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Resistance of several strawberry cultivars against three different pathogens

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Abstract

In addition to the agronomical characteristics of strawberries (*Fragaria × ananassa* Duch.), strawberry breeding programs should take into account its resistance to pathogens. The main goal of this study was to evaluate the resistance of strawberry cultivars against crown and root rot caused by *Phytophthora cactorum* (Lebert & Cohn) J. Schröt., Verticillium wilt caused by *Verticillium dahliae* Kleb. and *V. albo-atrum* Reinke et Berth., and angular leaf spot caused by *Xanthomonas fragariae* Kennedy and King, all of which are important diseases in strawberry plant nurseries in Spain. Ten strawberry cultivars were used for resistance testing against *P. cactorum*; nine cultivars were used for resistance testing against *V. dahliae* and *V. albo-atrum*; and eight strawberry cultivars were used for resistance testing against two strains of *X. fragariae*, IVIA349.9a and NCPPB 1469. These assays were conducted under greenhouse conditions for *P. cactorum*, and under growth chamber conditions for *V. dahliae* and *V. albo-atrum* and *X. fragariae*. 'Sabrosa', was classified as being resistant to *P. cactorum* and *V. dahliae* and *V. albo-atrum* and only 'Sieger' was classified as being resistant to both strains of *X. fragariae*. These results emphasize the importance of screening for disease resistance before using strawberry cultivars in commercial strawberry production in Spain.

Additional key words: angular leaf spot; crown rot; disease resistance; soil-borne pathogens; Verticillium wilt.

Resumen

Resistencia de varios cultivares de fresa frente a tres patógenos diferentes

Los programas de mejora de la fresa (*Fragaria × ananassa* Duch.) deberían tener en cuenta, además de las características agronómicas de la fresa, su resistencia a patógenos. El objetivo principal de este estudio fue evaluar la resistencia de varios cultivares de fresa contra la podredumbre de corona y radicular causada por *Phytophthora cactorum* (Lebert and Cohn) J. Schröt., la Verticilosis causada por *Verticillium dahliae* Kleb. y *V. albo-atrum* Reinke et Berth., y la mancha angular causada por *Xanthomonas fragariae* Kennedy and King, todas las cuales son importantes enfermedades en los viveros de fresa en España. Diez cultivares de fresa, se utilizaron para evaluar la resistencia frente a *P. cactorum*; nueve cultivares se utilizaron para evaluar la resistencia frente a *V. dahliae* y *V. albo-atrum* y ocho cultivares fueron utilizados para probar la resistencia frente a dos cepas de *X. fragariae*, IVIA349.9a y NCPPB 1469. Estos ensayos se realizaron en condiciones de invernadero en el caso de *P. cactorum*, y en condiciones de cámara de crecimiento en los casos de *V. dahliae* y *V. albo-atrum* y *X. fragariae.* 'Sabrosa' fue clasificada como resistente a *P. cactorum* y *V. dahliae* y *V. albo-atrum,* mientras que sólo 'Sieger' se clasificó como resistente a ambas cepas de *X. fragariae*. Estos resultados señalan la importancia de evaluar la resistencia de los cultivares de fresa a las diferentes enfermedades presentes en España antes de utilizarlos en la producción comercial de fresas en España.

Palabras clave adicionales: mancha angular; patógenos de suelo; podredumbre de corona; resistencia a enfermedades; Verticilosis.

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Introduction

Strawberry is an important crop in Spain. In fact, Spain is the main European producer of fresh strawberries, whose production was 274,600 tonnes in 2010 (FAO, 2011; MARM, 2011). Californian and Floridian commercial nurseries in the USA supply more than 50% of the mother plants that are used in Spanish nurseries (Medina-Mínguez, 2011). Each year, strawberry runners for transplanting are produced in nurseries in Castilla-León (north-central Spain), from where they are transported to the fruit production fields in other regions of Spain and to those in other European countries. The cultivation of strawberry plants (runners) is annual, with planting between April and May and the digging of fresh commercial strawberry runners throughout October (De Cal *et al.,* 2004). In Spanish strawberry nurseries, pre-plant soil fumigation with mixtures of methyl bromide and chloropicrin was commonly used to the control of soil-borne diseases (López-Aranda *et al.*, 2009). Since use of methyl bromide in Europe is phase-out (MBTOC, 2007), Spanish strawberry growers are under considerable pressure to seek an alternative method of efficient disease control of their strawberry crops. One potential solution to this phase-out is to develop disease-resistant strawberry cultivars.

In Spanish strawberry breeding programs, agronomical characteristics are usually considered but resistance to main pathogens is only considered when strawberry cultivars have been previously selected by its agronomical characteristics. Furthermore the selection of new cultivars has been made in the last decades in soils where methyl bromide was largely used as soil disinfectant method, and then soils are free of the main soilborne pathogens (Duhart *et al.,* 2000). Therefore nowadays the Spanish strawberry breeding industry should consider the development of resistant cultivars to main pathogens while considering the agronomical characteristics of cultivars.

The oomycete *P. cactorum* is the causative microorganism for crown and root rot in cultivated strawberries, and the causative agent of leather rot of fruit in wild (*Fragaria vesca* L.) and commercial (*F.* × *ananassa*) strawberries (Mass, 1998). During the last few years, this soil-borne pathogen has become widespread, especially in Europe (Eikemo *et al.*, 2000; Duncan, 2002), and has become a limiting factor for successful strawberry production. Three reasons have been submitted to explain the increased incidence of this disease: (i) the extensive use of a few highly susceptible strawberry genotypes in most strawberry-producing areas (Harris *et al.*, 1997; Stensvand *et al.*, 1999), (ii) the introduction of the pathogen into pathogen-free strawberry nurseries by asymptomatic infected plants, and (iii) the limited effect of new soil fumigants, other than methyl bromide, against this pathogen (De Cal *et al.*, 2004; López-Aranda *et al.*, 2009). Crown rot resistance in strawberries is now well-documented, and several screening methods to identify such cultivars have been developed (van Rijbroek *et al.*, 1997; Redondo *et al.*, 2009).

The two fungi *V. dahliae* and *V. albo-atrum* are also important strawberry pathogens in Spain. Verticillium wilt is difficult to completely control even with soil fumigation (Martín & Bull, 2002), and repeated soil fumigations may be necessary to control the pathogen. Since their microsclerotia can survive in the soil for many years, crop rotation is less effective for controlling Verticillium wilt than other control methods, such as soil fumigation. Cross-infection does occur and the existence of pathotypes of *V. dahliae* and *V. albo-atrum* with different levels of pathogenicity has been described (Horiuchi *et al.*, 1990). It is also known that quantitative resistance to *Verticillium* spp. is now present in some strawberry cultivars, and that this resistance originated from wild, non-cultivated strawberry species, such as *F. chiloensis* L., *F. virginiana* Duch., and *F. ovalis* (Lehm.) Rydb. (Ebihara *et al.*, 2010).

