A Newly Developed Real-Time PCR Assay for Detection and Quantification of *Fusarium oxysporum* and Its Use in Compatible and Incompatible Interactions with Grafted Melon Genotypes

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ABSTRACT

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A reliable and species-specific real-time quantitative polymerase chain reaction (qPCR) assay was developed for detection of the complex soil-borne anamorphic fungus *Fusarium oxysporum*. The new primer pair, designed on the translation elongation factor 1- α gene with an amplicon of 142 bp, was highly specific to *F. oxysporum* without cross reactions with other *Fusarium* spp. The protocol was applied to grafted melon plants for the detection and quantification of *F. oxysporum* f. sp. *melonis*, a devastating pathogen of this cucurbit. Grafting technologies are widely used in melon to confer resistance against new virulent races of *F. oxysporum* f. sp. *melonis*, while maintaining the properties of valuable commercial varieties. However, the effects on the vascular pathogen colonization have not been fully investigated. Analyses were performed on

'Charentais-T' (susceptible) and 'Nad-1' (resistant) melon cultivars, both used either as rootstock and scion, and inoculated with *F. oxysporum* f. sp. *melonis* race 1 and race 1,2. Pathogen development was compared using qPCR and isolations from stem tissues. Early asymptomatic melon infections were detected with a quantification limit of 1 pg of fungal DNA. The qPCR protocol clearly showed that fungal development was highly affected by host–pathogen interaction (compatible or incompatible) and time (days postinoculation). The principal significant effect ($P \leq 0.01$) on fungal development was due to the melon genotype used as rootstock, and this effect had a significant interaction with time and *F. oxysporum* f. sp. *melonis* race. In particular, the amount of race 1,2 DNA was significantly higher compared with that estimated for race 1 in the incompatible interaction at 18 days postinoculation. The two fungal races were always present in both the rootstock and scion of grafted plants in either the compatible or incompatible interaction.

Additional keywords: *Cucumis melo*, Fusarium wilt, SYBR green.

Fusarium oxysporum Schltdl. is a fungal soilborne facultative parasite present worldwide. The species includes nonpathogenic and pathogenic strains, the latter causing vascular wilt and root rot on many economically important crops. The identification of pathogenic *F. oxysporum* isolates is traditionally based on pathogenicity testing, which is time consuming and laborious. Pathogenic *F. oxysporum* strains have been subdivided into over 100 different host-specific forms (formae speciales) which are morphologically indistinguishable and represent intraspecific groups of strains with similar or identical host ranges (6,21,27). A forma specialis can be further subdivided into races on the basis of characteristic virulence patterns on differential host cultivars (14). *F. oxysporum* f. sp. *melonis* W. C. Snyder & H. N. Hansen is specific to melon (*Cucumis melo* L.). Four races of the pathogen (races 0, 1, 2, and 1,2) have been defined according to host resistance genes overcome by variants of the pathogen (33).

Once introduced into the field, *F. oxysporum* f. sp. *melonis* can persist in soil over extended periods on crop residues and nonhost crops, and by forming chlamydospores (15). Although many *Fusarium* spp. can penetrate into the cortical tissue of roots, only

host-specific strains can penetrate the vascular elements by mycelial growth and form microconidia transported in the sap stream (6,25). Therefore, effective control can be achieved only through host resistance. Two dominant, independently-inherited resistance (*R*) genes (*Fom-1* and *Fom-2*) provide resistance to races 0 and 2, and races 0 and 1, respectively (33). Resistance to race 1,2 is complex and appears to be controlled by multiple recessive genes. Partial resistance was introgressed into 'Isabelle' melon (30), from which two doubled-haploid (DH) resistant lines, 'Nad-1' and 'Nad-2' were derived, which are resistant to *F. oxysporum* f. sp. *melonis* race 1 and race 1,2 (10). At present, only partially resistant genotypes are commercially available, mainly as rootstocks, to protect against race 1,2 damage. Grafting represents an effective tool for controlling *F. oxysporum* f. sp. *melonis* races (3), particularly applied against race 1,2, the most widespread and harmful *F. oxysporum* f. sp. *melonis* race (10,37). Research in grafting of melon started in the late 1920s (34). The main purpose was to reduce the damage of Fusarium wilt using resistant rootstocks. Successively, *Fom-1* and *Fom-2* resistant genes were extensively incorporated into melon cultivars, reducing the need to use grafting technologies in melon for some time. When *F. oxysporum* f. sp. *melonis* race 1,2 became widespread, melon rootstock cultivars with multiple disease resistances were developed and used extensively to avoid Fusarium wilt damage (34). Symptoms do not occur on susceptible scions when grafted on a resis-

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tant rootstock. Nevertheless, *F. oxysporum* f. sp. *melonis* is reported to be present inside the host in either the compatible or incompatible interaction, and has been reisolated from the stem of resistant melon genotypes (3,37,40).

Isolations from infected plants are commonly used to verify the presence of pathogens in the host at any given time point of pathogenesis. Further information on the pathogen spreading inside the host has been reported in literature with the employment of green fluorescent protein (GFP) as a marker, which clearly documented the progress of the infection for xylem-colonizing *F. oxysporum* strains (6,16,40), but failed to quantify the amount of fungal biomass in host tissues. During the last decade, new DNA-based technology, such as the polymerase chain reaction (PCR) assay, has been developed to support morphology-based identifications of phytopathogenic fungi. Two specific PCR assays have been developed for the detection of *F. oxysporum*, but none of them were quantitative in nature (8,26). Today, quantitative real-time PCR (qPCR) has become the most reliable methodology for detection and quantification of a variety of plant pathogens (7,19,36). In this context, the species-specific PCR primers developed by Mishra et al. (26) on the internal transcribed spacer ribosomal DNA region were successively adapted to qPCR and tested for their specificity against several *Fusarium* spp. or *F. oxysporum* formae speciales, including one isolate of *F. oxysporum* f. sp. *melonis* (18). Recently, a sequence-characterized amplified region (SCAR)-based real-time TaqMan PCR has been developed for *F. oxysporum* (23) and applied to *F. oxysporum* f. sp. *melonis*. Nevertheless, the specificity of this method was tested on few fungal strains, which do not represent the broad variability of the *F. oxysporum* species complex.

Different DNA regions have been used to design real-time PCR primers for identification of fungi. In particular, translation elongation factor α (TEF-1 α) is extensively used in *Fusarium* phylogeny, because it is highly informative at the species level, nonorthologous copies of the gene have not been detected in the genus, and universal primers have been designed (13,22). In this study, we report on a species-specific DNA primer pair developed on TEF-1 α . The newly developed protocol was used to detect and assess fungal DNA amount in grafted melon plants infected by *F. oxysporum* f. sp. *melonis* race 1 or race 1,2, in either compatible or incompatible interactions. Tissue stem fragments were taken at different heights and times in both the rootstock and the scion and used for either fungal reisolations or fungal biomass detection and quantification by qPCR.

