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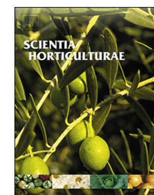

A TaqMan real-time polymerase chain reaction assay for accurate detection and quantification of *Fusarium solani* in strawberry plants and soil

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ABSTRACT

A new TaqMan real-time polymerase chain reaction (qPCR) was developed to accurately detect and quantify the soil-borne fungus *Fusarium solani*, pathogen to strawberry, in plant and soil samples. The assay was designed using sequences from the translation elongation factor 1 alpha (*EF-1α*) gene. The assay is highly specific: it specifically detected *F. solani* isolates when tested against 95 fungal isolates from 27 different species. The detection limit of the assay was 50 fg for *F. solani* genomic DNA and 10^2 conidia/g soil. A significant correlation ($P = 0.0002$) was observed between the amount of genomic DNA of *F. solani* detected by qPCR and the number of fungal propagules present in artificially inoculated soils. The effectiveness of the assay was validated by comparing it to traditional methods for the detection of *F. solani* in diseased strawberry plants and pre-planting soils from strawberry fields. A substantial and moderate correlation was found between the qPCR-based and traditional detection methods in diseased plants and soils. The amount of *F. solani* DNA estimated in roots and crowns of symptomatic strawberry plants ranged from 16 to 190 pg/mg fresh tissue. Inoculum densities in pre-planting soils varied between 3.1×10^2 and 1.3×10^5 colony-forming units per gram (CFU/g) of soil. Effectiveness was also evaluated by assessing the ability of the assay to detect decreasing levels of *F. solani* populations during biosolarization treatment. Taken together, this novel qPCR assay represents a useful tool for rapid assessment of pre-planting soils and nursery plants to prevent *F. solani* infection and production losses.

1. Introduction

Fusarium solani (Mart.) Appel & Wollenw. is a ubiquitous soil-borne fungus that can infect a wide range of plant species (Kolattukudy and Gamble 1995). *F. solani* has been described as the causal agent of crown and root rot in strawberry—a disease that causes stunted growth, wilting and/or plant death (Pastrana et al., 2014). The fungus was detected in soils and asymptomatic plant material from strawberry nurseries, and in soils and diseased plants from fruit production fields in Spain (Pastrana et al., 2014; Redondo et al., 2012) and causing fruit rot in Pakistan (Mehmood et al., 2017).

Strawberry crop in Spain is also threatened by another soil-borne pathogen of the genus Fusarium, *F. oxysporum* f. sp. *fragariae* (*Fof*), detected as a point outbreak in soilless grown strawberries in 2008 (Arroyo et al., 2009). Several years later, only one isolation was reported from plants cultivated on soil in prospections made in nurseries and fruit production fields along 2011–2014 seasons (Pastrana et al., 2017a). New focuses of *F of* have recently appeared in fruit production fields, probably related to the phasing out of most of the chemical soil fumigants used in European Union (Borrero et al., 2017).

F. solani is well adapted to long-term survival in soil as it can form resistant structures named chlamydospores (Schippers et al., 1981). It can also persist in organic plant residues (Booth, 1971), and asymptomatically colonize other plant species (Molinero-Ruiz et al., 2011; Pastrana et al., 2017b). These features make eradication quite difficult because chlamydospores, conidia and mycelia may serve as source of inoculum for infection in subsequent growing seasons. Specifically, in strawberry crops, infected plants from nurseries and not effectively disinfested soils could serve as sources of infection in fruit production plants (Pastrana et al., 2017a).

Studies on current incidence in strawberry nurseries and fruit production fields and inoculum density in soils are scarce because it was only recently described as pathogen for strawberry. Methods to detect and identify the fungus are currently based on plate culturing of small

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pieces of plant tissues and soil dilutions, followed by morphological characterization. These traditional methods are laborious, time-consuming and not always accurate due to the presence of other *Fusarium* species in propagative plant material and soil, and due to the irregular distribution of the fungus in these samples. In contrast, molecular methods based in quantitative real-time polymerase chain reaction (qPCR) enable the accurate identification and quantification of target samples and, due to its feasibility and high throughput, the rapid analysis of a large number of samples. This technique has been successfully applied for the specific detection of members of *Fusarium solani* species complex (FSSC), such as *F. virguliforme,* which is the causal agent of soybean sudden death syndrome (Gao et al., 2004; Li et al., 2008; Mbofung et al., 2011); and *F. solani* f. sp. *phaseoli,* the causal agent of bean root rot (Filion et al., 2003). It has also been used clinically for the detection of *F. solani*, which can cause keratitis of the eye in humans (Bernal-Martínez et al., 2012; Muraosa et al., 2014).

Control strategies such as biosolarization are emerging as sustainable alternatives to the use of chemicals for soil disinfection in strawberry crop management (Chamorro et al., 2015). The use of the solarization technique combined with the application of organic amendments, such as fresh chicken manure, has provided promising results in terms of soil productivity and crop yields (De los Santos et al., 2017). To date, no study exists that has reported the effect of biosolarization on *F. solani* inoculum density in strawberry soils and its effect on disease control and plant yields. Furthermore, no study exists that details the use of qPCR for this purpose. Therefore, the objectives of this work were (1) to develop a duplex qPCR assay to detect and quantify *F. solani* in strawberry plant tissues and soil samples, including the detection of an internal positive control (IPC); (2) to validate the developed qPCR assay by comparing it with traditional detection methods (plant tissue cultures and soil plate-counting techniques); and (3) to apply the qPCR assay in a real-world scenario by assessing the efficiency of biosolarization to increase strawberry fruit production.

