Potential Inoculum Sources and Incidence of Strawberry Soilborne Pathogens in Spain

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Abstract

The decline and death of strawberry plants in Spanish fruit production fields have mainly been attributed to the soilborne pathogens *Macrophomina phaseolina*, *Phytophthora cactorum*, and *Fusarium* spp. Inoculum sources of *M. phaseolina* and *P. cactorum*, and the incidence all three genera, were investigated in nurseries and fruit production fields over three consecutive seasons. *M. phaseolina* inoculum sources consisted of fumigated preplant fruit production soils (50%) and fumigated nursery soils (47%), although the pathogen could not be detected in nursery mother and runner plants. *P. cactorum* inoculum sources included nursery (20%) and preplant fruit production (17%) fumigated soils, and

nursery runner plants (up to 15%). In fruit production plants, the average incidence of *M. phaseolina* and *P. cactorum* were 4.2 and 3.7%, respectively. *Fusarium* spp. inoculum sources could not be accessed extensively due to the lack of effective quantitative real-time PCR assays. Limited testing of nursery plants showed that *Fusarium oxysporum* f. sp. *fragariae* (Fof) was absent. In field production plants and soil, *F. solani* was the main pathogenic *Fusarium* spp., with Fof only identified once in a fruit production plant. Ineffectively fumigated soils in nurseries and production fields, along with infected runner plants, can be inoculum sources of soilborne strawberry pathogens in Spain.

Spain is the largest producer of strawberry (*Fragaria × ananassa* Duch.) plants and fruits in Europe. Strawberry runners from mother plants are grown in high-elevation nurseries in northern Spain, mainly Segovia, Ávila, and Valladolid provinces, where optimal agroclimatic conditions prevail from March to September. Runner plants from nurseries are uprooted and transplanted into fruit production fields located mainly in southwestern Spain (Huelva Province), where plants develop and fruit from October to May-June, mainly under plastic macrotunnels.

Reduced yields due to plant diseases have increased alarmingly in these strawberry fruit production fields in recent years (Chamorro et al. 2015; Pérez-Jiménez et al. 2012). The losses are reportedly the result of soilborne pathogens such as *Macrophomina phaseolina* (Tassi) Goid., the causal agent of charcoal rot (Avilés et al. 2008); *Phytophthora cactorum* (Leb. & Cohn), causing strawberry crown rot (de los Santos et al. 2002) and leather rot of fruit (Maas 1998); *Fusarium oxysporum* f. sp. *fragariae* Winks & Y.N. Williams (Fof), causing Fusarium wilt (Arroyo et al. 2009); and *F. solani* (Mart.) Sacc. causing crown and root rot (Pastrana et al. 2014). The recent emergence of these fungal diseases in strawberry has been related largely to the phasing out of more than 70% of chemical soil fumigants used in the European Union, including methyl bromide (MeBr), in accordance with the Montreal Protocol (Martin 2003).

The detection and accurate identification of plant pathogens are among the most important strategies to control plant diseases and to develop and apply preventative control measures (Capote et al. 2012). In strawberry crop management, special attention should be given to the early detection of pathogens in propagation plant material (mother and runner plants) to avoid the introduction and further spread of pathogens in a growing area where the disease is not present yet. The ability of many soilborne fungi and oomycetes to form resistant structures that survive in the soil for long periods causes difficulties in eradicating the pathogens once they have become established, making infested soils another main source of pathogen inoculum. Traditional methods to detect and identify pathogens have

often relied on visual inspection of symptoms or on pathogen isolation using selective media. However, the visual symptoms of *M. phaseolina*, *P. cactorum*, and *Fusarium* spp. in strawberry plants are nearly indistinguishable, consisting of plant wilting, chlorosis, drying of leaves, and eventual collapse of plants. Morphological identifications of plant pathogenic fungi are time-consuming and rely on labor-intensive techniques and taxonomic expertise. These limitations have led to the development of new diagnostic methods and protocols for detecting plant pathogens based on molecular techniques. In addition, the quantification of pathogens represents an important aspect of plant disease management (Mirmajlessi et al. 2015). For this reason, quantitative real-time PCR (qPCR) has emerged as the gold standard method for identifying and quantifying fungal and oomycete pathogens, due to its accuracy, speed, and high throughput (Capote et al. 2012).

qPCR TaqMan-based methods have been developed to detect and quantify *M. phaseolina* and *P. cactorum* in plant and soil samples (Babu et al. 2011; Li et al. 2013). To improve the above-cited protocols and use them as effective and rapid diagnostic techniques, we developed a simple and direct sample-preparation method based on the dilution of crude plant extracts. The protocol excludes the DNA-extraction step, saving labor and time while facilitating the diagnosis of large numbers of plant samples. In the current study, the aforementioned detection and quantification protocols were used to determine the possible sources of inoculum (nursery mother and runner plants, fumigated nursery soils, and fumigated preplant field production soils) of *M. phaseolina* and *P. cactorum* in strawberries in Spain. The incidence of these pathogens was also determined in field production soils at the end of the cropping season (postharvest) and in field production plants.

No qPCR method has yet been successfully developed to specifically detect *F. oxysporum* f. sp. *fragariae*. The *F. oxysporum* species complex includes pathogenic and nonpathogenic strains that are morphologically indistinguishable (O'Donnell et al. 2009). To differentiate between pathogenic and nonpathogenic isolates, Suga et al. (2013) developed PCR primers (FofraF and FofraR) to amplify a specific DNA sequence from *F. oxysporum* f. sp. *fragariae*. However, a subset of isolates diagnosed as pathogenic by PCR did not produce symptoms in strawberry in pathogenicity tests, and vice versa (Henry et al. 2017; Suga et al. 2013). Li et al. (2014) also developed qPCR primers that target the *FOW1*-gene involved in *F. oxysporum* pathogenesis that encodes a mitochondrial carrier protein required specifically for the colonization of plant-root tissue by *F. oxysporum*. However, the study did not perform any test to verify whether this

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method discriminates between pathogenic and nonpathogenic *F. oxysporum* isolates. Due to the lack of a qPCR method to specifically detect *Fof* and because of the recent report of *F. solani* also contributing to the decline and death of strawberry plants under cultivation (Pastrana et al. 2014), isolation studies and pathogenicity tests must be conducted to determine the importance of *Fusarium* spp. in strawberries. Therefore, the aim of the current study was to assess the importance of *Fusarium* species in contributing to the *Fusarium* diseases in Spanish strawberry crops. *Fusarium* isolates, mainly obtained from fruit production plants and soil, were evaluated for pathogenicity, with a limited number of isolates from nursery plants also being tested. The *Fof* PCR primer pair of Suga et al. (2013) was also evaluated on the *F. oxysporum* isolates included in the pathogenicity tests, to determine if the assay might have potential for identifying *Fof* in Spanish strawberry production regions. Altogether, the information can be used in future to facilitate the development of methods for evaluating the potential inoculum sources of *Fusarium* spp. in strawberries in Spain.

Materials and Methods

Soil sampling and assessment. *Soil samples from nurseries.* Soil samples were collected at three high-elevation nurseries over three consecutive seasons (2011–12, 2012–13, and 2013–14) for the detection and quantification of *M. phaseolina* and *P. cactorum*. The nurseries were located in the provinces of Segovia, Ávila, and Valladolid (Spain). For each nursery, two fumigated and two nonfumigated plots were analyzed, except for the Ávila nurseries where two fumigated soils were analyzed the first season and one fumigated and one nonfumigated soil the following two seasons. A total of 15 fumigated and 13 nonfumigated soils were thus investigated in the three localities over the three seasons. Plots from the Segovia and Valladolid nurseries were fumigated with 1,3-dichloropropene:chloropicrin (1,3D:CP) (50:50, v/v), whereas 3,5-dimethyl-1,3,5-thiadiazinane-2-thione (Dazomet) was used in the Ávila plots. Fumigation was conducted by injection of 1,3D:CP or Dazomet (500 liters/ha) into the soil at 20 to 25 cm depth under a plastic mulch. Due to the 3-year crop rotation applied at the nurseries, analyzed fumigated and nonfumigated plots were not the same each season. Nonfumigated plots were cultivated with strawberry plants the previous season. Soil samples were collected 1 month after fumigation from fumigated and nonfumigated plots, which was approximately 1 to 2 weeks prior to planting of the strawberry runner plants. Twelve samples (0 to 20 cm depth, approximately 100 g) taken at random in each plot and season were pooled and mixed as a composite sample. Soil samples were air-dried for 7 to 10 days at room temperature, passed through a 1 mm mesh sieve, and stored at 5°C until used. Three replicates per plot were analyzed.