The objectives of this research were to develop a species-specific and sensitive detection method for *F. oxysporum* to apply in melon plants, with the aim of quantifying *F. oxysporum* f. sp. *melonis* DNA within compatible and incompatible interactions in either grafted or ungrafted plants, and to understand the impact of grafting on the development of *F. oxysporum* f. sp. *melonis* inside the host.

MATERIALS AND METHODS

Host material. Two melon genotypes 'Charentais-T' (CHT-T), fully susceptible, and Nad-1, resistant to *F. oxysporum* f. sp. *melonis* race 1 and race 1,2, were grafted (3) in both reciprocal combinations and inoculated either with *F. oxysporum* f. sp. *melonis* race 1 (strain ISPaVe1070) or *F. oxysporum* f. sp. *melonis* race 1,2 (strain ISPaVe1018) obtained from a single spore culture. Inoculation was carried on at the four- to five-true-leaf stage by dipping the plant roots in a suspension of 1×10^6 conidia/ml for 30 min, according to Sestili et al. (37). Plants were kept in greenhouse under controlled conditions (10,37).

In preliminary experiments, grafted and ungrafted melon plants were analyzed for *F. oxysporum* f. sp. *melonis* reisolations at 2, 4, 14, and 18 days postinoculation (dpi), and 2 and 18 dpi were the most significant time points in stressing differences between the

two *F. oxysporum* f. sp. *melonis* races, as reported by Sestili et al. (37). Hence, only 2 and 18 dpi were further considered for either reisolations or qPCR analyses. At 18 dpi, all plants undergoing the compatible interaction displayed obvious wilting symptoms. Hence, melon plants grafted in both combinations were inoculated with either race 1 or race 1,2 and collected at 2 and 18 dpi. Water-inoculated grafted and ungrafted plants were used as controls. Three replicate plants (biological replicates) for each treatment (melon-genotype grafting combination $\times$ *F. oxysporum* f. sp. *melonis* race or water) and collection time were used for re-isolation assays or for qPCR analysis, while inoculated ungrafted plants of the same genotypes were used for comparison in qPCR only. All inoculations were repeated three times. A completely randomized distribution was adopted for the melon plants kept in the greenhouse under controlled conditions at $25 \pm 2^\circ\text{C}$ with 80 to 90% relative humidity and a 16-h photoperiod (10,37).

Analyses were performed on melon stems which were prepared by removing roots and discarding the basal 15 mm. Successively, they were abundantly washed under running tap water, surface disinfested in 1% NaOCl for 30 s followed by 2×30 s in sterile deionized water, and dried on sterile filter paper sheets in a laminar flow hood. The whole stem length considered was ≈ 70 mm and sections were excised from top to bottom (37).

Reisolations were performed only on grafted melon plants (CHT-T scion/Nad-1 rootstock and Nad-1/CHT-T). Stems were cut in six sections above and six below the grafting point for a total of 12 stem segments (≈ 5 mm long) per plant. Sections were placed in petri dishes onto potato dextrose agar (PDA; Oxoid, Basingstoke, UK) in an ordered manner (37). A completely randomized distribution was adopted for petri dishes incubated at room temperature for ≈ 7 days and examined for the appearance of fungal outgrowths.

For the qPCR assay tissue, two fragments above 45 to 70 mm and two below 15 to 40 mm of the stem length were excised in grafted and ungrafted melon plants. For ungrafted plants of Nad-1, only the late time point (18 dpi) was considered because the number of plants available was not sufficient to include the 2-dpi analysis.

DNA extraction. For DNA extraction, both grafted (CHT-T/Nad-1 and Nad-1/CHT-T) and ungrafted (Nad-1 and CHT-T) melon plants were considered. Stem sections were ground to a fine powder under liquid nitrogen with a sterile mortar and pestle. DNA was extracted and purified according to the protocol of Wizard genomic DNA purification kit (Promega Corp., Madison, WI). The concentration of DNA was measured spectrophotometrically (Nanodrop ND-1000; Thermo Fisher Scientific Inc., Waltham, MA). Special care was taken to obtain accurate concentrations of total DNA by quantifying each DNA sample in triplicate in two independent microplates. As an internal control of DNA quantification, a DNA sample of known concentration was included in each quantification plate.

Primer design and qPCR assay. A primer pair specific to *F. oxysporum* was designed by comparison of TEF-1 α gene sequences generated by EF1/EF2 primer pair amplification (28) of *Fusarium* spp. listed in Table 1. Additional sequences of *Fusarium* spp. were retrieved from GenBank and used for an in silico comparative analysis (Table 2). Sequences were aligned using CLUSTALX (38). Specific primers were designed by the program PRIMER3 (<http://primer3.sourceforge.net/>). Primer selection was based on a minimum formation of secondary structure and an amplicon size of 100 to 200 bp.

The specificity of the newly designed primer pair was tested in conventional PCR against DNA extracted from the fungal isolates listed in Table 1. All reactions always included positive (*F. oxysporum* f. sp. *melonis* DNA, isolate ISPaVe1018 or ISPaVe1070) and negative controls and were analyzed by gel electrophoresis to confirm that only one PCR product was present and no amplification product was obtained from negative controls.

To optimize the qPCR reaction conditions, annealing temperature, MgCl₂, and primer concentrations were adjusted experimentally. qPCR was carried out in the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) on the three biological replicates of each treatment, with three technical replicates

each. A *Fusarium*-free melon plant DNA sample was used as negative control. A dissociation curve was included at the end of the qPCR program to evaluate potential primer-dimers and non-specific amplification products. DNA standard curves for qPCR assays were obtained from 10-fold dilutions of *F. oxysporum* f. sp.