Angular leaf spot incited by *X. fragariae* is another important disease in strawberry nursery production. This disease affects only *Fragaria* spp., and is most commonly transmitted by planting infected stock (Maas *et al.*, 1995). No effective chemical control is available, and the disease can only be managed by avoiding the introduction of contaminated planting material into nurseries (Maas *et al.*, 1995). The European and Mediterranean Plant Protection Organization (EPPO) have listed *X. fragariae* as an A2 quarantine pathogen (EPPO, 2010). Therefore, nurseries that wish to export strawberry plants to European countries must maintain high phytosanitary standards in order to exclude *X. fragariae*. Resistance to *X. fragariae* has been reported in some *Fragariae* spp. (Lewers *et al.*, 2003), and strawberry cultivars reportedly vary in their susceptibility to angular leaf spot (Maas *et al.*, 2000).

The main goal of this study was to evaluate the resistance of strawberry cultivars against crown and root rot caused by *P. cactorum*, Verticillium wilt caused by *V. dahliae* and *V. albo-atrum* and angular leaf spot

caused by *X. fragariae*, all of which are important diseases in strawberry plant nurseries in Spain.

Material and methods

Plant material and plant micropropagation

The strawberry plants used in this study were obtained from the Fragaria germplasm collection of the Instituto Andaluz de Investigación y Formación Agraria y Pesquera (IFAPA)-Centro de Churriana, Málaga, Spain. Resistance testing was done using different strawberry cultivars (Table 1). The cultivars 'Frau Mieze Schindler', 'Sieger', 'Deutsch Evern', and 'Africa' are historical or pioneer cultivars that are not in current use, but whose genetic base is more expanded than the cultivars currently grown, namely 'Camarosa', 'Ventana', 'Aguedilla', and 'Sabrosa' (Candonga®) (Gil-Ariza *et al.*, 2009). Two cultivars were used as controls for each pathogen. These control cultivars were different for each pathogen according to their susceptibility or resistance to each pathogen. For *P. cactorum*, 'Senga Sengana' was used as the crown rot-resistant cultivar (Stensvad *et al.*, 1999; Eikemo *et al.*, 2000) and 'Surprise Halles' was used as the crown rot-susceptible cultivar (Pitrat & Risser, 1977). 'Pandora' was used as the Verticillium wilt-resistant cultivar and 'Carisma' was used as the Verticillium wilt-susceptible cultivar (Redondo *et al.,* 2009). For *X. fragariae* only, 'Camarosa' was used as the control because of its high susceptibility to this pathogen (Xue *et al.,* 2005).

The strawberry plants were propagated *in vitro* using a previously described protocol (López-Aranda *et al.*, 1994). The use of micropropagated material can be allowed to initiate *in vitro* cultures at any time of year because it does not depend on the production of runners from the mother plant. Furthermore *in vitro* propagation for continuous clonal plant production was initiated from meristematic tissues, which were free from viruses and other potential pests or pathogens. For each cultivar, strawberry shoots were subcultivated every six weeks. At each subculture, the new plants were first separated, and then individually cultured. This process was repeated three times until the required number of plants for each assay was reached. The individual *in vitro* micropropagated plants were carefully washed in running water, and then transplanted into 4 cm × 4 cm polystyrene trays containing a 1:1 (by volume) mix of sterile peat (Gebr. BRILL substrate, GmbH & Co. KG, Germany) and perlite (Perlita expandida, Europerlita Española S.A., Barcelona). The plants were acclimatised to *ex vitro* conditions in a greenhouse for resistance testing against *P. cactorum* and in a growth chamber for resistance testing against Verticillium wilt and *X. fragariae*. For this purpose, the trays were first placed inside a polyethylene tunnel, whose relative humidity (RH) was 100% for two weeks. At the end of the two weeks, the tunnel was slowly demolished over two weeks by progressive removal of the polyethylene tunnel so that the RH was same as that of the room.

Table 1. Strawberry cultivars used in this study and their origins

Name	Type	Pedigree[1]	Developer[2]	Country
Africa	Historical cultivar	Unknown	1870	France
Deutsch Evern	Historical cultivar	Unknown	Johannes Böttner 1902	Germany
Frau Mieze Schindler	Historical cultivar	Lucida Perfekta × Johannes Müller	Frau Mieze Schindler 1919	Germany
Sieger	Historical cultivar	Kaiser SamLing × Laxton's Noble	Johannes Böttner 1897	Germany
Aguedilla	Commercial cultivar	Camarosa × Sel.r67-35(167)	IFAPA-INIA-IVIA FNM-AEVPF 1998	Spain
Camarosa	Commercial cultivar	Douglas × Cal 85.218-605	UCA 1992	USA
	Susceptible *X. fragariae*			
Sabrosa (Candonga®)	Commercial cultivar	Sel. 9238 × Sel. 86032	PLANASA	Spain
Ventana	Commercial cultivar	Cal 93.170-606 × Cal 92.35-601	UCA 1997	USA
Surprise Halles	Susceptible to *P. cactorum*	Unknown	Guyot of Dijon 1925	France
Senga Sengana	Tolerant to *P. cactorum*	Sieger × Makee (or reverse)	Sengbush 1954	Germany
Carisma	Susceptible to *Verticillium* spp.	Oso grande × Vilanova	IFAPA-INIA-IVIA 1998	Spain
Pandora	Tolerant to *Verticillium* spp.	(Von Humboldt × Redstar) × Merton Down	1988	United Kingdom

[1] Information from Darrow (1966). [2] UCA, University of California; IFAPA, Instituto Andaluz de Investigación y Formación Agraria y Pesquera, Málaga; INIA, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid; IVIA, Instituto Valenciano de Investigaciones Agrarias, Valencia; FNM, Fresas Nuevos Materiales, Huelva; AEVPF, Asociación Española de Viveristas de Plantas de Fresa, Madrid; PLANASA, Plantas de Navarra SA, Valtierra.

The plants were then transplanted into plastic pots that contained the identical substrate, and grown in the greenhouse or in growth chambers until required for resistance testing.