For DNA extraction, soil samples were homogenized in a mortar. DNA was extracted from 250 mg soil, with three extraction replicates per sample using the MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories, Solana Beach, CA) following the manufacturers' instructions. The DNA concentration was determined on a ND-1000 NanoDrop spectrophotometer (NanoDrop Products, Wilmington, DE). Five microliters of the extracted DNA was used as template DNA for qPCR detection and quantification of *M. phaseolina* and *P. cactorum* (see below). The results of qPCR (pg pathogen DNA/g of soil) analyses are presented as the average and standard error of fumigated or nonfumigated plots in each province and season.

Soil samples from fruit production fields. Soil samples were collected in two strawberry fruit production fields situated in different geographic locations in Huelva Province, namely Cartaya and Palos de la Frontera (hereafter, Palos). Soils were fumigated in August with 1,3:CP (500 liters/ha) through the drip irrigation system under beds covered with a black polyethylene plastic film. The same three macrotunnels per field were sampled each season. From the strawberry beds, 30 soil samples (0 to 20 cm in depth, approximately 100 g) per macrotunnel were taken in 1,3:CP fumigated soils in October, approximately 2 months after fumigation and 1 or 2 weeks prior to planting and at the end of the harvest season (May–June). All the samples from each macrotunnel were pooled and used as a composite

soil sample for further analysis. Three replicates per macrotunnel were analyzed for qPCR-detection and quantification of *M. phaseolina* and *P. cactorum* as described for the nursery soil samples. Results of qPCR (pg pathogen DNA/g of soil) analyses are presented as the average and standard error of three replicates in the three macrotunnels analyzed per field and season.

For soil assays for *Fusarium* spp. isolation, 5 g of fumigated or nonfumigated soil were added to a flask containing 150 ml of 0.1% water agar (WA) (Difco) and placed on a stir plate for 1 min. Before the stirring ended, 1 ml of the suspension was removed and spread on V8 agar plates (5 plates/soil sample) and incubated at 25°C for 2 days in darkness and 3 additional days with a 12 h photoperiod (OSRAM L 18 W / 21-840 Hellweiss Lumilux Cool White, 75 $\mu\text{E m}^{-2} \text{s}^{-1}$). *Fusarium*-like colonies were transferred to PDA plates and single-spore isolates were obtained for further morphological, molecular, and pathogenicity characterization.

Plant sampling and assessment. *Plant samples from nurseries.* Asymptomatic mother plants were collected from the same nurseries from which soil samples were taken in the Segovia, Ávila, and Valladolid provinces. A total of 314, 180, and 207 mother plants were analyzed, respectively, in the 2011–12, 2012–13, and 2013–14 seasons for *M. phaseolina* and *P. cactorum* through qPCR-assays, and using plate-culture isolations for *Fusarium* spp. The mother plants analyzed corresponded to 12 different strawberry cultivars (Amiga, Benicia, Candonga, Camarosa, Merced, Sabrina, Favette, Festival, Florida Fortuna, Primoris, San Andreas, and Viva Patricia).

Plant samples were washed under tap water and cut longitudinally in half. One half (0.5 to 2 g of fresh weight) was ground into individual extraction bags (Bioreba, Switzerland) in the presence of 1:10 (w/v) 1× PBS buffer, pH 7.2 supplemented with 2% (w/v) polyvinyl pyrrolidone (PVP-40) and 0.2% (w/v) sodium diethyl dithiocarbamate (DIECA). Plant tissue was homogenized with a hand roller to obtain a crude extract. Crude extracts were incubated for 10 min at 95°C and frozen at –80°C until used. Dilutions of plant crude extracts in sterile distilled water (1:20 v/v) were directly used as the template DNA (5 μl) for qPCR reactions for the specific detection of *M. phaseolina* and *P. cactorum* (see below). The other longitudinal half part of the plant was used for plate culture isolations of *Fusarium* spp. Crown, root, and petiole segments (0.5 to 1 cm) were surface sterilized in 1% sodium hypochlorite for 2 min, rinsed in sterile distilled water, air dried, placed onto PDA, and incubated at 25°C for 2 days in darkness and 5 days at a 12 h photoperiod. Colonies identified as *Fusarium* spp. on PDA were streaked on water agar, and single-spore subcultures were stored at –80°C until further characterization.

Asymptomatic runner plants were collected from the same three strawberry nurseries from which mother plants were sampled. An additional 20 other nurseries were also sampled that were located in Segovia, Ávila, and Valladolid provinces. A total of 480, 200, and 340 runner plants were analyzed, respectively, in the three respective seasons, as described for the mother plants. The plants analyzed corresponded to 13 different strawberry cultivars (Amiga, Antilla, Benicia, Camarosa, Candonga, Festival, Florida Fortuna, Honor, Merced, Primoris, Sabrina, San Andreas, and Splendor).

Plant samples from fruit production fields. Symptomatic (declining and dead) fruit-production plants cv. Sabrina, Florida Fortuna, Splendor, and Primoris were sampled throughout Huelva Province, mainly Cartaya and Palos, over three production seasons during 2011–12, 2012–13, and 2013–14. A total of 452, 1,249, and 719 plants were analyzed, respectively, using plate-culture isolations of *M. phaseolina*, *P. cactorum*, and *Fusarium* spp., as described above for *Fusarium* spp.

Detection and quantification of *M. phaseolina* and *P. cactorum* by qPCR. Five microliters (μl) of DNA extracted from nursery and fruit producing field soils (three replicates/sample), or 5 μl diluted plant crude extract from mother and runner plants (two replicates/plant) were used to detect and quantify *M. phaseolina* and *P. cactorum*. The qPCR reactions were performed in 96-well plates in a Chromo 4 thermocycler (Bio-Rad). Reaction cocktails contained 1× SensiMix (SensiMix Probe No-Rox Kit, Bioline), 900 nM each forward and reverse primers, 150 nM TaqMan probe (Table 1),

and 1 mg/ml BSA. Amplifications were carried out at 95°C for 10 min, and 45 cycles of 15 s at 95°C and 60 s at 61°C for both pathogens. Distilled water was used for negative controls. Standard curves were constructed by amplifying 10-fold serial dilutions (50 ng to 50 fg) of genomic DNA from *M. phaseolina* M-16 or *P. cactorum* PF-305 diluted in healthy plant crude extract (1:10 dilution) or in DNA from sterile soil (20 ng/μl) for detection and quantification of the pathogens in plant material and soil samples, respectively. The efficiency of the reactions and R^2 values were calculated from the slope of the plant and soil DNA standard curves, and these were all within acceptable norms for the *M. phaseolina* (efficiency = 101.2%; R^2 = 0.998) and *P. cactorum* (efficiency = 99.7%; R^2 = 0.991) assays. An internal positive control (IPC) was used to rule out false negative amplifications. For the IPC assay, 0.05 ng of lambda (λ) bacteriophage DNA was added to each sample and amplified in parallel SYBRGreen-qPCR reactions. The IPC reaction cocktail contained 1× SensiMix (SensiMix SYBRGreen No-Rox, Bioline), 50 nM λ primers (Table 1) that were designed using Primer Express version 2.0, 1 mg/ml BSA, and 5 μl of diluted plant crude extract or genomic DNA from soil samples. Amplification conditions were the same as for *M. phaseolina* and *P. cactorum*.

Morphological and molecular characterization of *Fusarium* spp., *M. phaseolina*, and *P. cactorum* isolates. Single-spore cultures from 88 selected *Fusarium* spp. isolates recovered from preplanting and postharvest production field soils (five isolates), and nursery (12 isolates) and fruit production plants (71 isolates) (Table 2) were cultured on PDA and on 0.6% KCl agar (Corpas-Hervias et al. 2006) at 25°C for 2 days in darkness, and 8 additional days at a 12-h photoperiod. Colony morphology and morphological characteristics were recorded under an optical microscope to identify isolates to the genus or species level using standard descriptions (Leslie and Summerell 2006). For molecular characterization, DNA from single-spore cultures from the 88 selected isolates was isolated using the Isolate II Plant DNA Kit (Bioline, London) following the manufacturer's instructions with some modifications. This first consisted of a small amount of fungal mycelium growing on PDA being scraped from the plate and placed into a centrifuge tube containing lysis buffer (Bioline), followed by homogenization with a pistil coupled to an electric drill. The subsequent DNA extraction steps were performed as described in the instruction manual. The DNA concentration was determined on an ND-1000 NanoDrop spectrophotometer (NanoDrop Products, Wilmington, DE). *Fusarium* spp. isolates were identified by PCR amplification and sequencing of a portion of the *EF-1α* gene using primers EF1 and EF2 (O'Donnell et al. 1998). The PCR reaction contained 1× Hi-Fi Buffer (Bioline), 2 mM MgCl₂, 0.25 mM each dNTP, 0.4 μM each primer, 1 unit of velocity DNA polymerase (Bioline), and 50 to 100 ng of genomic DNA. Amplifications were carried out at 95°C for 2 min, and 35 cycles of 30 s at 95°C, 30 s at 59°C, and 15 s at 72°C. Sequences were compared with published sequences in GenBank and *Fusarium*-ID databases by BLAST analysis.