TABLE 1. List of fungal strains used in this study

Isolate	Host	Race	Origin
<i>Fusarium oxysporum</i> f. sp. <i>melonis</i> ISPaVe673	Melon	0	Italy
<i>F. oxysporum</i> f. sp. <i>melonis</i> ISPaVe289	Melon	0	Italy
<i>F. oxysporum</i> f. sp. <i>melonis</i> ISPaVe676	Melon	0	Italy
<i>F. oxysporum</i> f. sp. <i>melonis</i> ISPaVe1069	Melon	1	Italy
<i>F. oxysporum</i> f. sp. <i>melonis</i> ISPaVe1070	Melon	1	Italy
<i>F. oxysporum</i> f. sp. <i>melonis</i> ISPaVe1074	Melon	1	Italy
<i>F. oxysporum</i> f. sp. <i>melonis</i> ISPaVe1077	Melon	1	Italy
<i>F. oxysporum</i> f. sp. <i>melonis</i> ISPaVe1085	Melon	1	Italy
<i>F. oxysporum</i> f. sp. <i>melonis</i> ISPaVe1297	Melon	1	Italy
<i>F. oxysporum</i> f. sp. <i>melonis</i> ISPaVe1404 ^a	Melon	1	United States
<i>F. oxysporum</i> f. sp. <i>melonis</i> ISPaVe1018	Melon	1,2	Italy
<i>F. oxysporum</i> f. sp. <i>melonis</i> ISPaVe1083	Melon	1,2	Italy
<i>F. oxysporum</i> f. sp. <i>melonis</i> ISPaVe1680	Melon	1,2	Italy
<i>F. oxysporum</i> f. sp. <i>melonis</i> ISPaVe1681	Melon	1,2	Italy
<i>F. oxysporum</i> f. sp. <i>melonis</i> ISPaVe1684	Melon	1,2	Italy
<i>F. oxysporum</i> f. sp. <i>melonis</i> ISPaVe1689	Melon	1,2	Italy
<i>F. oxysporum</i> f. sp. <i>melonis</i> ISPaVe1692	Melon	1,2	Italy
<i>F. oxysporum</i> f. sp. <i>melonis</i> ISPaVe1693	Melon	1,2	Italy
<i>F. oxysporum</i> f. sp. <i>melonis</i> ISPaVe1694	Melon	1,2	Italy
<i>F. oxysporum</i> f. sp. <i>melonis</i> ISPaVe1697	Melon	1,2	Italy
<i>F. oxysporum</i> f. sp. <i>melonis</i> ISPaVe1698	Melon	1,2	Italy
<i>F. oxysporum</i> f. sp. <i>melonis</i> ISPaVe1700	Melon	1,2	Italy
<i>F. oxysporum</i> f. sp. <i>melonis</i> ISPaVe1406 ^a	Melon	2	United States
<i>F. oxysporum</i> f. sp. <i>melonis</i> ISPaVe1408 ^a	Melon	2	United States
<i>F. oxysporum</i> f. sp. <i>melonis</i> ISPaVe2068 ^b	Melon	2	Israel
<i>F. oxysporum</i> f. sp. <i>melonis</i> ISPaVe2069 ^b	Melon	2	Israel
<i>F. oxysporum</i> f. sp. <i>basilici</i> ISPaVe2097 ^c	Basil	–	Switzerland
<i>F. oxysporum</i> f. sp. <i>cucumerinum</i> ISPaVe2098 ^d	Watermelon	–	Greece
<i>F. oxysporum</i> f. sp. <i>radicis-cucumerinum</i> ISPaVe2100 ^d	Watermelon	–	Greece
<i>F. oxysporum</i> f. sp. <i>lycopersici</i> ISPaVe2102	Tomato	–	Italy
<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i> ER1553 ^c	Tomato	–	Italy
<i>F. crookwellense</i> ISPaVe1831	Wheat	–	Italy
<i>F. crookwellense</i> ISPaVe1857	Potato	–	Italy
<i>F. culmorum</i> ISPaVe1522	Durum wheat	–	Italy
<i>F. culmorum</i> ISPaVe1559	Durum wheat	–	Italy
<i>F. equiseti</i> ISPaVe1754	Durum wheat	–	Italy
<i>F. equiseti</i> ISPaVe1811	Durum wheat	–	Italy
<i>F. lateritium</i> ISPaVe1960	Walnut	–	Italy
<i>F. lateritium</i> ISPaVe2002	Hazelnut	–	Italy
<i>F. oxysporum</i> ISPaVe18	Artichoke	–	Italy
<i>F. oxysporum</i> ISPaVe904	Watermelon	–	Italy
<i>F. poae</i> ISPaVe1704	Durum wheat	–	Italy
<i>F. proliferatum</i> ISPaVe1110	Sorghum	–	Italy
<i>F. proliferatum</i> ISPaVe921	Melon	–	Italy
<i>F. redolens</i> ER1276 ^f	Lentil	–	Italy
<i>F. redolens</i> ER1277 ^f	Lentil	–	Algeria
<i>F. semitectum</i> ISPaVe1946	Walnut	–	Italy
<i>F. semitectum</i> ISPaVe2096	Walnut	–	Italy
<i>F. solani</i> ISPaVe2057	Gardenia	–	Italy
<i>F. solani</i> ISPaVe1962	Walnut	–	Italy
<i>F. solani</i> f. sp. <i>cucurbitae</i> ISPaVe931	Zucchini	1	Italy
<i>Plectosporium tabacinum</i> ISPaVe835	Zucchini	–	Italy
<i>Monosporascus cannonballus</i> ER1327 ^c	Melon	–	Italy
<i>M. cannonballus</i> ER1328 ^c	Melon	–	Italy
<i>M. cannonballus</i> ER1329 ^c	Melon	–	Italy
<i>Acremonium cucurbitacearum</i> ER1179 ^c	Melon	–	Italy
<i>A. cucurbitacearum</i> ER1516 ^c	Watermelon	–	Italy
<i>A. cucurbitacearum</i> ER1517 ^c	Melon	–	Italy
<i>Rhizopycnis vagum</i> ER925 ^c	Tomato	–	Italy
<i>Pyrenochaeta lycopersici</i> ER1320 ^c	Tomato	–	Spain
<i>P. lycopersici</i> ER1278 ^c	Tomato	–	Italy
<i>P. lycopersici</i> ER876 ^c	Tomato	–	Italy

^a Strains supplied by T. R. Gordon, Department of Plant Pathology, University of California, Davis.

^b Strains supplied by R. Perl-Treves, The Mina & Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan, Israel.

^c Strain supplied by G. Tamietti, Università di Torino, DIVAPRA, Torino, Italy.

^d Strain supplied by D. J. Vakalounakis, National Agricultural Research Foundation, Plant Protection Institute, Heraklion, Greece.

^e Strain supplied by A. Infantino, CRA-Centre of Plant Pathology Research, Rome.

^f Strain supplied by L. Riccioni, CRA-Centre of Plant Pathology Research, Rome.

melonis isolate ISPaVe1018 or ISPaVe1070. For this purpose, fungal DNA (10 ng/μl) was serially diluted (1:1, 1:10, 1:10², 1:10³, and 1:10⁴) in sterile ultrapure water as well as in a fixed background of plant DNA (50 ng) extracted from stem. Standard curves were generated by plotting logarithmic values of known quantities of target DNA versus the corresponding cycle threshold (Ct) values. After optimization, fungal DNA amount was assessed in both grafted melon plants and ungrafted genotypes for comparative purposes.

