Journal of Phytopathology

ORIGINAL ARTICLE

Fusarium Species from the *Fusarium fujikuroi* Species Complex Involved in Mixed Infections of Maize in Northern Sinaloa, Mexico

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Keywords
calmodulin, elongation factor, *Fusarium*, *Fusarium fujikuroi* species complex, maize

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Received: April 28, 2014; accepted: October 5, 2014.

doi: 10.1111/jph.12346

Abstract

Fusarium species belonging to the *Fusarium fujikuroi* species complex (FFSC) are associated with maize in northern Mexico and cause *Fusarium* ear and root rot. In order to assess the diversity of FFSC fungal species involved in this destructive disease in Sinaloa, Mexico, a collection of 108 fungal isolates was obtained from maize plants in 2007–2011. DNA sequence analysis of the calmodulin and elongation factor 1α genes identified four species: *Fusarium verticillioides*, *F. nygamai*, *F. andiyazi* and *F. thapsinum* (comprising 79, 23, 4 and 2 isolates, respectively). Differential distribution of *Fusarium* species in maize organs was observed, that is *F. verticillioides* was the most frequently isolated species from maize seeds, while *F. nygamai* predominated on maize roots. Mixed infections with *F. verticillioides*/*F. thapsinum* and *F. verticillioides*/*F. nygamai* were detected in maize seeds and roots, respectively. Pathogenicity assay demonstrated the ability of the four species to infect maize seedlings and induce different levels of disease severity, reflecting variation in aggressiveness, plant height and root biomass. Isolates of *F. verticillioides* and *F. nygamai* were the most aggressive. These species were able to colonize all root tissues, from the epidermis to the vascular vessels, while infection by *F. andiyazi* and *F. thapsinum* was restricted to the epidermis and adjacent cortical cells. This is the first report of *F. nygamai*, *F. andiyazi* and *F. thapsinum* infecting maize in Mexico and co-infecting with *F. verticillioides*. Mixed infections should be taken into consideration due to the production and/or accumulation of diverse mycotoxins in maize grain.

Introduction

The genus *Fusarium* comprises some of the most detrimental fungal pathogens in agriculture (Bacon and Yates 2006; Summerell et al. 2010). Species of the *Fusarium fujikuroi* species complex (FFSC) and *Fusarium graminearum* species complex (FGSC) are the causative agents of ear and root rot in maize, with *F. verticillioides* being the most common species associated with this disease (Zainuddin et al. 2011; Covarelli et al. 2012; Madania et al. 2013). This fungus is hemibiotrophic and can be transmitted either vertically (seed-borne) or horizontally (soil- or aerial-borne, or through wounds) to its host, where it

produces symptomatic or asymptomatic systemic infections (Munkvold 2003; Bacon and Yates 2006). Plants infected with *F. verticillioides* can show root, stalk and ear rot, as well as wilting, stalk thinning and reduced aerial and root growth (Oren et al. 2003; Wu et al. 2011). Other *Fusarium* species of the FFSC can be recovered from maize exhibiting the same symptomatology (Figueroa-Rivera et al. 2010; Reyes-Velázquez et al. 2011; Covarelli et al. 2012; Madania et al. 2013).

Species in the FFSC produce a wide range of mycotoxins that contaminate food and are harmful to human and animal health (Kvas et al. 2009). The taxonomy of this species complex is based on phylogenetic, biological and morphological species concepts (Kvas et al. 2009**;** Summerell et al. 2010). Reviews of the FFSC in the past 30 years have reported 45 phylogenetic species, ten biological species and 34 morphospecies (Kvas et al. 2009). This severely complicates the identification of new isolates based only on morphological characters, and as a consequence, this has led to misclassification and underestimation of *Fusarium* diversity.