Isolates

Four *P. cactorum* isolates, two isolates of *V. dahlia*e and *V. albo-atrum*, and two *X. fragariae* isolates were used as inoculum in this study. The *P. cactorum* isolates, CH971 and CH972, were obtained from strawberry plants (*F. vesca*) with crown rot from Málaga, Andalusia, Spain. The *P. cactorum* isolates, CH1020 and CH1021, were obtained from strawberry plants (*F.* × *ananassa*) with crown rot from Huelva, Andalusia, Spain. All isolates were conserved in IFAPA's plant pathogen collection, and were stored on Oatmeal agar (OMA, Difco, Detroit, MI, USA) at 15°C for short-term storage, and underwater at 4°C for long-term storage.

The *V. dahliae* isolate, VER13, was obtained from strawberry plants (*F.* × *ananassa*) in Segovia, Spain, and the *V. albo-atrum* isolate 20103, was obtained from alfalfa (*Medicago sativa* L.) in Aragón, Spain and was kindly provided by Dr. Rafael González Torres, Servicio de Investigación Agraria (SIA), Aragón, Spain. These two isolates were maintained in 20% glycerol at –80°C for long-term storage, and were grown on potato dextrose agar (PDA) (Difco, Detroit, MI, USA) at 20 ± 2°C for seven days in the dark for production of the conidial inoculum.

The *X. fragariae* strain NCPPB 1469 was isolated from *F.* ×*ananassa* in the USA. The *X. fragariae* IVIA 349.9a strain was isolated from *F.* ×*ananassa* in Benifayo, Valencia, Spain (López *et al.*, 1985) and kindly provided by Dr. María Milagros López (IVIA, Valencia). Both strains were maintained in 30% glycerol at –80°C for long-term storage and grown on Wilbrink-N medium whose composition (per litre distilled water) was 10 g sucrose, 5 g protease peptone type 3, 0.5 g K_2HPO_4, 0.25 g $MgSO_4$, 0.25 g $NaNO_3$, and 15 g agar (Koike, 1965), and incubated at 24°C for three to five days for inoculum production.

Testing the resistance of strawberry cultivars against *P. cactorum*

Prior to testing for resistance, the strawberry plants were inoculated with the *P. cactorum* isolates, and the isolates were then re-isolated, as recommended by Eikemo *et al.* (2000). Each *P. cactorum* inoculum was prepared by growing each re-isolated *P. cactorum* isolate on OMA at 24°C for ten days in the dark. The methods that were used for inducing sporangial and zoospore production were identical to those described by Redondo *et al.* (2009). Briefly, 5-mm mycelial plugs of each 10-day-old isolate were transferred to Petri dishes that contained 20 mL of sterile Petri's mineral solution, and then maintained at 22°C for seven days under fluorescent lighting. Sterile Petri's mineral solution was added during the production of the zoospores if necessary (Redondo *et al.,* 2009). At the end of the incubations, the zoospore-containing solutions from the different isolates were each passed through a sterile glass cloth, and the filtrates from the different isolates were then pooled. The number of zoospores in the pooled filtrate was then counted using a cell counting chamber and a light microscope. The concentration of the zoospore-containing filtrate was then adjusted with Petri's mineral solution so that its final concentration was 10^4 zoospores mL^{-1}. The strawberry plants were inoculated with the isolates of *P. cactorum* by immersing their roots in the zoospore-containing filtrate for 30 min. Non-inoculated or control plants had their roots immersed in zoospore-free Petri's mineral solution for 30 min. Twenty inoculated and non-inoculated plants of each strawberry cultivar, 'Frau Mieze Schindler', 'Sieger', 'Deutsch Evern', 'Africa', 'Camarosa', 'Ventana', 'Aguedilla', 'Sabrosa', 'Senga Sengana', and 'Surprise Halles', were used for resistance testing. After inoculation, the plants were transplanted to plastic pots containing a 1:1 (by volume) mix of sterile peat and perlite, and then grown in a greenhouse at 18 to 24°C and 60 to 80% RH for ten days under natural lighting.

Disease severity in each plant was then scored for presence of disease every 3 days for ten days on a scale from 0 to 4, where a score of (a) 0 was given to healthy plants (0 to 24%); (b) 1 was given to plants with apparent wilting of their leaves (25 to 49%); (c) 2 was given to plants with severe wilting symptoms (50 to 74%); (d) 3 was given to plants with general wilting and several dry leaves (75 to 90%); and (e) 4 was given to dead plants (100%). In addition, the pathogen was re-isolated from the crown and root tissues using a selective medium, P_5ARP (Jeffers & Martin, 1986).Two independent resistance assays using the zoospore-containing filtrate were performed.

Testing the resistance of strawberry cultivars against Verticillium wilt

These assays were done using previously described protocols in Redondo *et al.* (2009). In this investigation, one isolate of *V. dahliae* VER13, and one isolate of *V. albo-atrum*, 20103, were used as the inoculum, which was prepared by incubating the two isolates on PDA in Petri dishes at 20 ± 2°C for seven days in the dark. A conidial suspension of the two isolates was prepared by first scratching the plates to detach the conidia from the PDA, and then flooding the plates with 10 mL of sterile distilled water. The strawberry plants were inoculated with the conidia by immersing their roots in the conidial suspensions (10^7-10^8 conidia mL^{-1}) for 30 min. Non-inoculated or control plants had their roots immersed in sterile distilled water for 30 min. Twenty inoculated and non-inoculated plants of each strawberry cultivar, 'Sieger', 'Deutsch Evern', 'Africa', 'Camarosa', 'Ventana', 'Aguedilla', 'Sabrosa', 'Carisma', and 'Pandora', were used for resistance testing.

After inoculation, each strawberry plant was first placed into pots that contained one litre of sterile peat, and then incubated in growth chambers at 18 to 24°C and 60 to 80% RH under fluorescent lighting (100 μE m^{-2} s, 16-hour photoperiod) for 35 days. The strawberry plants were watered with tap water for the duration of the assay. Therefore, disease severity in each plant was scored for presence of disease weekly on a scale of 0 to 5, where a score of (a) 0 was given to healthy plants (0%); (b) 1 was given to plants whose lower leaves were yellowing (1 to 24%); (c) 2 was given to plants whose lower leaves were dead (25 to 49%); (d) 3 was given to plants whose upper leaves showed signs of wilting (50 to 74%); (e) 4 was given to plants with necrotic leaves and were sometimes stunted in stature (75 to 99%); and (f) 5 was given to dead plants (100%). At the end of 35-day observation period, the crowns of all plants that showed signs of disease were cut into two pieces, and then cultured in an incubator for 7 to 15 days at 20 ± 2°C in the dark in order to recover the pathogen. The pathogens were identified by observing fungal structures under a light microscope. Two independent resistance assays were performed.