Statistical analysis. The isolation assay was a factorial experiment with three factors (time, melon genotype combination, and fungal race) and three replicates (plants). The section height on the stem cannot be considered as a further factor in an analysis of variance (ANOVA) design, because the sections were excised from the same plant (nonindependence of error). The presence or absence values obtained for the six sections excised above or the six sections excised below the grafting point were pooled into two percentage values of *F. oxysporum* f. sp. *melonis* presence calculated for each plant. Both sets of values, obtained either below or above the grafting point, were separately subjected to ANOVA. Because the two sets did not match the parametric ANOVA requirements with any transformation, the nonparametric approach of the Monte Carlo permutation test was used. To generate probabilities for the main effects, permutations were restricted within the levels of the other factor (9). Interactions were tested by unrestricted permutations after the calculation of residuals (1). Statistical tests used for main factors and interactions were the sum of squares between groups (Qb) and the pseudo F ratio ($F = Qb/Qw$, where Qw is the sum of squares within groups), respectively. In both ANOVAs, a significant interaction of melon genotype combination × time was detected; therefore, the effects of time and melon genotype combination were tested separately for each level of the other factor. Analyses were performed using Multiv software (31).

Data obtained by qPCR, calculated as pathogen DNA amount in 1 ng of plant DNA, were averaged for the two lower stem part sections (15 to 40 mm), and the same procedure was applied to data obtained from the two higher stem sections (45 to 70 mm).

The two data sets were further processed separately. To meet the parametric tests assumptions, data of each set were rank-transformed according to Conover and Iman (4) and analyzed through parametric ANOVA performed with SAS (35). Because the factorial design was not complete due to the lack of 2-dpi data for Nad-1 ungrafted plants, each data set was subjected to two factorial ANOVAs, with the first (AV1) considering two fungal races, four melon genotype combinations, and only one time (18 dpi) and the second (AV2) considering two fungal races, two times (2 and 18 dpi), and only three melon genotype combinations (the Nad-1 ungrafted plant data set was excluded). Standard regression lines obtained from fungal DNA diluted with either water or melon DNA were statistically compared for homogeneity ($P \geq 0.05$) of variance (Bartlett's test) and for equality of slopes and intercepts using an *F* test at $P < 0.05$. Tukey's multiple range test ($P \leq 0.01$) was performed on means of pathogen DNA amounts estimated either below or above the grafting point or in ungrafted melon plants.

RESULTS

Presence of *F. oxysporum* f. sp. *melonis* race 1 or 1,2 by isolations from infected grafted melon plants. Results of the isolation analysis for presence or absence of *F. oxysporum* f. sp. *melonis* along the stem during compatible and incompatible interaction with melon grafted plants are shown in Figure 1. The reaction of the whole grafted plant was conditioned by the genotype used as rootstock. In the compatible interaction, there were no significant differences between the two races for the colonization pattern (Fig. 1). In fact, Nad-1/CHT-T grafted plants (compatible interaction) were totally colonized at 18 dpi by both *F. oxysporum* f. sp. *melonis* races (Fig. 1) and all plants were symptomatic. Grafted CHT-T/Nad-1 plants (incompatible interaction) responded to infection by both *F. oxysporum* f. sp. *melonis* races by remaining symptomless, as if the whole plant were a resistant genotype (i.e., Nad-1). Nevertheless, isolations were positive and a different colonization profile was evident between *F. oxysporum* f. sp. *melonis* race 1 (ISPaVe1070) and race 1,2 (ISPaVe1018), especially concerning the time needed for colonizing the scion

Fig. 1. Isolation assay. *Fusarium oxysporum* f. sp. *melonis* presence (%) above or below the grafting point (six sections each) in either the incompatible ('Charentais-T' [CHT-T]/Nad-1') or compatible (Nad-1/CHT-T) interaction at 2 and 18 days post inoculation (dpi) with *F. oxysporum* f. sp. *melonis* race 1 (ISPaVe1070) or *F. oxysporum* f. sp. *melonis* race 1,2 (ISPaVe1018). Graphs indicate mean values ± standard error of the mean. Values equal to 0 are indicated with a black star. Significant ($P \leq 0.01$) effects were found both below and above the grafting point for the interaction of melon-genotype grafting combination × time, main effect of melon-genotype grafting combination at 18 dpi, and main effect of time in compatible interactions only.

(Fig. 1). Race 1 was more frequent at the early phase (2 dpi) while race 1,2 was more frequent at the late time point (18 dpi). With time, race 1 reduced its presence in CHT-T/Nad-1 stems, whereas race 1,2 slightly increased. Consequently, at 18 dpi, the effect of rootstock was highly significant ($P \leq 0.01$). Fungal colonization of the melon stem was significantly affected by the type of rootstock and by time. The incompatible combination was less colonized than the compatible one. No fungal colonies were reisolated from water inoculated plants.

Quantification of *F. oxysporum* f. sp. *melonis* race 1 or race 1,2 in inoculated grafted and ungrafted melon plants by qPCR. To quantify *F. oxysporum* biomass, the primer pair Fef1F (5'-TAGTCACTTCCCTTCAATCGC-3') and Fef2R (5'-CTC AAGTGGCGGGTAAGT-3') was specifically designed to yield a species-specific PCR product of 142 bp with no cross reaction against other *Fusarium* spp. or other fungal pathogens attacking melon (Fig. 2). The optimized qPCR mixture consisted of total reaction volumes of 15 µl containing 6 µl of total DNA (10 ng/µl), 7.5 µl of 2× Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA), and 0.2 µM each primer. The PCR program consisted of: 50°C for 2 min, 95°C for 2 min, and 34 cycles of 95°C for 15 s and 57°C for 1 min.

