consequence, we recognise the need to undertake a study of the *Pythium* flora in natural cultivated soils in the region in order to understand the distribution of the species as a whole, as well as to elucidate the relative epidemiological importance of the different inoculum sources.

Secondly, the common occurrence of *Pythium* spp. propagules observed in the dust deposited over the plastic covers of the greenhouses of the Poniente region of Almería accords with the cosmopolitan distribution described for this genus (Plaats-Niterink, 1981) and corroborates the limited observations made previously within the region (Gómez, 1993a).

Lastly, the high mortality observed in the pathogenicity tests undoubtedly highlights the likely pathological importance of the dispersion of *Pythium* propagules by means of dust in Almería province (Aparicio *et al.*, 1995), an area of frequent winds, semi-arid climate and a desert landscape (Capel, 1990).

In addition, rainwater will transport these dust particles into the interior of greenhouses directly, either as a result of the frequent leaks that occur in the flexible plastic sheeting of the flat roofs of these greenhouses (Vasco, 1999), or indirectly via the irrigation of the plot using rainwater collected in irrigation ponds (Garzón *et al.*, 1999).

Thus, these factors must be taken into account in undertaking tests of the diseases of greenhouse crops, and above all in seedbeds. The following recommendations are examples of practices to reduce the incidence of disease in this sense: (*i*) design of greenhouses to adequately protect against the ingress of *Pythium* propagules transported by the wind (in atmospheric dust). Currently, greenhouses in Almería tend to have open

sides in the direction of the dominant winds, in order to achieve a reduction in the relative humidity within the greenhouse (Cuadrado *et al.*, 2000). The opening or closing of side strips can influence the development of various diseases such as *Botrytis* (Bravenboer and Strijbosch, 1975) or *Sclerotinia* (Cuadrado *et al.*, 2000); (*ii*) control of the irrigation water derived from washings from the greenhouse roofs. Various methods have been devised to overcome the problem of waters infested with *Pythium*. These include: (*i*) physical and chemical control: application of fungicides (nabam), addition of nonylphenol polyethoxylate wetting agents (Messiaen *et al.*, 1994), ozonization of the water (Runia, 1994), UV irradiation (Stanghellini *et al.*, 1984), or water filtration (Goldberg *et al.*, 1992); (*ii*) cultivation control: use of floating collector tubes to avoid the draw-off of sediments collected at the bottom of the reservoirs: (Shokes and McCarter, 1979), covering the reservoirs to stop dust and plant remains from falling into them (Aparicio *et al.*, 1995); (*iii*) biological control: use of bacteria such as *Pseudomonas fluorescens* (McCullagh *et al.*, 1996) or antagonistic fungi such as *S. griseofulvis* (Postma *et al.*, 1996), *Pythium oligandrum* (Thinggaard *et al.*, 1988) or *Gliocadium virens* (Lumsden *et al.*, 1996).

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