Testing the resistance of strawberry cultivars against *X. fragariae*

Resistance to *X. fragariae* of the different cultivars was evaluated on micropropagated strawberry plants that were generated, as described in subsection "*Testing the resistance of strawberry cultivars against P. cactorum"*. Each bacterial strain was grown in Wilbrink-N medium (Koike, 1965), as described in subsection "*Isolates*". The final optical density of the bacterial suspension was adjusted spectrophotometrically (SmartSpec™ Plus spectrophotometer, BioRad Laboratories Ltd., Ontario, Canada) to 0.1 at an optical density of 600 nm, which corresponded to approximately 10^8 colony-forming units mL^{-1}. The plants were inoculated by pressure infiltration of the abaxial surface of the leaflets using a previously described protocol (Maas *et al.,* 2000). Three plants each of the cultivars 'Sieger', 'Frau Mieze Schindler', 'Deutsch Evern', 'Africa', 'Camarosa', 'Ventana', 'Aguedilla', and 'Sabrosa', were inoculated with each bacterial strain on three different leaves. Each leaf was inoculated at four different points, for a total of 12 inoculation points per plant. The control for this assay was three plants of each cultivar that were inoculated with sterile water at four different inoculation points on three different leaves. After inoculation, plants were grown in growth chambers at 18 to 24°C and 60 to 80% RH under fluorescent lighting (100 μE m^{-2} s, 16-h photoperiod) for three weeks. The inoculation sites were graded every week using a previously described scoring system (Maas *et al.,* 2000): 0 - no reaction; 1 - water-soaked lesions at the inoculation site; 2 - central necrosis of the inoculation site; 3 - water-soaked lesions that extended beyond the inoculation site; 4 - necrosis that had spread beyond the inoculation site; 5 - extensive necrosis beyond the inoculation site. Two independent resistance assays to the two strains of *X. fragariae* were performed.

Data analysis

The disease severity of each plant caused by each pathogen in each experiment was plotted as a disease progress curve. The area under this disease progress curve (AUDPC) was calculated as described Campbell & Madden (1990) for each experiment and each pathogen using an Excel spreadsheet. Data were statistically analysed by analysis of variance. When the F-test was significant at $p = 0.05$, the means were compared by Student-Newman-Keul's multiple range test (Snedecor & Cochram, 1980). All experiments were repeated twice. When there were not significant differences between the results of the repeated experiments,

the data from each experiment were pooled. When there was quantitative significant difference between the results of the repeated experiments, the data from each experiment were not pooled and only one of them was shown.

The cultivars were classified into four resistance categories for each pathogen using values of AUDPC in the following way: (a) resistant (R) was given to cultivar whose AUDPC was not significantly different to that observed in disease-resistant control cultivar; (b) tolerant (T) was given to cultivars whose AUDPC was significantly greater than that observed in disease-resistant control cultivar and significantly lesser than that observed in disease-susceptible control cultivar; (c) susceptible (S) was given to cultivar whose AUDPC was not significantly different to that observed in disease-susceptible control cultivar; (d) highly susceptible (HS) was given to cultivars whose AUDPC was significantly greater than that observed in disease-susceptible control cultivar.

Results

Resistance of strawberry cultivars against *P. cactorum*

Significant differences in resistance to *P. cactorum* among the ten strawberry cultivars were observed. *P. cactorum* was isolated and identified as the cause of crown rot in the diseased plants at the end of the assay.

The susceptible ('Surprise Halles') and the resistant ('Senga Sengana') control cultivars reacted as expected. Specifically, the disease severity in the crown rot-susceptible cultivar, 'Surprise Halles' displayed greater than 80% at the end of the assay (Fig. 1) and the AUDPC was large (803.8) (Table 2). In contrast, the crown rot-resistant cultivar, 'Senga Sengana' displayed a percentage of disease severity lesser than 50% at the end of the assay (Fig. 1) and the AUDPC was small (321.9) (Table 2).

When the differential resistance of the strawberry cultivars to *P. cactorum* was determined based on AUDPC, 'Deutsch Evern' and 'Ventana' were categorized as being highly susceptible (HS) to *P. cactorum* (Table 2). On the other hand, 'Sabrosa' was categorized as being resistant (R) because its AUDPC was not significantly different to that of 'Senga Sengana', the tolerant control cultivar to *P. cactorum* (Table 2).The remaining of the tested cultivars were categorized as being tolerant (T) to *P. cactorum* (Table 2).

Resistance of strawberry cultivars against Verticillium wilt

The disease severity of Verticillium wilt in each of the nine strawberry cultivars is displayed in Fig. 2. Significant differences in resistance to Verticillium wilt among the nine strawberry cultivars were observed. The susceptible ('Carisma') and the resistant ('Pandora') controls reacted as expected. The first symptoms of Verticillium wilt were observed in 'Camarosa', seven days after inoculation. Disease severity in 'Camarosa' was always the highest during the 35-day observation period (Fig. 2). The disease severity for 'Africa', 'Aguedilla', and 'Carisma' was between 15 and 25% and for 'Deutsch Evern', 'Pandora', 'Sieger', and 'Ventana' was the lowest (< 10%) the duration of the assay (Fig. 2). *V. dahliae* and *V. albo-atrum* were isolated and identified as the cause of Verticillium wilt from the diseased plants that were grown for 35 days under controlled conditions.

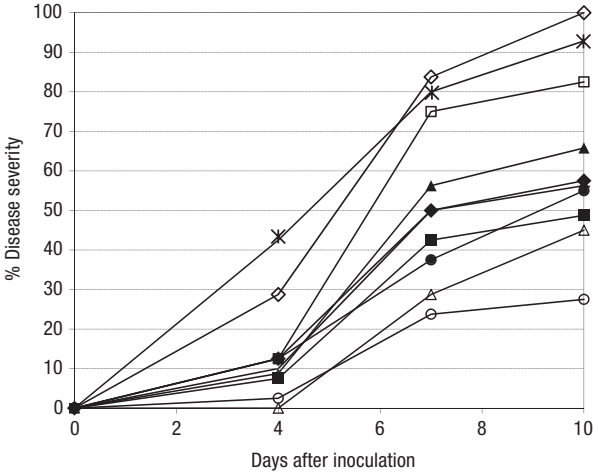

Figure 1. Disease severity (%) incited by a mixture of four isolates of *Phytophthora cactorum* (CH971, CH972, CH1020 and CH1021) in ten different strawberry cultivars, Africa (♦), Aguedilla (■), Camarosa (▲), Sabrosa (Candonga®) (○), Deutsch Evern (∗), Frau Mieze Schindler (●), Senga Sengana (△), Sieger (+), Surprise Halles (□), and Ventana (◇). Plants were grown in pots containing a 1:1 (v:v) mix of sterile peat and perlite in a greenhouse at 18 to 24°C and 60 to 80% relative humidity under natural light for ten days. Evaluations of disease severity were done at 4, 7 and 10 days. Disease severity was scored as 100% in the dead plants. Values are the mean of 20 replicates.