The standard curve showed a linear correlation between Ct values and fungal DNA, with a correlation coefficient (R^2) = 0.999 and an amplification efficiency of 99.9%, described by the equation $y = -3.313x + 23.925$ (Fig. 3). Statistical comparison of the standard regression lines of the source of the background DNA (water versus melon stem DNA) indicated lack of significant differences between slopes and significant differences ($P < 0.001$) between intercepts. Thus, a standard curve obtained by

TABLE 2. *Fusarium* spp. and GenBank accession numbers of translation elongation factor 1α gene sequences used for species-specific Fef1F/Fef2R primer pair design

Species	Accession number
<i>Fusarium andiyazi</i>	GU564308
<i>F. andiyazi</i>	EU620634
<i>F. armeniacum</i>	HM744692
<i>F. armeniacum</i>	GU116572
<i>F. nisikadoi</i>	AF324330
<i>Gibberella circinata</i>	AF160295
<i>G. circinata</i>	AF333930
<i>F. sacchari</i>	HM805097
<i>F. sacchari</i>	GU989189
<i>F. sacchari</i>	EF452969
<i>F. sacchari</i>	AF160278
<i>F. sacchari</i>	DQ465942
<i>F. sacchari</i>	DQ465935
<i>F. sacchari</i>	DQ465945
<i>F. sacchari</i>	GU377296
<i>F. subglutinans</i>	EU091074
<i>F. subglutinans</i>	AF160289
<i>G. moniliformis</i>	GU377299
<i>F. verticillioides</i>	FN179338
<i>F. redolens</i>	DQ854922
<i>F. redolens</i>	GU934577
<i>F. redolens</i>	HQ702586
<i>F. redolens</i>	HQ165857
<i>F. redolens</i>	FJ939707
<i>F. redolens</i>	GU250583
<i>F. redolens</i>	AF456449
<i>F. redolens</i>	AF324320
<i>F. oxysporum</i> f. sp. <i>melonis</i>	EF056790
<i>F. oxysporum</i> f. sp. <i>melonis</i> ISPaVe1018	HE585984
<i>F. oxysporum</i> f. sp. <i>melonis</i> ISPaVe1070	HE585983
<i>F. oxysporum</i> f. sp. <i>melonis</i>	DQ837696
<i>F. oxysporum</i> f. sp. <i>melonis</i>	AF008504
<i>F. oxysporum</i> f. sp. <i>melonis</i>	FJ985266
<i>F. oxysporum</i> f. sp. <i>melonis</i>	FJ664912
<i>F. oxysporum</i> f. sp. <i>melonis</i>	AF363403
<i>F. oxysporum</i>	JN222908
<i>F. oxysporum</i>	DQ852627

amplification of fungal DNA was normalized with the addition of melon stem DNA. The detection limit of the qPCR assay was fixed at a *F. oxysporum* DNA concentration of 1 pg or at a Ct value of 34 cycles, because *F. oxysporum* DNA was not accurately quantified at a concentration of 1 pg if fungal DNA was diluted in water or in DNA extracted from melon stem (Fig. 3).

The newly developed protocol was used to analyze the pathogen DNA amount in grafted plants—CHT-T/Nad-1 (incompatible interaction) and its reverse, Nad-1/CHT-T (compatible interaction)—and in the two ungrafted genotypes. In both combinations, both fungal races were detected along the stem at 2 dpi (Fig. 4). The amount of fungal DNA was highly affected by the type of interaction (compatible or incompatible) and time. *F. oxysporum* f. sp. *melonis* DNA content in grafted plants was much higher in the compatible interaction than in the incompatible one at 18 dpi. The principal significant effect ($P \leq 0.01$) on fungal development was due to melon genotype used as rootstock, and this effect had a significant interaction with time and race. In particular, in the incompatible interaction and at the late phase (18 dpi), the amount of race 1,2 was significantly higher compared with that estimated for race 1. Similarly, above the grafting point, the effect of the interaction fungal race × time was

Fig. 2. Specificity of Fef1F/Fef2R primer pair tested by conventional polymerase chain reaction against several species of *Fusarium* and melon fungal pathogens. Lane 1, *F. oxysporum* f. sp. *melonis* ISPaVe676; lane 2, *F. oxysporum* f. sp. *melonis* ISPaVe1069; lane 3, *F. oxysporum* f. sp. *melonis* ISPaVe1070; lane 4, *F. oxysporum* f. sp. *melonis* ISPaVe1018; lane 5, *F. oxysporum* f. sp. *melonis* ISPaVe1083; lane 6, *F. oxysporum* f. sp. *melonis* ISPaVe2069; lane 7, *F. crookwellense* ISPaVe1831; lane 8, *Rhizopycnis vagum* ER925; lane 9, *F. culmorum* ISPaVe1522; lane 10, *F. equiseti* ISPaVe1754; lane 11, *Monosporascus cannonballus* ER1327; lane 12, *F. lateritium* ISPaVe2002; lane 13, negative control with sterile distilled water; lane 14, *F. oxysporum* f. sp. *cucumerinum* ISPaVe2098; lane 15, *F. oxysporum* f. sp. *radicis-lycopersici* ER1553; lane 16, *F. oxysporum* f. sp. *radicis-cucumerinum* ISPaVe2100; lane 17, *F. oxysporum* f. sp. *basilici* ISPaVe2097; lane 18, *F. oxysporum* f. sp. *lycopersici* ISPaVe2102; lane 19, *F. oxysporum* ISPaVe18; lane 20, *F. oxysporum* ISPaVe904; lane 21, *F. poae* ISPaVe1704; lane 22, *F. redolens* ER1276; lane 23, *Pyrenochaeta lycopersici* ER1278; lane 24, *F. solani* f. sp. *cucurbitae* ISPaVe931; lane 25, *Acremonium cucurbitacearum* ER1517; lane 26, *Plectosporium tabacinum* ISPaVe835; lane M, 1-kb-plus ladder marker (Invitrogen); and lane m, 100-bp ladder marker (New England BioLabs).

also significant (Fig. 4). Tukey's multiple range test ($P \leq 0.01$) performed on the theses analyzed at both time points (AV2 statistical analysis) indicated that the amount of fungal DNA in the ungrafted CHT-T (0.041 ng below and 0.08 ng above 40 to 45 mm) was significantly higher than in Nad-1/CHT-T (0.0080 ng below and 0.0091 ng above the grafting point); and, in Nad-1/CHT-T (compatible interaction), it was significantly higher compared with that estimated in the incompatible interaction CHT-T/Nad-1 (0.000099 ng below and 0.00017 ng above the grafting point). Accordingly, when comparing data obtained exclusively at the 18 dpi (AV1 statistical analysis), the amount of fungal DNA in CHT-T/Nad-1 (0.000138 ng below and 0.000279 ng above the grafting point) was significantly lower than in Nad-1 (0.005806 ng below and 0.016573 ng above 40 to 45 mm) as well as in Nad-1/CHT-T (0.015823 ng below and 0.018087 above the grafting point), either below or above the grafting point.

The use of the qPCR protocol allowed the detection of *F. oxysporum* f. sp. *melonis* DNA in 100% of stem sections of the two melon genotypes regardless of resistance or susceptibility. No Fef1F/Fef2R amplification was obtained from water-inoculated plant samples.

As a general observation, the colonization of both *F. oxysporum* f. sp. *melonis* races was strongly inhibited in the incompatible interaction with CHT-T/Nad-1, thus confirming the dominance of the rootstock on the whole-plant response. Moreover, data showed that the simple presence of the resistant genotype, even when used as scion (Nad-1/CHT-T), decreased fungal development compared with CHT-T seedlings (Fig. 4).