M. phaseolina and *P. cactorum* single-spore isolates recovered from diseased fruit-production plants were also identified using morphological characteristics (Gallegly and Hong 2008; Sutton 1980), which was confirmed by sequencing of a portion of the *EF-1α* gene

for *M. phaseolina* using primers EF446f and EF1035r (Inderbitzin et al. 2005) and a portion of the internal transcribed spacer (ITS) of the nuclear ribosomal DNA for *P. cactorum* using primers ITS6 and ITS7 (Cooke et al. 2000). Sequences were compared using BLAST analyses in the GenBank database, where only published peer reviewed sequences were considered for reliable species identifications.

Pathogenic characterization of *Fusarium* spp. isolates. To assess pathogenicity, 70 out of 88 *Fusarium* spp. isolates recovered from preplanting and postharvest field production soils, field production plants, and nursery runner plants were tested (Table 2). Single-spore isolates were cultured in potato dextrose broth (PDB) in a rotary shaker (150 rpm) at 25°C with a 12-h photoperiod for 10 days. The spore suspension was filtered through four layers of cheesecloth and adjusted to 10⁶ conidia/ml with sterile distilled water using a hemocytometer. One-month-old strawberry seedlings cv. Camarosa from meristem cultures were inoculated by dipping roots and crowns in the conidial suspension for 30 min. Control plants were inoculated by immersion in PDB/sterile distilled water (1:1, v/v). Each plant was individually transplanted into a 13-cm pot containing sterilized 1:9 perlite/peat moss (v/v). Plants were maintained for 3 months at 25/18°C and 60/40% relative humidity (day/night) in a growth chamber with a daily 16-h photoperiod of fluorescent light, fertilized 15 days after inoculation with 0.3 g of Osmocote per pot and watered as required. Eight plants were inoculated for each *Fusarium* spp. isolate. Each pathogenicity test was conducted twice. Plants were placed in a completely randomized design. At the end of the experiment, plants were removed from the pots and infection severity was assessed according to: (i) percentage dead plants, (ii) percentage new roots, (iii) percentage crown rot, and (iv) plant fresh weight. Reisolation of the inoculated *Fusarium* spp. from roots, crowns, and petiole tissues was performed on PDA plates, as described above.

PCR identification of *F. oxysporum* f. sp. *fragariae* isolates. All isolates identified as *F. oxysporum* based on sequence analyses (Table 2) were also tested for PCR amplification of a 239-bp fragment specific for *F. oxysporum* f. sp. *fragariae* using primers FofraF (5'-CAGACTGGGGTGCTTAAAGTT-3') and FofraR (5'-AACCGCTAGGGTCGTAACAAA-3') (Suga et al. 2013). Amplification was conducted in a 10-μl volume with 1× BioTaq buffer (Bioline) 1×, 2 μM each primer, 2.5 units of BioTaq polymerase (Bioline), and 50 to 100 ng of genomic DNA. PCR was performed using the same cycling parameters described by Suga et al. (2013). PCR products were sized by electrophoresis in 2.5% agarose gels run in 0.5× TAE buffer, stained with RedSafe, and visualized and photographed over a UV transilluminator. Isolates previously confirmed by pathogenicity tests as *F. oxysporum* f. sp. *fragariae* in Spain (G2-4) (Arroyo et al. 2009), Australia (ATCC 18138), and South Carolina (NRRL 26438) (O'Donnell et al. 2009) were also included in the assays. A Japanese *Fof* isolate (MAFF 744009) was used as positive control for the reaction (Suga et al. 2013).

Statistical data analyses. Student's *t* test was used to compare variable means between fumigated and nonfumigated soils in nurseries and between preplanting and postharvest soils in fruit production fields from each studied season. The variables that were analyzed included the amount of *M. phaseolina* DNA/g soil and *P. cactorum* DNA/g soil.

Table 1. Primers and probes used to detect and quantify *Macrophomina phaseolina* and *Phytophthora cactorum* in soil samples and strawberry plant crude extracts by qPCR. Primers sequences for an internal positive control, based on lambda bacteriophage, are also included

Target species	Oligo name	Sequence (5'→3')	Amplicon size	Chemistry	Target gene	Reference
<i>Macrophomina phaseolina</i>	MpTq-F	GCCTTACAAGGGTCTCGTCAT	150 bp	TaqMan	SCAR	Babu et al. 2011
	MpTq-R	CCCTTGGCGATGCCGATA				
	MpTq-P	FAM-CAGGCCACAGGATCTT-MGB				
<i>Phytophthora cactorum</i>	Ypt-cac-F3	CATGGCATTATCGTGGTGTGA	122 bp	TaqMan	ras protein (<i>Ypt1</i>)	Li et al. 2013
	Ypt-cac-R3	GCTCTTTTCCGTCGGC				
	P-cac4	VIC-CGGACCAGGAGTCGTTCAACAAC-MGB				
Lambda bacteriophage	λ-F	CTGCGGCCTTTTACACATGA	71 bp	SYBR Green	λ genome	This work
	λ-R	AAACGGCAGGAGGTGTGTTAGC				

For the pathogenicity tests, analyses of variance (ANOVA) were used to test for significant effects of independent variables, following a completely randomized design. Data from repeated experiments were previously subjected to an ANOVA and, since no statistical differences were found, the data of the two trials were combined into a single set of data. Percentage data were transformed ($\arcsin \sqrt{Y/100}$) prior to analysis. Where significant *P* values were determined, means were compared using Fisher's LSD test at the 95% confidence level. All statistical analyses were performed with Statistix 9.0 (Analytical Software, Tallahassee, FL).

Results

Detection and quantification of *M. phaseolina* and *P. cactorum* in soil samples. Soil samples from nursery fields. *M. phaseolina* was detected in 47% (7 out of 15) of the fumigated soils and in 31% (6 out of 13) of the nonfumigated soils investigated in all localities and seasons. Average inoculum densities ranged from 1 to 30 pg DNA/g soil

in fumigated soils, and from 4 to 92 pg DNA/g soil in nonfumigated soils (Fig. 1A). In plots investigated in Segovia, *M. phaseolina* was detected only in 1,3D:CP fumigated soils in the last cropping season. However, the pathogen was detected in all three seasons at variable levels in Ávila and Valladolid, with no significant differences evident between fumigated and nonfumigated plots. The inoculum reached the highest average concentration (92 ± 37 pg DNA/g soil) in nonfumigated plots from the Valladolid nursery in the last analyzed season (Fig. 1A).