Table 2. Area under disease progress curves (AUDPC[1]) and resistance index (RI[2]) determined for the different strawberry cultivars that were artificially inoculated with *Phytophthora cactorum* or *Verticillium dahliae* and *V. albo-atrum* or one of *Xanthomonas fragariae*'s strains, IVIA 349.94a or NCPPB 1469

Cultivar[3]	*P. cactorum*		Verticillium wilt		*X. fragariae* IVIA 349.94a		*X. fragariae* NCPPB 1469	
	AUDPC	RI	AUDPC	RI	AUDPC	RI	AUDPC	RI
Africa	566.3 b	T	425.6 cd	S	34.5 ab	R	68.0 c	S
Deutsch Evern	1,038.8 d	HS	244.4 abc	T	67.5 d	S	60.5 c	S
Frau Mieze Schindler	477.5 b	T	NT		48.4 bcd	S	70.0 c	S
Sieger	548.8 b	T	245.7 abc	T	23.3 a	R	37.1 a	R
Aguedilla	463.8 b	T	594.7 d	S	30.2 ab	R	52.2 bc	T
Sabrosa (Candonga®)	250.6 a	R	87.6 ab	R	56.4 cd	S	62.9 c	S
Ventana	1,002.5 d	HS	184.4 abc	T	38.2 abc	T	44.5 ab	R
Camarosa	616.3 b	T	629.7 d	S	58.5 cd	S	67.4 c	S
Surprise Halles[3]	803.8 c	S	NT	–	NT	–	NT	–
Senga Sengana[3]	321.9 a	R	NT	–	NT	–	NT	–
Carisma[3]	NT	–	411.3 bcd	S	NT	–	NT	–
Pandora[3]	NT	–	55.9 a	R	NT	–	NT	–
MSE_{within}[4]	37,729.5		426,099		183.4		233.8	

[1] Data are the mean of 20 replicates at 10 or 35 days after inoculation with *P. cactorum* or *V. dahliae* and *V. albo-atrum*, respectively. Data for *X. fragariae* were obtained from the mean of four different inoculation sites on three different leaves of three plants per strawberry cultivar, 21 days after inoculation. Data followed by the same letter in each column are not significantly different ($p = 0.05$) from each other according to the Student-Newman-Keul's multiple range test. NT, not tested. [2] (S) susceptible not significantly different to disease-susceptible cultivar for each pathogen; (R) resistant not significantly different to disease-resistant cultivar for each pathogen; (T) tolerant significantly greater than disease-resistant cultivar and significantly lesser than disease-susceptible cultivar for each pathogen; (HS) highly susceptible significantly greater than disease-susceptible cultivar for each pathogen. [3] 'Surprise Halles' is the crown rot-susceptible cultivar against *P. cactorum*; 'Senga Sengana' is the crown rot-resistant cultivar against *P. cactorum*; 'Carisma' is the crown rot-susceptible cultivar against *V. dahliae* and *V. albo-atrum*; 'Pandora' is the crown rot-resistant cultivar against *V. dahliae* and *V. albo-atrum*; 'Camarosa' is the crown rot-susceptible cultivar against *X. fragariae*. [4] MSE_{within} = mean square error.

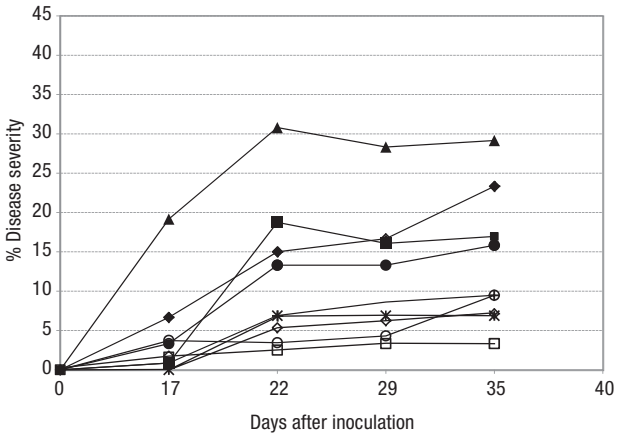

Figure 2. Disease severity (%) incited by a mixture of a *Verticillium dahliae* isolate (VER13) and a *V. albo-atrum* isolate (20103) in nine different strawberry cultivars, Africa (♦), Aguedilla (■), Camarosa (▲), Sabrosa (Candonga®) (○), Carisma (●), Deutsch Evern (∗), Pandora (□), Sieger (+), and Ventana (◇) that were grown in pots that contained one litre of sterile peat in growth chambers at 18 to 24°C and 60 to 80% relative humidity under fluorescent lighting (100 µE m^{-2} s, 16-h photoperiod) for 35 days. Evaluations of disease severity were done every seven days. Disease severity was scored as 100% in the dead plants. Values are the mean of 20 replicates.

When the differential resistance of the strawberry cultivars against Verticillium wilt was determined based on AUDPC, the most susceptible (S) cultivars were 'Africa', 'Aguedilla' and 'Camarosa' (Table 2). Of the nine tested cultivars, only 'Sabrosa' was categorized as being resistant (R) (Table 2). The remaining of the tested cultivars was categorized as being tolerant (T) cultivars to Verticilllium wilt (Table 2).

Resistance of strawberry cultivars against *X. fragariae*

Fig. 3 describes the disease progression curves for angular leaf spot disease caused by the *X. fragariae* strains, IVIA349.9a (Fig. 3A) and NCPPB 1469 (Fig. 3B), in the eight tested strawberry cultivars. Disease symptoms caused by *X. fragariae* strain IVIA 349.9a were first observed in 'Deutsch Evern', 'Camarosa', and 'Sabrosa', seven days after their inoculation (Fig. 3A). For *X. fragariae* NCPPB 1469, all cultivars, except 'Sieger', showed disease symptoms,

seven days after their inoculation (Fig. 3B). Significant differences in the AUDPCs of angular leaf spot disease caused by the two strains of *X. fragariae* were found (Table 2). 'Sieger', 'Aguedilla', and 'Africa' were categorized as being resistant (R) to *X. fragariae* strain IVIA 349.9a. One historical cultivar, 'Sieger', and one commercial cultivar, 'Ventana', were categorized as being resistant (R) to *X. fragariae* strain NCPPB 1469 (Table 2). Disease susceptibility to the two bacterial strains was the same for 'Camarosa', 'Sabrosa', 'Frau Mieze Schindler', and 'Deutsch Evern' when they were compared to susceptible control ('Camarosa') and were categorized as being susceptible (S) (Table 2).