DISCUSSION

The aim of this work was to develop a tool enabling the detection and quantification of *F. oxysporum* in plant tissue which was applied to the quantification of *F. oxysporum* f. sp. *melonis* in compatible and incompatible interactions with grafted and un-

grafted melon genotypes. We fulfilled the objectives, and the newly developed primers pair was highly specific to *F. oxysporum* without cross reactions with other *Fusarium* spp. and the common melon fungal pathogens. The protocol was highly sensitive and detected *F. oxysporum* in asymptomatic melon stems at the early infection stage, with a quantification limit of 1 pg of fungal DNA. Similar detection limits were found in other qPCR protocols for the detection of vascular-infecting fungi, including *Fusarium* spp. or other formae speciales of *F. oxysporum* in planta and soil (11,17,18,20,29,39). In the referenced protocols, the target sequence of the qPCR was within the ribosomal DNA that occurs in multiple copies, or the primers were developed on a SCAR-specific target sequence. Instead, we used the TEF-1 α gene for our primer design. This gene, which occurs as a single copy within the genome, is extensively used in *Fusarium* spp. phylogeny, because it is highly informative at the species level (13,27). The newly developed primer set, Fef1F and Fef2R, unambiguously differentiated *F. oxysporum* from other *Fusarium* spp., generating an amplicon shorter than 200 bp, which allows an efficient, reliable, and repeatable qPCR.

The use of this protocol allowed us to clearly detect *F. oxysporum*. Thereafter, colonies obtained from reisolations were confirmed as *F. oxysporum* f. sp. *melonis* race 1 or race 1,2 because they were reinoculated on CHT-T and Charentais-Fom2, respectively, for further investigations (data not shown). Moreover, no contaminations were obtained from water-inoculated melon plants used as controls, confirming that no other *Fusarium* spp. or *F. oxysporum* were present than the strains used as inoculum.

qPCR detected and quantified the amount of pathogen DNA in stem tissues at 2 dpi, when plants showed no or scarcely discernible symptoms. Because the limitation of a qPCR protocol, shared by all molecular detection and quantification methods, is the inability to distinguish viable from not viable fungal material, isolations from host tissue were performed, and the results supported differences in colonization profiles between *F. oxysporum*

Fig. 3. Standard regression lines of a five-point 10-fold serial dilution of *Fusarium oxysporum* f. sp. *melonis* (ISPaVe1018) DNA (10 ng/ μ l) diluted in sterile, ultrapure water (water series) or in DNA (50 ng) extracted from melon stems. Threshold cycles (Ct) were plotted against the log of genomic DNA standard curves of known concentrations.

f. sp. melonis races obtained by qPCR. Nevertheless, the DNA of both fungal races was assessed by qPCR at the early time point (2 dpi) above the grafting point, where a minimal fungal presence was revealed by reisolations in either the compatible or incompatible interaction, confirming the higher sensitivity of the qPCR methodology. The present study also provides new information on the effects of grafting. Though grafting is a widely applied system in commercial melon cultivation (3,5), knowledge is lacking on interactions between scion and rootstock genotypes and on their

effects on the vascular pathogen colonization. On this account, we investigated both grafted and ungrafted plants of CHT-T (susceptible) and Nad-1 (resistant). To our knowledge, this is the first study carried out to both detect and quantify fungal infection in grafted plants by means of real-time PCR.

In grafted plants, the response to the pathogen is affected by rootstock characteristics (i.e., the resistant rootstock Nad-1 makes the whole plant asymptomatic), which is the practical reason for the commercial use of resistant rootstocks. The general hypothe-

Fig. 4. Amounts of *Fusarium oxysporum* f. sp. *melonis* DNA assessed by quantitative polymerase chain reaction (fungal DNA [ng] present in 1 ng of DNA extracted from infected plants) using Fef1F/Fef2R primer pair in infected grafted melon plants ('Charentais-T' [CHT-T]/Nad-1') (incompatible interaction) or Nad-1/CHT-T (compatible interaction) or in ungrafted plants (Nad-1 resistant and CHT-T susceptible). Data are shown in log₁₀ scale. Graphs indicate mean values $\pm$ standard error of the mean. Significant effects ($P \leq 0.01$) were found both below and above the grafting point for melon-genotype grafting combination, time, fungal race $\times$ melon-genotype grafting combination, or time $\times$ melon-genotype grafting combination. Above the grafting point, the effect of the fungal race $\times$ time interaction was also significant ($P \leq 0.01$).

sis underlying this use is that the rootstock genotype constitutes a physical or biochemical barrier against the fungus that infects through roots; consequently, the pathogen cannot reach the susceptible scion or is drastically impeded (3). This general assumption has been partially supported by the present study. As a matter of fact, the biomass of both races was drastically reduced in the incompatible interaction; however, the pathogen was detected also in the scion with either reisolations or qPCR. As expected, both incompatible interactions, CHT-T/Nad-1 and Nad-1, remained completely symptomless. The influence of the resistant genotype also is detectable when it is used as a scion (i.e., Nad-1/CHT-T compatible interaction), inducing a significant reduction of fungal development compared with that assessed in CHT-T ungrafted plants. This result gives further support to the effect of the scion on the response of grafted plants and to the general indication that the resistance level achieved by grafting also depends on the scion (3).

Considering the differences between the two *F. oxysporum* f. sp. *melonis* races, race 1 was generally faster in colonizing the melon plant than race 1,2, but its presence and quantity was reduced with time in incompatible interactions (CHT-T/Nad-1) (2,37). This could be due to the absence of a stable or continuous colonization at the early stage of infection (2 dpi), so that the presence or quantity of race 1 decreases with time in the incompatible interaction. These results support the observations already reported by reisolations (37) or by a GFP-expressing strain (40). Conversely, race 1,2 affirms its presence at the late time point in the incompatible interactions. In fact, this race produced the highest amount of biomass assessed in the incompatible interaction (CHT-T/Nad-1 or in Nad-1 ungrafted plants) at 18 dpi (Fig. 4). The ability of race 1,2 to grow in resistant melon genotypes, without inducing symptoms, has already been reported by using a GFP-expressing strain (6,16,40).

Quantitative assessments obtained by the use of this newly developed protocol complete the descriptive picture obtained with GFP markers of the xylem colonization already reported for *F. oxysporum* (6,16,40). The GFP studies reported that the fungus colonized the plant and invaded xylem vessels, being essentially confined within them. Furthermore, *F. oxysporum* f. sp. *melonis* race 1,2 is able to invade resistant plants, although to a lesser extent than susceptible ones (40), thus supporting our results.