P. cactorum was detected in 20% (3 out of 15) of the fumigated soils and in 54% (7 out of 13) of the nonfumigated soils analyzed in the three provinces and seasons. Average inoculum densities were significantly higher in nonfumigated soils (ranging from 33 to 4,422 pg DNA/g soil) than in fumigated soils, having mean levels of between 183 and 986 pg DNA/g soil. In the first investigated season, *P. cactorum* was detected only in a nonfumigated plot from Valladolid province (207 ± 114 pg DNA/g soil) and not in fumigated soils. In

Table 2. *Fusarium* spp. isolates from soil and plant material obtained from strawberry nurseries and fruit production fields characterized in this study

Species	Isolate	Origin ^y	Location	Source	Cultivar	Pathogenicity ^z	Accession no.
<i>F. oxysporum</i>	TOR-2	FPF	Moguer	Preplanting soil	-	-	KX215004
	TOR-3	FPF	Moguer	Preplanting soil	-	-	KX215005
	TOR-4	FPF	Moguer	Root	Camarosa	-	KX215006
	TOR-6	FPF	Moguer	Postharvest soil	-	nt	KX215007
	TOR-12	FPF	Moguer	Root	Camarosa	nt	KX215008
	TOR-19	FPF	Moguer	Crown	Camarosa	-	KX215009
	TOR-25	FPF	Moguer	Root	Camarosa	nt	KX215010
	TOR-27	FPF	Moguer	Root	Camarosa	-	KX215011
	TOR-29	FPF	Moguer	Root	Camarosa	nt	KX215012
	TOR-30	FPF	Moguer	Root	Camarosa	nt	KX215013
	TOR-31	FPF	Moguer	Root	Camarosa	-	KX215014
	TOR-43	FPF	Palos	Root	Camarosa	-	KX215015
	TOR-44	FPF	Palos	Crown	Camarosa	nt	KX215016
	TOR-56	FPF	Palos	Root	Camarosa	-	KX215017
	TOR-60	FPF	Palos	Root	Camarosa	-	KX215018
	TOR-84	FPF	Moguer	Root	Camarosa	-	KX215019
	TOR-85	FPF	Moguer	Root	Camarosa	-	KX215020
	TOR-105	NRP	Segovia	Root	Splendor	nt	nd
	TOR-107	NRP	Segovia	Root	Splendor	nt	nd
	TOR-109 V	NRP	Segovia	Crown	Splendor	-	KX215021
	TOR-111	NRP	Valladolid	Crown	Amiga	-	nd
	TOR-112	NRP	Valladolid	Crown	Amiga	-	nd
	TOR-114	NRP	Ávila	Crown	Festival	-	nd
	TOR-115 V	NRP	Ávila	Crown	San Andreas	-	KX215022
	TOR-116 I	NRP	Ávila	Root	San Andreas	-	KX215023
	FPRE-1	NRP	Ávila	Crown	Camarosa	nt	KX215024
	FPOST-3	FPF	Palos	Root	Fortuna	-	KX215025
	FPOST-5	FPF	Palos	Root	Fortuna	nt	KX215026
	FPOST-6	FPF	Palos	Root	Fortuna	-	KX215027
	FPOST-11	FPF	Palos	Root	Fortuna	-	KX215028
	FPOST-13	FPF	Palos	Crown	Fortuna	-	KX215029
	FPOST-19	FPF	Palos	Root	Fortuna	-	KX215030
	FPOST-21	FPF	Palos	Root	Fortuna	-	KX215031
	FPOST-30	FPF	Palos	Root	Fortuna	-	KX215032
	FPOST-36	FPF	Palos	Root	Fortuna	-	KX215033
	FPOST-82	FPF	Cartaya	Crown	Sabrina	-	KX215034
	FPOST-83	FPF	Cartaya	Crown	Sabrina	-	KX215035
	FPOST-84	FPF	Cartaya	Crown	Sabrina	-	KX215036
	FPOST-85	FPF	Cartaya	Crown	Sabrina	-	KX215037
	FPOST-86	FPF	Cartaya	Root	Sabrina	-	KX215038
	FPOST-91	FPF	Cartaya	Crown	Sabrina	-	KX215039

(continued on next page)

^y FPF: Fruit production fields from Huelva province in southwestern Spain. NRP: Nursery runner plant. Nurseries were located at Segovia, Ávila, and Valladolid provinces in northern Spain.

^z Pathogenicity test results of *Fusarium* species isolates that were artificially inoculated on strawberry plants, whereafter plant growth was evaluated after 3 months of growth in a growth chamber. The results are from two repeated experiments. Isolates were identified as nonpathogenic (-) when they did not cause a significant reduction in plant growth parameters relative to the uninoculated control plants, + indicates isolates that were identified as pathogenic since they caused a significant reduction in growth parameters relative to the uninoculated control plants; nt: not tested; nd: not determined.

the two subsequent seasons, the pathogen was detected at highly variable levels in nonfumigated soils from Ávila (maximum average level $4,422 \pm 1,921$ pg DNA/g soil) and Segovia (maximum average level $2,221 \pm 204$ pg DNA/g soil) (Fig. 1B).

Soil samples from fruit production fields. *M. phaseolina* was detected in all fumigated preplant plots analyzed from Palos and showed a significant ($P \leq 0.05$) increase in inoculum density (measured as pg DNA/g soil) at the end of each cropping season. Although the pathogen was not found in preplant fumigated plots in Cartaya in the last season, it appeared in all plots at the end of the seasons, being significantly higher in soils postharvest than in fumigated soils preplant ($P \leq 0.05$) (Fig. 2A). *M. phaseolina* inoculum levels ranged from 35 to 373 pg DNA/g soil in fumigated soils and between 62 and 1,568 pg DNA/g of soil in soils postharvest at the end of the seasons (Fig. 2A).

P. cactorum was first detected in Palos at the end of the 2011–12 season. In the following seasons, at this location the pathogen was found in soil, both in preplanted fumigated soil and in soils postharvest. The same pattern was found in Cartaya one year later, where *P. cactorum* was detected for the first time in Cartaya soils at the

end of the 2012–13 season and, one year later, it was found in fumigated preplant soil and in soil of the postharvest plots (Fig. 2B). *P. cactorum* soil inoculum was lower than those of *M. phaseolina*, ranging from 2 to 3 pg DNA/g soil before planting in the fumigated soil, and from 1 to 6 pg DNA/g soil at the end of the season.

Fusarium spp. isolates, including *F. oxysporum* and *F. solani*, were recovered from preplant fumigated soil, and soils postharvest at the end of the cropping season in the three investigated seasons. Two *F. oxysporum* isolates recovered from soils postharvest were selected for pathogenicity testing, but none were pathogenic. However, the two selected *F. solani* isolates recovered from soils postharvest were pathogenic to strawberry (Table 2, see below).

Detection of *M. phaseolina*, *P. cactorum*, and *Fusarium* spp. in plant samples. *Plant samples from nurseries.* In mother plants, *M. phaseolina* and *P. cactorum* were not detected through qPCR analyses. However, isolates of *Fusarium* spp. were recovered by conventional isolation assays from 11.7, 9.4, and 12.1% of the analyzed mother plants in the 2011–12, 2012–13, and 2013–14 seasons, respectively (Table 3).

Table 2. (continued from preceding page)

Species	Isolate	Origin ^y	Location	Source	Cultivar	Pathogenicity ^z	Accession no.
<i>F. solani</i>	FPOST-92	FPF	Cartaya	Root	Sabrina	-	KX215040
	FPOST-140	FPF	Cartaya	Crown	Fortuna	+	KX215041
	FPOST-143	FPF	Cartaya	Crown	Fortuna	-	KX215042
	FPOST-162	FPF	Cartaya	Crown	Fortuna	-	KX215043
	FPOST-166	FPF	Cartaya	Crown	Sabrina	-	KX215044
	FPOST-170	FPF	Cartaya	Crown	Sabrina	-	KX215045
	FPOST-172	FPF	Cartaya	Crown	Sabrina	-	KX215046
	FPOST-179	FPF	Palos	Crown	Splendor	-	KX215047
	TOR-393	FPF	Moguer	Crown	Fortuna	+	KX215048
	TOR-397	FPF	Moguer	Postharvest soil	-	+	KX215049
	TOR-725	FPF	Moguer	Postharvest soil	-	+	KX215050
	E-III	FPF	Moguer	Crown	Camarosa	+	KX215056
	FPOST-164	FPF	Cartaya	Root	Fortuna	-	KX215052
	FPOST-169	FPF	Cartaya	Crown	Sabrina	+	KX215053
	FPOST-174	FPF	Palos	Crown	Splendor	+	KX215054
<i>F. avenaceum</i>	FPOST-176	FPF	Cartaya	Crown	Fortuna	+	KX215055
	FPOST-2	FPF	Palos	Crown	Fortuna	-	KX215057
	FPOST-7	FPF	Palos	Root	Fortuna	-	KX215058
	FPOST-10	FPF	Palos	Crown	Fortuna	-	KX215059
	FPOST-16	FPF	Palos	Crown	Fortuna	nt	KX215060
	FPOST-18	FPF	Palos	Crown	Fortuna	-	KX215061
	FPOST-20	FPF	Palos	Crown	Fortuna	nt	KX215062
	FPOST-35	FPF	Palos	Root	Fortuna	nt	KX215062
	FPOST-40	FPF	Palos	Crown	Fortuna	nt	KX215064
	FPOST-41	FPF	Palos	Crown	Fortuna	nt	KX215065
	FPOST-43	FPF	Palos	Root	Fortuna	nt	KX215066
	FPOST-48	FPF	Palos	Crown	Fortuna	nt	KX215067
<i>F. acuminatum</i>	TOR-22	FPF	Moguer	Crown	Camarosa	-	KX215068
	TOR-24	FPF	Moguer	Crown	Camarosa	nt	KX215069
	TOR-26	FPF	Moguer	Root	Camarosa	-	KX215070
	TOR-28	FPF	Moguer	Root	Camarosa	-	KX215071
	TOR-32	FPF	Moguer	Root	Camarosa	-	KX215072
	TOR-53	FPF	Moguer	Crown	Camarosa	-	KX215073
	TOR-57	FPF	Moguer	Root	Camarosa	-	KX215074
<i>F. proliferatum</i>	TOR-23	FPF	Moguer	Crown	Camarosa	-	KX215075
	TOR-55	FPF	Moguer	Root	Camarosa	-	KX215076
	TOR-115 III	NRP	Ávila	Crown	San Andreas	-	KX215077
	FPOST-90	FPF	Cartaya	Crown	Sabrina	-	KX215079
	FPOST-117	FPF	Cartaya	Crown	Fortuna	-	KX215080
<i>F. commune</i>	TOR-58	FPF	Moguer	Root	Camarosa	-	KX215081
	TOR-115 IV	NRP	Ávila	Crown	San Andreas	-	KX215082
	TOR-116 IV	NRP	Ávila	Root	San Andreas	-	KX215083
	FPOST-153	FPF	Cartaya	Crown	Fortuna	-	KX215084
<i>F. equiseti</i>	TOR-9	FPF	Moguer	Root	Camarosa	-	KX215087
	TOR-45	FPF	Moguer	Crown	Camarosa	-	KX215085
	FPOST-15	FPF	Palos	Crown	Fortuna	-	KX215086
<i>F. tricinctum</i>	TOR-17	FPF	Moguer	Root	Camarosa	-	KX215088