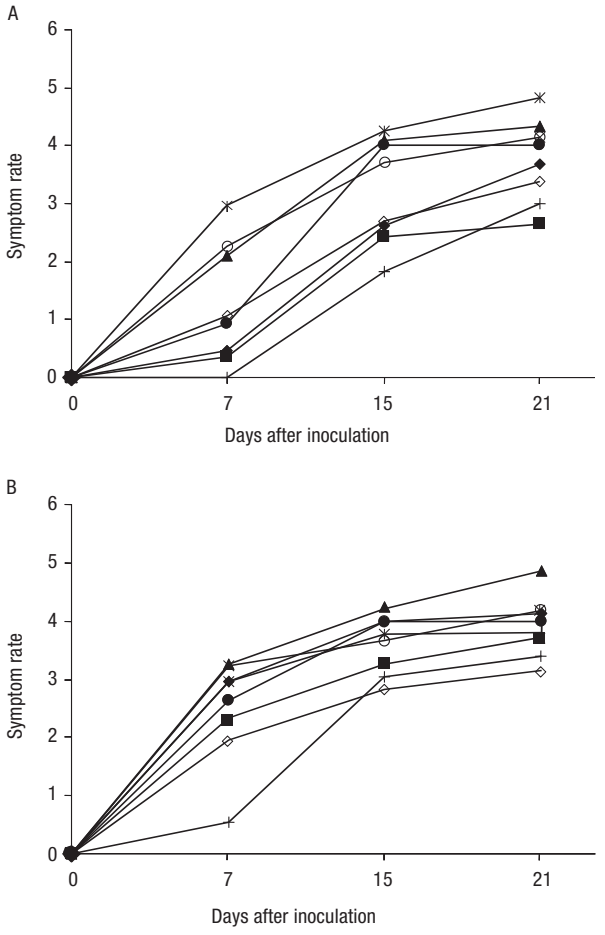

Figure 3. The disease severity curves of angular leaf spot disease caused by the *Xanthomonas fragariae* strains, IVIA 349.9a (A) and NCPPB 1469 (B) in eight strawberry cultivars, Africa (◆), Aguedilla (■), Camarosa (▲), Sabrosa (Candonga®) (○), Deutsch Evern (*), Frau Mieze Schindler (●), Sieger (+), and Ventana (◇) were grown in the growth chambers at 18 to 24°C and 60 to 80% relative humidity under fluorescent lighting (100 µE m^{-2} s, 16-h photoperiod) for three weeks. Evaluations of disease severity were done every seven days. Values are the mean symptom scores of 39 replicates.

Discussion

In this investigation, the differential resistance of various strawberry cultivars to three diseases that cause serious losses of strawberry crops in Spain was determined under greenhouse conditions for *P. cactorum* and under growth chamber conditions for Verticillium wilt and *X. fragariae*. None of the strawberry cultivars that underwent resistance testing against *P. cactorum*, *V. dahliae* and *V. albo-atrum,* and *X. fragariae* were resistant to each of the three tested pathogens. This failure to find a universal disease-resistant cultivar may be related to the limited size of the strawberry collection that we tested in this study. However, we found that (i) 'Sabrosa' (Candonga®) was resistant cultivar to both *P. cactorum* and Verticillium wilt, (ii) 'Frau Mieze Schindler', 'Sieger', 'Africa', 'Camarosa', and 'Aguedilla' were tolerant cultivars to *P. cactorum*, and (iii) 'Sieger' was resistant to *X. fragariae*.

Strawberry production in Spain involves a sequence of nursery propagation steps of which one step is preplant soil fumigation. In response to environmental and health concerns about the widespread use of pesticides, there is now considerable pressure to find alternative control strategies for crop diseases in integrated pest management (Reuveni, 1995). Plant resistance is the most powerful control measure against diseases and pests (Horiuchi *et al.*, 1990; Eikemo *et al.*, 2003). However, there are no strawberry cultivars with acceptable fruit quality and shelf life that are resistant to the diseases that were investigated in this study (Pertot *et al.,* 2008). Hence, the development of *Verticillium*, *Phytophthora*, and *Xanthomonas* resistant strawberry cultivars has now become a major breeding goal in Spain because of the European ban on the use of methyl bromide as a soil fumigant. Furthermore, commercially available alternatives to methyl bromide, such as chloropicrin or 1,3-dichloropropene (1,3-D) (De Cal *et al.*, 2004; López-Medina *et al.*, 2007; López-Aranda *et al.*, 2009), either used alone or combined, will also be banned in the near future as a result of these European Union regulations (García-Méndez *et al.*, 2008).

Another important task in disease control on strawberry is to identify sources of resistance with the purpose of using them in the creation of new commercial

cultivars. In this investigation, the four historical or pioneer cultivars, 'Frau Mieze Schindler', 'Sieger', 'Deutsch Evern', and 'Africa' were selected because they were commonly used in earlier European breeding programs. The four commercial cultivars that were used in the present study were selected because of their importance for Spanish strawberry industry: 'Camarosa' and 'Ventana' are the most produced cultivars, and 'Aguedilla' and 'Sabrosa' were developed for use in Spanish breeding programs by the Spanish Ministry of Agriculture and Planasa SA, respectively (López-Aranda *et al.*, 2005; Maxwell, 2010). According to our results, 'Sabrosa', 'Frau Mieze Schindler', 'Sieger', 'Africa', 'Camarosa', and 'Aguedilla' could be used as parents to increase the average resistance in the resulting offspring for newly-developed commercial strawberry cultivars against *P. cactorum*. In addition, 'Sabrosa' could also be used as a parent to increase the average resistance in the resulting offspring for newly-developed commercial strawberry cultivars against Verticillium wilt, and 'Sieger' could also be used as a parent to increase the average resistance in the resulting offspring for of newly-developed commercial strawberry cultivars against *X. fragariae*.

In this investigation was found that 'Sabrosa' was resistant to *P. cactorum.* We also found that the level of resistance against *P. cactorum* of three historical cultivars, 'Frau Mieze Schindler', 'Sieger', and 'Africa', and two commercial cultivars, 'Camarosa' and 'Aguedilla', was also high, although lower than that of the control, 'Senga Sengana'. Overall, the resistance responses to crown rot of the cultivars were comparable to those that have been previously reported (Pitrat & Risser, 1977; Bell *et al.*, 1997). Although some European strawberry breeding programs select for crown rot resistance when developing new cultivars (Eikemo *et al.*, 2003), information on the genetic control of strawberry resistance to the crown rot pathotype of *P. cactorum* is lacking. Finally, the degree of resistance against crown rot also seems to be dependent on the plant's age, the season of the year, and the physiological state of the plant (Pitrat & Risser, 1977; Eikemo *et al.*, 2000).