This study shows that, even in the incompatible melon genotype, both races are present but, in grafted plants, the maximum quantity reached by the pathogen is significantly lower than in the ungrafted plants. In any case, the resistant genotype (Nad-1) has an evident influence on both fungal races by reducing their development.

In all experiments, race 1,2 (ISPaVe1018) has the highest ability to grow in melon stems in the presence of host resistance. Interestingly, race 1,2 grew >0.05 ng/ng of plant DNA without inducing symptoms in the resistant Nad-1 ungrafted genotype whereas, in the compatible interaction, a lower amount of the fungus incited wilting symptoms. Therefore, it seems evident that, in the *F. oxysporum* f. sp. *melonis*-melon combination, the ability of *F. oxysporum* to colonize the plant is independent from the ability to induce symptoms.

The rapid and reliable detection of pathogens, particularly soil-borne, are important features in formulating strategies for disease management in either plant nurseries or open fields (23). The qPCR system is less time consuming than isolations and has higher sensitivity due to the ability to detect *F. oxysporum* f. sp. *melonis* in melon stems where no growth was found by culturing. The qPCR protocol might have direct applications in studies on microbial ecology and plant pathology, because it provides opportunities for analyzing *F. oxysporum* populations and their interactions with the host.

The different behavior observed between *F. oxysporum* f. sp. *melonis* race 1 and 1,2, in colonizing melon plants suggests a

different genetic background as a probable result of independent evolutionary processes (12,24,32). Further work will be performed to study this different genetic background by comparative genomic analyses, which might provide novel models of molecular diversification and genetic transmission to explain how fungal genomes adapt to pathogenic behavior.

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LITERATURE CITED

- Anderson, M. J., and Ter Braak, C. 2003. Permutation tests for multi-factorial analysis of variance. *J. Stat. Comput. Simul.* 73:85-113.
- Catalano, V., Haegi, A., Luongo, L., Ficcadenti, N., and Belisario, A. 2010. Differential colonization of resistant and susceptible melon genotypes infected by *Fusarium oxysporum* f. sp. *melonis*. *J. Plant Pathol.* 92:78.
- Cohen, R., Horev, C., Burger, Y., Shriber, S., Hershenhorn, J., Katan, J., and Edelstein, M. 2002. Horticultural and pathological aspects of *Fusarium* wilt management using grafted melons. *HortScience* 37:1069-1073.
- Conover, W. J., and Iman, R. L. 1981. Rank transformations as a bridge between parametric and nonparametric statistics. *Am. Stat.* 35:124-129.
- Crinò, P., lo Bianco, C., Roupael, Y., Colla, G., Saccardo, F., and Paratore, A. 2007. Evaluation of rootstock resistance to *Fusarium* wilt and gummy stem blight and effect on yield and quality of a grafted 'Inodorus' melon. *HortScience* 42:521-525.
- Di Pietro, A., Madrid, M. P., Caracul, Z., Delgado-Jarana, J., and Roncero, M. I. G. 2003. *Fusarium oxysporum*: exploring the molecular arsenal of a vascular wilt fungus. *Mol. Plant Pathol.* 4:315-325.
- Edel, V., Aim, S., Cordier, C., Olivain, C., Steinberg, C., and Alabouvette, C. 2011. Development of a strain-specific real-time PCR assay for the detection and quantification of the biological control agent Fo47 in root tissues. *FEMS Microbiol. Lett.* 322:34-40.
- Edel, V., Steinberg, C., Gautheron, N., and Alabouvette, C. 2000. Ribosomal DNA-targeted oligonucleotide probe and PCR assay specific for *Fusarium oxysporum*. *Mycol. Res.* 104:518-526.
- Edgington, E. S., and Onghena, P. 2007. Randomization Tests, Fourth ed. Chapman and Hall/CRC Press, Boca Raton, FL.
- Ficcadenti, N., Sestili, S., Annibali, S., Campanelli, G., Belisario, A., Maccaroni, M., and Corazza, L. 2002. Resistance to *Fusarium oxysporum* f. sp. *melonis* race 1,2 in muskmelon lines Nad-1 and Nad-2. *Plant Dis.* 86:897-900.
- Filion, M., St. Arnaud, M., and Jabaji-Hare, S. H. 2003. Quantification of *Fusarium solani* f. sp. *phaseoli* in mycorrhizal bean plants and surrounding mycorrhizosphere soil using real-time polymerase chain reaction and direct isolations on selective media. *Phytopathology* 93:229-235.
- Fourie, G., Steenkamp, E. T., Ploetz, R. C., Gordon, T. R., and Viljoen, A. 2011. Current status of the taxonomic position of *Fusarium oxysporum* formae specialis *cubense* within the *Fusarium oxysporum* complex. *Infect. Genet. Evol.* 11:533-542.
- Geiser, D. M., Jiménez-Gasco, M. M., Kang, S., Makalowska, I., Veeraraghavan, N., Ward, T. J., Zhang, N., Kuldau, G. A., and O'Donnell, K. 2004. FUSARIUM-ID v. 1.0: A DNA sequence database for identifying *Fusarium*. *Eur. J. Plant Pathol.* 110:473-479.
- Gordon, T. R., and Martyn, R. D. 1997. The evolutionary biology of *Fusarium oxysporum*. *Annu. Rev. Phytopathol.* 35:11-128.
- Gordon, T. R., Okamoto, D., and Jacobson, D. J. 1989. Colonization of muskmelon and non susceptible crops by *Fusarium oxysporum* f. sp. *melonis* and other species of *Fusarium*. *Phytopathology* 79:1095-1100.
- Inoue, I., Namiki, F., and Tsuge, T. 2002. Plant colonization by vascular wilt fungus *Fusarium oxysporum* requires FOW1, a gene encoding a mitochondrial protein. *Plant Cell* 14:1869-1883.
- Jimenez-Fernandez, D., Montes-Borrego, M., Jiménez-Díaz, R. M., Navas-Cortés, J. A., and Landa, B. B. 2011. In planta and soil quantification of *Fusarium oxysporum* f. sp. *ciceris* and evaluation of *Fusarium* wilt resistance in chickpea with a newly developed quantitative polymerase chain reaction assay. *Phytopathology* 101:250-262.
- Jimenez-Fernandez, D., Montes-Borrego, M., Navas-Cortés, J. A., Jiménez-Díaz, R. M., and Landa, B. B. 2010. Identification and quantification of *Fusarium oxysporum* in planta and soil by means of an improved specific and quantitative PCR assay. *Appl. Soil Ecol.* 46:372-382.