Analyses of runner plants showed that *M. phaseolina* was not present, based on qPCR assays, in any of the investigated seasons (Table 3). In contrast, *P. cactorum* was detected in 1.2, 3.0, and 15.4% of runner plants in the 2011–12, 2012–13, and 2013–14 seasons, respectively (Table 3). *Fusarium* spp. isolates were recovered from conventional isolation assays in 16.9, 16.5, and 30% of the runner plants analyzed in the three seasons (Table 3). Nine of the *Fusarium* spp. isolates (six *F. oxysporum*, one *F. proliferatum*, and two *F. commune*) were included in the pathogenicity assays, but none were pathogenic (Table 2, see below).

Plants from fruit production fields. Over the study period, several isolates of *M. phaseolina*, *P. cactorum*, and *Fusarium* spp. were recovered by plate culturing from declining or dead plants, mainly from the selected macrotunnels in Cartaya and Palos, but also from different locations in Huelva province. The total percentage of symptomatic plants infected by *M. phaseolina* reached 4.2% in the investigated macrotunnels in the three seasons analyzed, reaching a maximum of 9.3% of plants infected with *M. phaseolina* in the Palos production field in the 2012–13 season. *P. cactorum* was isolated from 3.7% of the analyzed plants with maximum infection occurring in the Cartaya plots in the 2013–14 season (21.1%). Seven percent of the plants contained *Fusarium* spp., with a maximum of 29.6% plants identified in the Cartaya plots in the 2013–14 season (Table 4). A total of 57 *Fusarium* spp. isolates were tested for pathogenicity, of which one *F. oxysporum* and five *F. solani* isolates were pathogenic (Table 2, see below).

Morphological and molecular characterization of *Fusarium* spp. Isolates identified by morphological characteristics were confirmed by

sequencing of a portion of the *EF-1 α* gene. Fifty three percent of the 88 evaluated *Fusarium* spp. isolates were identified as *F. oxysporum*, 13% as *F. solani*, 12% as *F. avenaceum*, 7.6% as *F. acuminatum*, 5.4% as *F. proliferatum*, 4.3% as *F. commune*, 3.3% as *F. equiseti*, and 1.1% as *F. tricinctum* (Table 2).

Pathogenic characterization of *Fusarium* spp. isolates. Noninoculated control plants showed no symptoms during the experiments and no pathogens were reisolated. FPOST-140 was the only *F. oxysporum* isolate, obtained from a fruit production plant, which caused disease in strawberry plants from the 39 *F. oxysporum* isolates tested (Table 2). Thirty seven percent of the plants died when inoculated with FPOST-140, and symptoms included a decrease in the development of new roots (27%), crown necrosis (16%), and a reduction in plant fresh weight (33%) that differed significantly from the uninoculated control (Table 5). At the end of the experiment, FPOST-140 was reisolated from root, crown, and petiole tissues.

F. avenaceum, *F. acuminatum*, *F. commune*, *F. equiseti*, *F. proliferatum*, and *F. tricinctum* isolates did not cause disease symptoms in inoculated strawberry plants. Percentage of new roots, percentage of crown necrosis, and fresh plant weight of the plants inoculated with these isolates did not significantly differ from uninoculated controls (data not shown), although all were recovered from plant tissues at the end of the experiment. However, seven out of eight *F. solani* isolates recovered from fruit production field soils (TOR-393 and TOR-725) and diseased plants (TOR-397, FPOST-169, FPOST-174, FPOST-176, and E-III) caused a significant reduction in the growth of strawberry plants (Table 6). The most aggressive *F. solani* isolate was TOR-393, which caused 100% mortality of the inoculated

Fig. 1. Quantification of *Macrophomina phaseolina* (A) and *Phytophthora cactorum* (B) (pg DNA/g soil) in nursery soils from Segovia, Ávila, and Valladolid provinces in three consecutive strawberry seasons (2011–12, 2012–13, and 2013–14) by qPCR. Each bar is the average of two fumigated and two nonfumigated plots per location and season, except for Ávila in 2012–13 and 2013–14 where just one fumigated and one nonfumigated plot was analyzed. NF: nonfumigated soils; 1,3D+CP: soils fumigated with 1,3-dichloropropene:chloropicrin; Dazomet: soils fumigated with 3,5-dimethyl-1,3,5-thiadiazinane-2-thione. nt: not tested. Bars containing "*" differed significantly ($P = 0.05$) from the other treatments. Pairwise comparisons were done between fumigated and nonfumigated soils per location and season.

plants, a 99% reduction of new roots, an 87% increase of crown rot, and a 34% decrease of fresh plant weight; all these parameters significantly different from uninoculated controls. Isolates TOR-725 and FPOST-169 caused 75% plant mortality, and isolate TOR-397 caused 62.5% plant mortality. The rest of the *F. solani* isolates caused mortality in 38 to 50% of plants. This resulted in a significant decrease (50 to 75%) in the percentage of new roots after transplanting and a significant reduction of fresh weight (64 to 81%), compared with noninoculated control plants. The percentage of crown necrosis ranged from 21 to 87%, being significantly higher than the uninoculated control (Table 6). Pathogenic *F. solani* isolates were reisolated from root, crown, and petiole tissues.

Specific PCR detection of *F. oxysporum* f. sp. *fragariae*. The PCR assay yielded the expected 239 bp band for the Japanese *Fof* reference isolate (MAFF 744009) (data not shown). None of the *F. oxysporum* isolates from declining strawberry plants from the current study were amplified using PCR primers *FofraF* and *FofraR*, which included the pathogenic *Fof* isolate FPOST-140. No amplifications were also obtained for the reference *Fof* isolates from Spain (G2-4), Australia (ATCC 18138), and South Carolina (NRRL 26438).

Discussion

The inoculum sources of the strawberry soilborne pathogens *M. phaseolina* and *P. cactorum* and the importance of *F. oxysporum*

Fig. 2. Quantification of *Macrophomina phaseolina* (A) and *Phytophthora cactorum* (B) (pg DNA/g soil) in soils from two strawberry production fields of the Huelva province (Palos de la Frontera and Cartaya) in three consecutive seasons (2011–12, 2012–13, and 2013–14) by qPCR. Each bar is the average of three analyzed macrotunnels per location and season. FUM: Fumigated soils before planting (September–October); POST: Postharvest soils at the end of the production season (May–June). Bars containing "*" were significantly different ($P = 0.05$) from the other treatments. Pairwise comparisons were done between fumigated and nonfumigated soils per location and season.