In this study, 'Sabrosa' was classified as Verticillium-resistant cultivar because it AUDPC was not significantly different to that of 'Pandora', the Verticillium wilt-resistant cultivar control. Redondo *et al.* (2009) previously reported 'Aguedilla' resulted more resistant to *V. dahliae* than 'Camarosa'. Here, the results showed both of cultivars as being susceptible to a mixture of *V. dahliae* and *V. albo-atrum*. Other researchers have also reported varietal differences for resistance to Verticillium wilt (Ebihara *et al.*, 2010). In previous studies, Shaw *et al.* (1997) suggested large differences among the plants treated with different conidial concentrations, so genotypes originally classified as intermediate in resistance performed more like susceptible types at higher conidial concentration (10^6 conidia mL^{-1}). In this work, the conidial concentration was 10^7-10^8 conidia mL^{-1}.

The results showed differences in the level of the resistance of cultivars against the two strains of *X. fragariae*. Specifically, 'Sieger' was classified as being resistant (R) against both strains of *X. fragariae*, whereas 'Aguedilla' and 'Africa' were classified as being resistant against *X. fragariae* strain IVIA 349.94a and 'Ventana' was classified as being resistant against *X. fragariae* strain NCPPB 1469. The strain NCPPB 1469 of *X. fragariae* was more aggressive than the strain IVIA 349.94a of *X. fragariae*. Resistance to *X. fragariae* has been reported in other *Fragaria* species. Kennedy & King (1962a, b) found that the disease severity of the diploid *F. vesca* cultivar 'Alpine' is low when compared to that of other *Fragaria* spp., when determined under greenhouse and field conditions. Hazel (1981) reported that of the several *Fragaria* spp. that were evaluated by inoculation, only *F. moschata* Duch. are disease-resistant and some *F. virginiana* clones displayed moderate degrees of tolerance. More recently, Maas *et al.* (2000, 2002) reported that two genotypes, a native *F. virginiana* and an *F. virginiana* × *F.* × *ananassa* hybrid, were highly resistant to the four pathogenic strains of *X. fragariae* that were identified by Pooler *et al.* (1996). These data suggest that resistance to angular leaf spot can be incorporated into cultivars by recurrent selection (Maas *et al.*, 2000).

'Sabrosa' is the most important strawberry cultivar in Spain, and in this study this cultivar has been categorized as being susceptible to both strains of *X. fragariae*. 'Camarosa' is used in Spanish production areas because of its good agronomical characteristics, although this cultivar was highly susceptible to the strawberry pathogens that were assayed in this study. 'Aguedilla' was the first strawberry cultivar produced in the research project, INIA CC01-0008-C3 with increased adaptability to the environmental conditions of Huelva and other Spanish production areas. In the present work, this cultivar was categorized as being susceptible to Verticillium wilt, resistant to *P. cactorum* and *X. fragariae* IVIA349.94a, and tolerant to *X. fragariae* NCPPB 1469.

Therefore, we emphasize the importance of considering both the agronomical characteristics of strawberries and the resistance of strawberries to their main pathogens in commercial strawberry breeding programmes. These studies will require the use of naturally infected soils in order to determine whether the observed resistance of the strawberry cultivars that we found in this investigation is adequate for their commercial production.

In conclusion, the role of susceptible cultivars in the spread of diseases underlies the importance of screening for disease resistance before the introduction and use of strawberry cultivars in commercial strawberry production. In addition, the planting of disease-resistant strawberry cultivars will reduce the number of chemical treatments in the commercial production of strawberries.

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References

Bell JA, Simpson DW, Harris DC, 1997. Development of a method for screening strawberry germplasm for resistance to *Phytophthora cactorum*. Acta Hort 439: 175-179.

Campbell CL, Madden LV, 1990. Introduction to plant disease epidemiology. Wiley, NY. 532 pp.

De Cal A, Martínez-Treceño A, López-Aranda JM, Melgarejo P, 2004. Chemical alternatives to methyl bromide in Spanish strawberry nurseries. Plant Dis 88: 210-214.

Darrow GM, 1966. The strawberry, history, breeding and physiology. Available on line in http://www.nal.usda.gov/speccoll/findaids/darrow/strawberry/book. [3 May 2012].

Duhart ME, Montes F, Páez JI, Vega M, 2000. Principales enfermedades de la fresa en Huelva. Agr Verg 218: 117-130. [In Spanish].

Duncan JM, 2002. Prospects for integrated control of Phytophthora diseases of strawberry. Acta Hort 567: 603-610.

Ebihara Y, Uematsu S, Nomiya S, 2010. Control of *Verticillium dahliae* at a strawberry nursery by paddy-upland rotation. J Gen Plant Pathol 76: 7-20.

Eikemo H, Stensvand A, Tronsmo AM, 2000. Evaluation of methods of screening strawberry cultivars for resistance to crown rot caused by *Phytophthora cactorum*. Ann Appl Biol 137: 237-244.

Eikemo H, Stensvand A, Davik J, Tronsmo AM, 2003. Resistance to crown rot (*Phytophthora cactorum*) in strawberry cultivars and in offspring from crosses between cultivars differing in susceptibility to the disease. Ann Appl Biol 142: 83-89.

EPPO, 2010. EPPO Alert List. European and Mediterranean Plant Protection Organization. Available on line in http://www.eppo.org/QUARANTINE/quarantine.htm. [21 January 2012]

FAO, 2011. FAOSTAT. Food and Agriculture Organization of the United Nations. Available on line in http://faostat.fao.org/site/567/default.aspx#ancor. [21 December 2011].

García-Méndez E, García-Sinovas D, Becerril M, De Cal A, Melgarejo P, Martínez-Treceño A, Fennimore SA, Soria C, Medina JJ, López-Aranda JM, 2008. Chemical alternatives to methyl bromide for weed control and runner plant production in strawberry nurseries. HortScience 43: 177-182.

Gil-Ariza DJ, Amaya I, López-Aranda JM, Sánchez-Sevilla JF, Botella MA, Valpuesta V, 2009. Impact of breeding on the genetic diversity of cultivated strawberry as revealed by expressed sequence Tag-derived simple sequence repeat markers. J Am Soc Hort Sci 134: 337-347.

Harris DC, Simpson DW, Bell JA, 1997. Studies on the possible role of micropropagation in the dissemination of the strawberry crown rot pathogen *Phytophthora cactorum*. J Hort Sci 72: 125-133.

Hazel WJ, 1981. *Xanthomonas fragariae*, cause of strawberry angular leaf spot, its growth, symptomatology, bacteriophages, and control. Doctoral thesis. Univ Maryland, College Park, USA.