19. Lievens, B., Brouwer, M., Vanachter, A. C. R. C., Cammue, B. P. A., and Thomma, B. P. H. J. 2006. Real-time PCR for detection and quantification of fungal and oomycete tomato pathogens in plant and soil samples. *Plant Sci.* 171:155-165.
20. Lievens, B., Claes, L., Vakalounakis, D. J., Vanachter, A. C. R. C., and Thomma, B. P. H. J. 2007. A robust identification and detection assay to discriminate the cucumber pathogens *Fusarium oxysporum* f. sp. *cucumerinum* and f. sp. *radicis-cucumerinum*. *Environ. Microbiol.* 9:2145-2161.
21. Lievens, B., Rep, M., and Thomma, B. 2008. Recent developments in the molecular discrimination of formae speciales of *Fusarium oxysporum*. *Pest Manage. Sci.* Online publication. doi:10.1002/ps
22. Lievens, B., van Baarlen, P., Verreth, C., van Kerckhove, S., Rep, M., and Thomma, B. 2009. Evolutionary relationships between *Fusarium oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *radicis-lycopersici* isolates inferred from mating type, elongation factor-1 α and exopolysaccharuronase sequences. *Mycol. Res.* 113:1181-1191.
23. Lopez-Mondejar, R., Beaulieu, R., Ros, M., and Pascual, J. A. 2012. SCAR-based real-time TaqMan PCR for early detection of *Fusarium oxysporum* in melon seedlings under greenhouse nursery conditions. *Crop Prot.* 33:1-6.
24. Ma, L. J., van der Does, H. C., Borkovich, K. A., Coleman, J. J., Daboussi, M.-J., Di Pietro, A., Dufresne, M., Freitag, M., Grabherr, M., Henrissat, B., Houterman, P. M., Kang, S., Shim, W.-B., Woloshuk, C., Xie, X., Xu, J.-R., Antoniw, J., Baker, S. E., Bluhm, B. H., Breakspear, A., Brown, D. W., Butchko, R. A. E., Chapman, S., Coulson, R., Coutinho, P. M., Danchin, E. G. J., Diener, A., Gale, L. R., Gardiner, D. M., Goff, S., Hammond-Kosack, K. E., Hilburn, K., Hua-Van, A., Jonkers, W., Kazan, K., Kodira, C. D., Koehrsen, M., Kumar, L., Lee, Y.-H., Li, L., Manners, J. M., Miranda-Saavedra, D., Mukherjee, M., Park, G., Park, J., Park, S.-Y., Proctor, R. H., Regev, A., Ruiz-Roldan, M. C., Sain, D., Sakthikumar, S., Sykes, S., Schwartz, D. C., Turgeon, B. G., Wapinski, I., Yoder, O., Young, S., Zeng, Q., Zhou, S., Galagan, J., Cuomo, C. A., Kistler, H. C., and Rep, M. 2010. Comparative genomics reveals mobile pathogenicity chromosomes in *Fusarium*. *Nature* 464:367-373.
25. Michielse, C. B., and Rep, M. 2009. Pathogen profile update: *Fusarium oxysporum*. *Mol. Plant Pathol.* 10:311-324.
26. Mishra, P. K., Fox, R. T. V., and Culham, A. 2003. Development of a PCR-based assay for rapid and reliable identification of pathogenic Fusaria. *FEMS Microbiol. Lett.* 218:329-332.
27. O'Donnell, K., Gueidan, C., Sink, S., Johnston, P. R., Crous, P. W., Glenn, A., Riley, R., Zitomer, N. C., Colyer, P., Waalwijk, C., van der Lee, T., Moretti, A., Kang, S., Kim, H. S., Geiser, D. M., Juba, J. H., Baayen, R. P., Crome, M. G., Bithel, S., Sutton, D. A., Skovgaard, K., Ploetz, R., Kistler, H. C., Elliott, M., Davis, M., and Sarver, B. A. J. 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.
28. O'Donnell, K., Kistler, H. C., Cigelnik, E., and Ploetz, R. C. 1998. Multiple evolutionary origin of the fungus causing Panama disease of banana; Concordant evidence from nuclear and mitochondrial gene genealogies. *Proc. Natl. Acad. Sci. USA* 95:2044-2049.
29. Pasquali, M., Piatti, P., Gullino, M. L., and Garibaldi, A. 2006. Development of a real-time polymerase chain reaction for the detection of *Fusarium oxysporum* f. sp. *basilici* from basil seed and roots. *J. Phytopathol.* 154:632-636.
30. Perchepe, L., and Pitrat, M. 2004. Polygenic inheritance of partial resistance to *Fusarium oxysporum* f. sp. *melonis* race 1,2 in melon. *Phytopathology* 94:1331-1336.
31. Pillar De Patta, V. 2006. MULTIV. Multivariate Exploratory Analysis, Randomization Testing and Bootstrap Resampling. User's Guide v. 2.4. Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.
32. Rep, M., and Kistler, H. C. 2010. The genomic organization of plant pathogenicity in *Fusarium* species. *Curr. Opin. Plant Biol.* 13:420-426.
33. Risser, G., Banihashemi, Z., and Davis, D. W. 1976. A proposed nomenclature of *Fusarium oxysporum* f. sp. *melonis* races and resistance genes in *Cucumis melo*. *Phytopathology* 66:1105-1106.
34. Sakata, Y., Ohara, T., and Sugiyama, M. 2008. The history of melon and cucumber grafting in Japan. *Acta Hort.* 767:217-228.
35. SAS. 1985. Users Guide: Statistics. SAS Institute, Cary, NC.
36. Schena, L., Nigro, F., Ippolito, A., and Gallitelli, D. 2004. Real-time quantitative PCR: A new technology to detect and study phytopathogenic and antagonistic fungi. *Eur. J. Plant Pathol.* 110:893-908.
37. Sestili, S., Polverari, A., Luongo, L., Ferrarini, A., Scotton, M., Hussain, J., Delledonne, M., Ficcacanti, N., and Belisario, A. 2011. Distinct colonization patterns and cDNA-AFLP transcriptome profiles in compatible and incompatible interactions between melon and different races of *Fusarium oxysporum* f. sp. *melonis*. *BMC Genomics* 12:122. Online publication. doi:10.1186/1471-2164-12-122
38. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. 1997. The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25:4876-4882.
39. Zhang, Z., Zhang, J., Wang, Y., and Zheng, X. 2005. Molecular detection of *Fusarium oxysporum* f. sp. *niveum* and *Mycosphaerella melonis* in infected plant tissues and soils. *FEMS Microbiol. Lett.* 249:39-46.
40. Zvirin, T., Herman, R., Brotman, Y., Denisov, Y., Belausov, E., Freeman, S., and Perl-Treves, R. 2010. Differential colonization and defence responses of resistant and susceptible melon lines infected by *Fusarium oxysporum* race 1-2. *Plant Pathol.* 59:576-585.