2. Material and methods

2.1. Isolation of Fusarium solani from plant and soil

A total of 66 *F. solani* isolates were collected from 93 diseased strawberry plants and 26 soil samples from strawberry fruit producing fields at Huelva province in Southwestern Spain during 2014–2016 strawberry seasons (Table 1).

Sampled diseased plants were carefully washed under tap water and cut longitudinally in half. Small pieces from one half (crowns and roots) were surface disinfested in 1% sodium hypochlorite for 2 min, rinsed in sterile distilled water, air-dried, placed on potato dextrose agar (PDA) (Difco) plates, and incubated at 25 °C for 2 days in darkness and 5 days at a 12 h photoperiod (OSRAM L 18 W/21-840 Hellweiss Lumilux Cool White, 75 $\mu E\, m^{-2} s^{-1}$). The other longitudinal half of the plant was used for DNA extraction and further detection and quantification of *F. solani* by qPCR (see below).

Soil samples were collected one month before standard soil fumigation. Twelve samples (0–20 cm depth, approximately 100 g) taken at random in each plot were pooled and mixed as a composite sample. Soil samples were air-dried for 7–10 days at room temperature, passed through a 1 mm-mesh sieve and stored at 5 °C until used. Five grams of the composite soil sample were passed through a 1 mm-mesh sieve, 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 dilutions (1:1, 1:10 and 1:100) were spread on V8 agar plates (5 plates/soil sample) and incubated at 25 °C for two days in darkness and three additional days with a 12 h photoperiod. Three replicates per plot were analyzed.

2.2. Characterization of F. solani isolates

Fusarium solani-like colonies from plant material and soils were selected and identified based in morphological characteristics (Leslie and Summerell, 2006). DNA from single spore cultures was isolated using the Isolate II Plant DNA Kit (Bioline, London UK), while following the manufacturers' instructions with some modifications: Fungal mycelium grown on PDA was scraped from the plate, placed into a centrifuge tube containing lysis buffer (Bioline) and homogenized 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 using a ND-1000 NanoDrop spectrophotometer (NanoDrop Products, Wilmington, DE). Species identification was confirmed by sequencing of a portion of the translation elongation factor-1 alpha (*EF-1α*) gene using primers EF1 and EF2 (O'Donnell et al., 1998). Fungal cultures were stored in 20% (v/v) glycerol at −80 °C until use.

2.3. Design of duplex qPCR assay

Partial sequences of the *EF-1α* gene from *F. solani* isolates obtained during this study (Table 1), (Acc. No. KF275032-KF275034, KY486652-KY486705, and KX215048- KX215056) were aligned with *EF-1α* partial sequences from *Fusarium* species reported on GeneBank using MEGA 7.0 software (Kumar et al., 2016). Nucleotide sequences that showed specific consensus for FSSC Clade 3 (but not for the rest of *Fusarium* spp.) were selected and used with Primer Express software v2.0 (Applied Biosystems) to design forward/reverse primers and a TaqMan probe (Table 2). The 5′ end of the *F. solani* TaqMan probe was labeled with 6-FAM (fluorescein) and the 3′ end harbored a Non-Fluorescent Quencher (NFQ) and a Minor Groove Binder (MGB) molecule (Life Technology) to increase specificity. To discard false negative amplifications, an internal positive control (IPC) was designed consisting of 1 pg of lambda (λ) bacteriophage DNA added to each sample and amplified in duplex qPCR reactions. Primer Express software (v 2.0) was used to design primers λ-F and λ-R, and a TaqMan probe labeled with Cy5 fluorophore at the 5′ end and a BlackBerry Quencher (BBQ) quencher in the 3′ end (Table 2).

2.4. Specificity and sensitivity of the duplex TaqMan PCR assay

qPCR reactions were performed in 96-well plates in a Chromo 4™ thermocycler (Bio-Rad). Reaction cocktails contained 5 µL of DNA template (extracted from plant material or from artificially or naturally infested soils, see below), 1x SensiMix (SensiMix™ Probe No-Rox Kit, Bioline), 1 µM forward and reverse primers for *F. solani* (Fs-F and Fs-R), 100 nM forward and reverse primers for IPC (λ-F and λ-R), 250 nM TaqMan probe for *F. solani* (FAM-Fs-P), 100 nM TaqMan probe for IPC (Cy5-λ-P), 1 pg λ genomic DNA and 1 mg/ml BSA. Amplifications were carried out at 95 °C for 10 min, then 50 cycles of 10 s at 95 °C, followed by 1 min at 60 °C. Sterile distilled water was used as a non-template control.

For species specificity, *F. solani* primers and probe were tested on 95 described isolates, most of them commonly associated to strawberry diseases. Of these, 66 were *F. solani* from strawberry, 16 were *Fusarium* spp. and 10 were fungal species that were not related to *Fusarium* spp. (Table 1).

To evaluate the sensitivity and detection limit of the assay for *F. solani* in plant material, qPCR reactions were carried out with the designed primers and probe on decreasing concentration of DNA obtained from *F. solani* TOR-1 isolate (50 ng–50 fg of total genomic DNA) diluted in DNA from a healthy plant. Three replicates per sample were amplified and values obtained for each dilution series were used to generate standard curves for quantification of *F. solani* in strawberry plants.

To evaluate the sensitivity and detection limit of the assay for *F. solani* in soils, 5 g of sterile soil was artificially inoculated with 5 ml of *F. solani* TOR-1 conidial suspension serial dilutions (10^7, 10^6, 10^5, 10^4,

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