Horiuchi S, Hagiwara H, Takeuchi S, 1990. Host specificity of isolates of *Verticillium dahliae* towards cruciferous and solanaceous plants. In: Biological control of soil-borne plant pathogens (Hornby D, ed). CAB Intnal, Wallingford, UK. pp: 285-298.

Jeffers SN, Martin SB, 1986. Comparison of two media selective for *Phytophthora* and *Pythium* species. Plant Dis 70: 1038-1043.

Kennedy BW, King TH, 1962a. Angular leaf spot of strawberry caused by *Xanthomonas fragariae* sp. Nov Phytopathology 52: 873- 875.

Kennedy BW, King TH, 1962b. Studies on epidemiology of bacterial angular leafspot on strawberry. Plant Dis Rpt 46: 360-363.

Koike H, 1965. The aluminium-cap method for testing sugarcane varieties against leaf scald disease. Phytopathology 55: 317-319.

Lewers KS, Mass JL, Hokanson SC, Gouin C, Hartung JS, 2003. Inheritance of resistance in strawberry to bacterial

angular leafspot disease caused by *Xanthomonas fragariae*. J Am Soc Hort Sci 128: 209-212.

López MM, Aramburu JM, Cambra M, 1985. *Xanthomonas fragariae*: detection and identification in Spain. An INIA Ser Agric 28: 245-259. [In Spanish].

López-Aranda JM, Pliego-Alfaro F, López-Navidad I, Barceló-Muñoz M, 1994. Micropropagation of strawberry (*Fragaria* × ananassa Duch.). Effect of mineral salts, benziladenine levels and number of subcultures on the in vitro and field behaviour of the obtained microplants and the fruiting capacity of their progeny. J Hort Sci 69: 625-637.

López-Aranda JM, Miranda L, Medina JJ, Soria C, De Los Santos B, Romero F, Pérez-Jiménez RM, Talavera M, Fennimore SA, Santos BM, 2009. Methyl bromide alternatives for high tunnel strawberry production in Southern Spain. HortTechnology 9: 187-192.

López-Aranda JM, Soria C, Miranda L, Sánchez-Sevilla JF, Galvez J, Villalba R, Romero F, De Los Santos B, Medina JJ, Palacios J *et al.,* 2005. 'Aguedilla' Strawberry. HortScience 40: 2197-2199.

López-Medina J, López-Aranda JM, Medina JJ, Miranda L, Soria C, Domínguez F, Vázquez-Ortiz E, Flores F, 2007. Strawberry production from transplants fumigated with methyl bromide alternatives. Span J Agric Res 5: 407-416.

Maas JL, 1998. Compendium of strawberry diseases, 2nd ed. APS Press, St. Paul, MN, USA.

Maas JL, Pooler MR, Galleta GJ, 1995. Bacterial angular leaf spot disease of strawberry, present status and prospects for control. Adv Strawberry Res 14: 18-42.

Maas JL, Gouin-Behe C, Hartung JS, Hokanson SC, 2000. Source of resistance for two differentially pathogenic strains of *Xanthomonas fragariae* in *Fragaria* genotypes. HortScience 35: 128-131.

Maas JL, Gouin-Behe C, Hokanson SC, Hartung JS, 2002. Strawberry parent clones US4808 and US4809 resistant to bacterial angular leaf spot disease caused by *Xanthomonas fragariae*. HortScience 37: 716-717.

MARM, 2011. Ministerio de Agricultura, Alimentación y Medio Ambiente. Available on line in http://www.marm.es/es/estadistica/temas/avances-de-superficies-y-producciones-de-cultivos [Noviembre 2011]. [In Spanish].

Martín FN, Bull CT, 2002. Biological approaches for control of root pathogens of strawberry. Phytopathology 92: 1356-1362.

Maxwell S, 2010. Planasa prepares new varieties. Eurofruit Magazine 425.

MBTOC, 2007. Report of the technology and economic assessment panel. Evaluations of 2007 critical use nominations for methyl bromide and related matters. UNEP, Nairobi, Kenya.

Medina-Mínguez JJ, 2011. RAEA. Resultados de los ensayos de variedades comerciales de fresa. Campaña 2010/11. Junta de Andalucía, IFAPA (ed.), Córdoba, Spain. [In Spanish].

MERCADOS, 2011. Nuevas variedades se incorporan al campo onubense. Revista Mercados 93. 1. Berries. Available on line in http://www.revistamercados.com. [Feb. 2011]. [In Spanish].

Pertot I, Zasso R, Amsalem L, Baldessari M, Angeli G, Elad Y, 2008. Integrating biocontrol agents in strawberry powdery mildew control strategies in high tunnel growing systems. Crop Protection 27: 622-631.

Pitrat M, Risser G, 1977. Étude de la sensibilité varietale du Fraiser *Phytophthora cactorum* après contamination provoqué. Annales de l'Amelioration des Plantes 27: 49-60. [In French].

Pooler MR, Ritchie DF, Hartung JS, 1996. Genetic relationship among strains of *Xanthomonas fragariae* based on random amplified polymorphic DNA PCR, repetitive extragenic palindromic PCR, and enterobacterial repetitive intergenic consensus PCR data and generation of multiplexed PCR primers useful for the identification of this phytopathogen. Appl Environ Microbiol 62: 3121- 3127.

Redondo C, De Cal A, Martínez-Treceño A, Becerril M, López-Aranda JM, Melgarejo P, 2009. Evaluation of resistance of several strawberry selections against main fungal pathogens. Acta Hort 842: 211-214.

Reuveni R (ed), 1995. Novel approaches to integrated pest management. Lewis Publ, CRC Press, Boca Raton, FL, USA. 383 pp.

Shaw DV, Gubler WD, Hansen J, 1997. Field resistance of California strawberries to *Verticillium dahliae* at three conidial inoculum concentrations. HortScience 32: 711-713.

Snedecor GW, Cochran WG, 1980. Statistical methods, 7th ed. Iowa St Univ Press, Ames, IA, USA. 593 pp.

Stensvand A, Herrero ML, Talgo V, 1999. Crown rot caused by *Phytophthora cactorum* in Norwegian strawberry production. Bulletin OEPP 29: 155-158.

Van Rijbroek PCL, Meulenbroek EJ, Van De Lindeloof CPJ, 1997. Development of a screening method for resistance to *Phytophthora cactorum*. Acta Hort 439: 81-183.

Xue S, Bors RS, Strelkov SE, 2005. Resistance sources to *Xanthomonas fragariae* in non-octoploid strawberry species. HortScience 40: 1653-1656.