Table 3. Percentage of nursery mother and runner plants from which *Macrophomina phaseolina* (MP) and *Phytophthora cactorum* (PC) were detected by qPCR, and from which *Fusarium* spp. (F) was obtained through isolation studies

	Nursery province	2011–12				2012–13				2013–14			
		No. plants analyzed	MP	PC	F	No. plants analyzed	MP	PC	F	No. plants analyzed	MP	PC	F
Mother plants	Segovia	100	0	0	6.0% (6/100)	40	0	0	7.5% (3/40)	42	0	0	14.3% (6/42)
	Valladolid	54	0	0	22.2% (12/54)	60	0	0	10.0% (6/60)	0	-	-	-
	Ávila	160	0	0	11.9% (19/160)	80	0	0	10.0% (8/80)	165	0	0	11.5% (19/165)
	Total	314	0	0	11.7% (37/314)	180	0	0	9.4% (17/180)	207	0	0	12.1% (25/207)
Runner plants	Segovia	131	0	2.3% (3/131)	22.1% (29/131)	70	0	4.3% (3/70)	14.3% (10/70)	216	0	16.2% (35/216)	35.2% (76/216)
	Valladolid	66	0	0.0% (0/66)	15.5% (10/66)	20	0	5.0% (1/20)	5.0% (1/20)	41	0	7.3% (3/41)	31.7% (13/41)
	Ávila	283	0	1.1% (3/283)	14.8% (42/283)	110	0	1.8% (2/110)	20.0% (22/110)	74	0	17.5% (13/74)	17.6% (13/74)
	Total	480	0	1.2% (6/480)	16.9% (81/480)	200	0	3.0% (6/200)	16.5% (33/200)	340	0	15.4% (51/340)	30.0% (102/340)

f. sp. *fragariae* and other *Fusarium* spp. in Spanish strawberry crops were investigated in soils and plant samples from Spanish nurseries and fruit production fields over three consecutive seasons. Sensitive and high throughput qPCR methods were applied for the specific detection and quantification of *M. phaseolina* and *P. cactorum*. The study indicated that ineffectively fumigated soils from nurseries and fruit production fields, and infected nursery runner plants, are the main sources of inoculum for some of these pathogens. *F. solani* was identified as the most important *Fusarium* spp. in fruit production fields.

M. phaseolina was detected in fumigated soils from nurseries and fruit production fields but not in nursery mother or runner plants. The fact that this pathogen could not be detected in runner plants could be due to inadequate environmental conditions and/or that the low inoculum density detected in nursery soils were below levels required for

M. phaseolina to cause infections. This phenomenon has previously been reported for *F. oxysporum* f. sp. *fragariae* (Koike and Gordon 2015), and it is thus possible that *M. phaseolina* could be present in the soil for several years without diseased plants being detected due to fumigation maintaining inoculum levels below levels that can cause disease. It could also be possible that the pathogen was not detected in runner plants since if the incidence in runner plants was very low, the sampling strategy would have resulted in infected runner plants not being sampled. For example, if 1,020 plants were tested, infection levels below 0.1% of plants would not be detected.

The presence of this pathogen in nursery soils may continue to be problematic, due to rotation crops used in nurseries, such as sunflower and leguminous species including soybean, being common hosts of this pathogen (Atiq et al. 2001; Dhingra and Sinclair

Table 4. Percentage of declining and dead strawberry plants from fruit production fields from which *Macrophomina phaseolina*, *Phytophthora cactorum*, and *Fusarium* spp. were detected through isolation studies

Locality	Season	No. plants sampled	No. symptomatic plants	Percentage of infected plants ^z		
				<i>M. phaseolina</i>	<i>P. cactorum</i>	<i>Fusarium</i> spp.
Cartaya	2011–12	4,300	253	5.8 (249/4,300)	0.0 (0/4,300)	2.3 (101/4,300)
	2012–13	6,450	829	3.2 (208/6,450)	4.0 (259/6,450)	8.8 (567/6,450)
	2013–14	2,150	713	3.3 (72/2,150)	21.1 (453/2,150)	29.6 (637/2,150)
Palos	2011–12	2,150	199	3.1 (66/2,150)	7.4 (160/2,150)	9.0 (194/2,150)
	2012–13	4,300	420	9.3 (399/4,300)	0.0 (0/4,300)	3.5 (151/4,300)
	2013–14	4,300	6	0.0 (0/4,300)	0.1 (2/4,300)	0.1 (3/4,300)
TOTAL		23,650	2,420	4.2	3.7	7.0

^z Plants infected with *M. phaseolina*, *P. cactorum*, and *Fusarium* spp. in single or mixed infections.

Table 5. Mean disease incidence, percentage of newly emitted roots, percentage of crown rot, and plant fresh weight of ‘Camarosa’ strawberry plants inoculated with *Fusarium* spp. isolates grown under growth-chamber conditions^y

Isolates	Dead plants (%)	New roots (%) ^z (<i>P</i> = 0.0001)	Crown rot (%) ^z (<i>P</i> = 0.0001)	Plant fresh weight (g) ^z (<i>P</i> = 0.0001)
Control	0	43.8 ± 3.2 ab	0.0 ± 0.0 a	8.2 ± 0.8 a
<i>F. commune</i>				
FPOST-153	0	38.8 ± 3.9 abc	0.0 ± 0.0 a	6.3 ± 0.7 bcd
<i>F. oxysporum</i>				
MAFF 744009	100	16.9 ± 5.0 d	66.25 ± 16.5 c	1.6 ± 0.2 f
NRRL 26438	62.5	20.0 ± 3.5 cd	18.8 ± 14.0 b	1.5 ± 0.3 f
FPOST-140	37.5	31.9 ± 9.3 cd	16.3 ± 12.1 b	5.5 ± 0.7 cde
FPOST-143	0	38.1 ± 3.9 abc	0.0 ± 0.0 a	7.3 ± 0.6 abc
FPOST-162	0	53.8 ± 4.5 a	0.0 ± 0.0 a	7.0 ± 0.5 abcd
FPOST-170	0	30.0 ± 4.2 bc	0.0 ± 0.0 a	4.4 ± 0.4 e
FPOST-172	0	43.1 ± 3.0 ab	0.0 ± 0.0 a	6.8 ± 0.6 abcd
FPOST-179	0	43.1 ± 3.9 ab	0.0 ± 0.0 a	5.4 ± 0.6 de
<i>F. proliferatum</i>				
FPOST-117	0	53.8 ± 3.2 a	4.4 ± 4.4 ab	7.6 ± 0.8 ab

^y Growth chamber adjusted at 25/18°C and 60/40% relative humidity (day/night) and 16-h photoperiod over 3 months.

^z Values are means ± standard error of two experiments with eight plants per isolate. Different letters within columns indicate significant differences among treatments according to the LSD test (*P* = 0.05).

Table 6. Mean disease incidence, percentage of newly emitted roots, percentage of crown rot, and plant fresh weight of ‘Camarosa’ strawberry plants inoculated with *Fusarium solani* isolates grown under growth-chamber conditions^y

Isolates	Dead plants (%)	New roots (%) ^z (<i>P</i> = 0.0003)	Crown rot (%) ^z (<i>P</i> = 0.0001)	Plant fresh weight (g) ^z (<i>P</i> = 0.0001)
Control	0 (0/16)	99.5 ± 0.0 a	0.0 ± 0.0 a	35.0 ± 1.6 a
TOR-393	100 (16/16)	0.5 ± 0.0 d	86.8 ± 7.1 d	2.3 ± 0.2 c
TOR-397	62.5 (10/16)	37.6 ± 18.1 cd	43.8 ± 14.1 bc	9.4 ± 3.8 bc
TOR-725	75 (12/16)	25.3 ± 16.2 cd	53.1 ± 14.6 bc	6.8 ± 2.9 bc
FPOST-164	0 (0/16)	87.1 ± 10.5 ab	1.1 ± 0.6 a	34.0 ± 2.6 a
FPOST-169	75 (12/16)	25.3 ± 16.2 cd	46.3 ± 14.3 bc	6.9 ± 2.9 bc
FPOST-174	50 (8/16)	47.1 ± 17.7 c	53.0 ± 17.7 cd	12.1 ± 4.1 b
FPOST-176	50 (8/16)	50.0 ± 18.7 bc	53.1 ± 14.7 bcd	9.0 ± 2.4 bc
E-III	50 (8/16)	50.0 ± 50.0 bc	21.3 ± 9.6 ab	12.7 ± 3.5 b

^y Growth chamber adjusted at 25/18°C and 60/40% relative humidity (day/night) and 16-h photoperiod over 3 months.

^z Values are means ± standard error of two experiments with eight plants per isolate. Different letters within columns indicate significant differences among treatments according to the LSD test (*P* = 0.05).

1973) and thus acting as natural reservoirs. This is the first time *M. phaseolina* has been reported in nursery soils in Spain.

P. cactorum was detected in fumigated nursery and fruit production soils and also in nursery runner plants. The presence of *P. cactorum* in runner plants highlights the fact that these plants could be infected through ineffectively fumigated nursery soils and could thus be another source of inoculum for *P. cactorum*. An additional source of inoculum could be irrigation water, which was not investigated in the current study. Irrigation water has been shown in other crops to be an inoculum source of *P. cactorum* (Bienapfl and Balci 2014; Rivero et al. 2011).

Spanish certified strawberry plants, under the rules of the technical regulation of control and certification of nursery-grown fruit-bearing plants (BOE, 12 February 2011), are under legal obligation to be free of *P. cactorum* and other pathogens before sales. Due to this successful certification, all the analyzed mother plants were negative for *P. cactorum*. Although not identified in any of the mother plants analyzed, *P. cactorum* was detected in an increasing percentage of runner plants over the three seasons studied, which is a pathway for introduction of this pathogen into fruit production fields and which means that runner plants were infested through *P. cactorum*-contaminated nursery soils. In the present study, we have showed that the use of an easy sample preparation method joined to the sensitivity of qPCR could be a powerful tool for the preventative detection of *P. cactorum* in mother and runner plants by the regulatory authorities.

Fusarium spp. were detected in all the fruit production field soils analyzed and in a high percentage of plant material from nurseries and fruit production fields. This is not surprising since *Fusarium* spp. are common soil inhabitants and endophytes in plants (Kuldau and Yates 2000). The inoculum sources of *Fusarium* spp. could not be elucidated since most isolates tested for pathogenicity were obtained from diseased field production plants. The most abundant *Fusarium* species detected was *F. oxysporum* (56% of total *Fusarium* spp. analyzed) (Table 2). *F. oxysporum* f. sp. *fragariae* has been associated with crown and root disease of strawberry in Australia (Winks and Williams 1965), Asia (Okamoto et al. 1970), North America (Koike et al. 2009), and Europe (Stanković et al. 2014), causing devastating losses. In Spain, *F. oxysporum* f. sp. *fragariae* was reported in 2007, when wilted and dead strawberry plants were observed in a soilless culture system by Arroyo et al. (2009). Currently, the incidence of *F. oxysporum* f. sp. *fragariae* in Spanish strawberry crops seems to be low, since only one of 39 *F. oxysporum* isolates tested in this study proved pathogenic to strawberry, even when a susceptible cultivar was used (Fang et al. 2012). The pathogenic isolate was recovered from the crown of a diseased plant from a fruit production field, but its inoculum source is unknown and could have originated from the production field soil or nursery plants. Plant material for generating mother plants comes largely from the U.S., where *F. oxysporum* f. sp. *fragariae* is a widespread pathogen that causes severe losses (Islas 2012; Koike and Gordon 2015). Since the use of infected plant material is one of the major causes of introducing a new pathogen into a cropping area, fast, sensitive, and specific detection methods are critical. Suga et al. (2013) developed a PCR assay to differentiate between strawberry-pathogenic and non-pathogenic *F. oxysporum* isolates. However, the only strawberry-pathogenic isolate identified in the present study, FPOST-140, did not yield the diagnostic fragment described as being specific for *F. oxysporum* f. sp. *fragariae*. Similarly, other strawberry-pathogenic isolates from culture collections analyzed in the present study including ATCC 18138 (Australia), G2-4 (Spain), and NRRL 26438 (South Carolina) did not yield the diagnostic amplicon. This result is consistent with findings of other studies where some strains that were identified as pathogens by pathogenicity testing did not yield the diagnostic fragment by PCR, and vice versa (Henry et al. 2017; Suga et al. 2013). Therefore, this molecular marker has proved to be useful for only a subset of *F. oxysporum* f. sp. *fragariae* populations. In addition, the PCR assay requires DNA extraction and agarose gel analyses, making it impractical for the analysis of a large number of samples. However, a quick and accurate method, as the described for

M. phaseolina and *P. cactorum* in this study, could be valuable for *F. oxysporum* f. sp. *fragariae* once a specific marker is described.

Our results on the pathogenic characterization of *Fusarium* spp. isolates recovered from field production plants and soil are important since it determined that *F. solani* is a new threat to strawberry production in Spain. Although most of the *Fusarium* species tested in this study did not cause disease in strawberry, special attention should be paid to the emerging pathogen *F. solani* to determine its current incidence and origin in Spain and other countries. This fungus, recently described as pathogenic to strawberry, was isolated from soil, asymptomatic runner plants from nurseries, and symptomatic plants from crop fields (Pastrana et al. 2014). The current study supports this finding, since a high percentage of *F. solani* isolates from fruit-production soil and diseased field plants were pathogenic to strawberry.

Early diagnosis and detection of strawberry pathogens in nursery soils and mother and runner plants is critical for disease control in fruit production fields to avoid the introduction, establishment, and spread of soilborne pathogens. In this study, qPCR methods were applied to soil samples and plant material for the specific detection and quantification of *M. phaseolina* and *P. cactorum*. The developed assays used DNA from soil and diluted crude plant extracts from asymptomatic or diseased plants. Inoculum levels in soil samples were measured as the amount of pathogen DNA/g of soil, which were able to detect levels of inoculum as low as 1 pg DNA/g soil for *M. phaseolina* and *P. cactorum*. These low inoculum levels most likely originated from viable pathogen propagules, since DNA from dead fungi has been reported to be rapidly degraded and would thus have a limited contribution to the total DNA level (Herdina et al. 2004). The use of crude plant extracts constitutes a simple and direct sample-preparation method for real-time PCR, avoiding the DNA-extraction step and allowing the high throughput analysis of a large number of plants. The use of a positive internal control in qPCR assays prevents the detection of false negatives, i.e., samples not amplified due to the presence of PCR inhibitors in the soil or plant extracts.

We conclude that ineffectively fumigated soils in nurseries and fruit production fields and infected runner plants can be sources of inoculum of some, but not all soilborne fungi and oomycetes that cause strawberry diseases in Spain. Preventive control measures such as the use of powerful molecular tools for the early and high throughput detection of fungal pathogens in mother and runner plants would be an effective strategy to decrease the introduction of pathogens into production fields that reduce yields. In addition, an integrated management strategy of these strawberry diseases should be considered including the use of resistant varieties (Avilés et al. 2009; Eikemo and Stensvand 2015), anaerobic disinfection methods (Shennan et al. 2016), and biological control agents (Pastrana et al. 2016), which have shown potential for being effective nonchemical alternatives.

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Literature Cited

- Arroyo, F. T., Llergo, Y., Aguado, A., and Romero, F. 2009. First report of *Fusarium* wilt caused by *Fusarium oxysporum* on strawberry in Spain. *Plant Dis.* 93:323.
- Atiq, M., Shabeer, A., and Ahmed, I. 2001. Pathogenic and cultural variation in *Macrophomina phaseolina*, the cause of charcoal rot in sunflower. *Sarhad J. Agric.* 2:253-255.
- Avilés, M., Castillo, S., Bascón, J., Zea-Bonilla, T., Martín-Sánchez, P. M., and Pérez-Jiménez, R. M. 2008. First report of *Macrophomina phaseolina* causing crown and root rot of strawberry in Spain. *Plant Pathol.* 57:382.

- Avilés, M., Castillo, S., Borrero, C., Castillo, M. L., Zea-Bonilla, T., and Pérez-Jiménez, R. M. 2009. Response of Strawberry Cultivars: 'Camarosa', 'Candonga' and 'Ventana' to Inoculation with Isolates of *Macrophomina phaseolina*. Pages 291-294 in: VI International Strawberry Symposium, 842.
- Babu, B. K., Mesapogu, S., Sharma, A., Somasani, S. R., and Arora, D. K. 2011. Quantitative real-time PCR assay for rapid detection of plant and human pathogenic *Macrophomina phaseolina* from field and environmental samples. *Mycologia* 103:466-473.
- Bienapfl, J. C., and Balci, Y. 2014. Movement of *Phytophthora* spp. in Maryland's nursery trade. *Plant Dis.* 98:134-144.
- Capote, N., Pastrana, A. M., Aguado, A., and Sánchez-Torres, P. 2012. Molecular tools for detection of plant pathogenic fungi and fungicide resistance. Pages 151-202 in: *Plant Pathology*. C. J. Cumagun, ed. InTech, Rijeka, Croatia.
- Chamorro, M., Miranda, L., Domínguez, P., Medina, J. J., Soria, C., Romero, F., López-Aranda, J. M., and De los Santos, B. 2015. Evaluation of biosolarization for the control of charcoal rot disease (*Macrophomina phaseolina*) in strawberry. *Crop Prot.* 67:279-286.
- Cooke, D. E., Drenth, A., Duncan, J. M., Wagels, G., and Brasier, C. M. 2000. A molecular phylogeny of *Phytophthora* and related oomycetes. *Fungal Genet. Biol.* 30:17-32.
- Corpas-Hervias, C., Melero-Vara, J. M., Molinero-Ruiz, M. L., Zurera-Muñoz, C., and Basallote-Ureba, M. J. 2006. Characterization of isolates of *Fusarium* spp. obtained from *asparagus* in Spain. *Plant Dis.* 90:1441-1451.
- de los Santos, B., Porras, M., Blanco, C., Barrau, C., and Romero, F. 2002. First report of *Phytophthora cactorum* on strawberry plants in Spain. *Plant Dis.* 86:1051.
- Dhingra, O. D., and Sinclair, J. B. 1973. Location of *Macrophomina phaseolina* on soybean plants related to cultural characteristics and virulence. *Phytopathology* 63:934-936.
- Eikemo, H., and Stensvand, A. 2015. Resistance of strawberry genotypes to leather rot and crown rot caused by *Phytophthora cactorum*. *Eur. J. Plant Pathol.* 143: 407-413.
- Fang, X. L., Kuo, J., You, M. P., Finnegan, P. M., and Barbetti, M. J. 2012. Comparative root colonisation of strawberry cultivars Camarosa and Festival by *Fusarium oxysporum* f. sp. *fragariae*. *Plant Soil* 358:75-89.
- Gallegly, M. E., and Hong, C. 2008. Page 30 in: *Phytophthora. Identifying Species by Morphology and DNA Fingerprints*. APS Press, St. Paul, MN.
- Henry, P. M., Kirkpatrick, S. C., Islas, C. M., Pastrana, A. M., Yoshisato, J., Koike, S. T., Daugovich, O., and Gordon, T. R. 2017. The population of *Fusarium oxysporum* f. sp. *fragariae*, cause of Fusarium wilt of strawberry, in California. *Plant Dis.* (in press). doi:10.1094/PDIS-07-16-1058-RE
- Herdina, N. S., Neate, S., Jabaji-Hare, S., and Ophel-Keller, K. 2004. Presistence of DNA of *Gaeumannomyces graminis* var. *tritici* in soil as measured by DNA-based assay. *FEMS Microbiol. Ecol.* 47:143-152.
- Inderbitzin, P., Harkness, J., Turgeon, B. G., and Berbee, M. L. 2005. Lateral transfer of mating system in *Stemphylium*. *Proc. Natl. Acad. Sci. USA* 102: 11390-11395.
- Islas, C. M. 2012. Fusarium wilt caused by *Fusarium oxysporum* f. sp. *fragariae*: An emerging disease of strawberry in California. Thesis, Master of Science. University of California, Davis.
- Koike, S. T., and Gordon, T. R. 2015. Management of Fusarium wilt of strawberry. *Crop Prot.* 73:67-72.
- Koike, S. T., Kirkpatrick, S. C., and Gordon, T. R. 2009. Fusarium wilt of strawberry caused by *Fusarium oxysporum* in California. *Plant Dis.* 93:1077.
- Kuldau, G. A., and Yates, I. E. 2000. Evidence for *Fusarium* endophytes in cultivated and wild plants. Pages 85-117 in: *Microbial endophytes*. C. W. Bacon and J. F. White Jr., eds. Marcel Dekker, New York.
- Leslie, J. F., and Summerell, B. A., eds. 2006. *The Fusarium laboratory manual*, 1st Ed. Backwell Publishing, Ames, IA.
- Li, M., Inada, M., Watanabe, H., Suga, H., and Kageyama, K. 2013. Simultaneous detection and quantification of *Phytophthora nicotianae* and *P. cactorum*, and distribution analyses in strawberry greenhouses by duplex real-time PCR. *Microbes Environ.* 28:195-203.
- Li, Y., Mao, L., Yan, D., Ma, T., Shen, J., Guo, M., Wang, Q., Ouyang, C., and Cao, A. 2014. Quantification of *Fusarium oxysporum* in fumigated soils by a newly developed real-time PCR assay to assess the efficacy of fumigants for Fusarium wilt disease in strawberry plants. *Pest Manag. Sci.* 70:1669-1675.
- Maas, J. L. 1998. *Compendium of Strawberry Diseases*, 2nd ed. APS Press, St. Paul, MN.
- Martin, F. N. 2003. Development of alternative strategies for management of soilborne pathogens currently controlled with methyl bromide. *Annu. Rev. Phytopathol.* 41:325-350.
- Mirmajlessi, S. M., Destefanis, M., Gottsberger, R. A., Mänd, M., and Loit, E. 2015. PCR-based specific techniques used for detecting the most important pathogens on strawberry: a systematic review. *Syst. Rev.* 4:9.
- O'Donnell, K., Gueidan, C., Sink, S., Johnston, P. R., Crous, P. W., Glenn, A., Riley, R., Zitomer, N. C., Colyer, P., Waalwijk, C., Lee, T., Moretti, A., Kang, S., Kim, H. S., Geiser, D. M., Juba, J. H., Baayen, R. P., Crome, M. G., Bithell, S., Sutton, D. A., Skovgaard, K., Ploetz, R., Corby Kistler, H., Elliott, M., Davis, M., and Sarver, B. A. 2009. A two-locus DNA sequence database for typing plant and human pathogens within the *Fusarium oxysporum* species complex. *Fungal Genet. Biol.* 46:936-948.
- O'Donnell, K., Kistler, H. C., Cigelnik, E., and Ploetz, R. C. 1998. Multiple evolutionary origins of the fungus causing Panama disease of banana: concordant evidence from nuclear and mitochondrial gene genealogies. *Proc. Natl. Acad. Sci. USA* 95:2044-2049.
- Okamoto, H., Fujii, S., Kato, K., and Yoshioka, A. 1970. A new strawberry disease 'Fusarium wilt'. *Plant Prot.* 24:231-235.
- Pastrana, A. M., Basallote-Ureba, M. J., Aguado, A., Akdi, K., and Capote, N. 2016. Biological Control of strawberry soil-borne pathogens *Macrophomina phaseolina* and *Fusarium solani*, using *Trichoderma asperellum* and *Bacillus* spp. *Phytopathol. Mediterr.* 55:109-120.
- Pastrana, A. M., Capote, N., De los Santos, B., Romero, F., and Basallote-Ureba, M. J. 2014. First report of *Fusarium solani* causing crown and root rot on strawberry crops in Southwestern Spain. *Plant Dis.* 98:161.
- Pérez-Jiménez, R. M., De Cal, A., Melgarejo, P., Cubero, J., Soria, C., Zea-Bonilla, T., and Larena, I. 2012. Resistance of several strawberry cultivars against three different pathogens. *SJAR* 10:502-512.
- Rivero, V. I., Giayetto, A., Rossini, M., and Vera, D. 2011. Detection of *Phytophthora cactorum* in the irrigation water in commercial orchards of 'Bartlett' pear in Villa Regina, Rio Negro, Argentina. *Acta Hortic.* 909:521-526.
- Shennan, C., Muramoto, J., Baird, G., Zavatta, M., Toyama, L., Mazzola, M., and Koike, S. T. 2016. Anaerobic soil disinfestation (ASD): a strategy for control of soil borne diseases in strawberry production. *Acta Hortic.* 1137:113-120.
- Stanković, I., Ristić, D., Vučurović, A., Milojević, K., Nikolić, D., Krstić, B., and Bulajić, A. 2014. First report of Fusarium wilt of strawberry caused by *Fusarium oxysporum* in Serbia. *Plant Dis.* 98:1435.
- Suga, H., Hirayama, Y., Morishima, M., Suzuki, T., Kageyama, K., and Hyakumachi, M. 2013. Development of PCR Primers to Identify *Fusarium oxysporum* f. sp. *fragariae*. *Plant Dis.* 97:619-625.
- Sutton, B. C. 1980. *The Coelomycetes*. CAB International, Kew, Surrey, U.K.
- Winks, B. L., and Williams, Y. N. 1965. A wilt of strawberry caused by a new form of *Fusarium oxysporum*. *Queensl. J. Agric. Anim. Sci.* 22:475-479.