Presently, taxonomy in this species complex is based primarily on DNA sequence analysis. O'Donnell et al. (2000) established that calmodulin, elongation factor 1α (EF-1α) and β-tubulin are capable of resolving most of the species in the FFSC, while the ITS region, 28S rDNA and mtSSU genes are not. By contrast, several studies in Mexico have identified *Fusarium* species that cause ear rot purely on the basis of characterization of morphological traits or sequencing of rDNA genes (Figueroa-Rivera et al. 2010; Reyes-Velázquez et al. 2011).

The aim of our study was to identify those *Fusarium* species belonging to the FFSC that infect commercial maize crops in northern Sinaloa, one of the most important maize-producing regions in northwest Mexico. A phylogenetic approach was used by analyzing partial sequences of the calmodulin and EF-1α genes. Finally, pathogenicity and aggressiveness of each *Fusarium* species were assessed in maize roots by two distinct assays. These results will contribute to our understanding of fungal infectivity in this agriculturally important crop.

Materials and Methods

Fungal isolates

A collection of 108 *Fusarium* isolates were used for this study (Table S1). Forty-three isolates were obtained from commercial maize seeds of different hybrids cultivated in Sinaloa, Mexico from 2007–2009 using the freezing blotter test (Warham et al. 1996) and were morphologically identified as *F. verticillioides* (F4–F81; García-Espinoza 2009). Three isolates (P01–P03) obtained from maize seeds, stalk and roots in 2009 were donated by Dr. R. Félix-Gastélum from the Junta Local de Sanidad Vegetal del Valle del Fuerte (Sinaloa, Mexico). Twenty-eight isolates (F87–F136) were obtained from symptomatic maize ears during 2008–2009. Ears were shelled and kernels were surface-sterilized with a 2% sodium hypochlorite solution. Disinfected kernels were placed in water agar plates containing ampicillin (200 ppm) and incubated at 25°C for 3 days. Fungal colonies were purified and maintained in assay tubes with sterile river sand (Gómez-Gómez 2014). All isolates were initially identified as *F. verticillioides* using the DNA sequence of the ITS region (Figueroa-López 2011; Gómez-Gómez 2014). Additionally, thirty-four isolates were obtained from roots of symptomatic maize plants during 2011, from seven different cultivation areas in northern Sinaloa (Table S1). Roots were cut into small pieces (2 mm), surface-sterilized with a 1.5% sodium hypochlorite solution for five min and rinsed three times with sterile distilled water. Tissue pieces were placed in Nash–Snyder agar plates (Leslie and Summerell 2006) and incubated at 25°C for 2 days. Fungal colonies were transferred to potato dextrose agar plates (PDA; BD Bioxon, Estado de Mexico, Mexico, Cat. No. 211900) and incubated at 25°C for 14 days. PDA cultures of all isolates were used to obtain monoconidial cultures by serial dilutions (Cañedo and Ames 2004). Monoconidial cultures were grown in Spezieller Nährstoffarmer Agar plates (SNA) for 14 days at 25°C. Mycelium and conidia were harvested, placed in potato dextrose broth (PD; BD Difco, Le Pont de Claix, France, Cat. No. 254920) with 15% glycerol and cryopreserved at −70°C.

DNA extraction

Fungal cultures, mycelium harvesting and grinding were performed as described by Leyva-Madrigal et al. (2014). Genomic DNA was extracted from 10 mg of frozen mycelium using the DNeasy Blood and Tissue Kit (QIAGEN, Cat. No. 69506, Hannover, Germany), according to the manufacturer's protocol adapted for yeast. Genomic DNA quality was verified by electrophoresis in 1% agarose gel and quantified using the Quant**-**iT dsDNA HS kit (Life technologies, Cat. No. Q32854, Grand Island, NY, USA**)**.

PCR amplification

Calmodulin was amplified using the primer pair Ver1 and Ver2 (Mulé et al. 2004), while EF-1α was amplified with the EF1 and EF2 primer pair (O'Donnell et al. 1998). PCR was performed in a volume of 25 μl containing 1 ng DNA template, 1.5 mM $MgCl_2$, 0.5 mM of each dNTP, 0.4 μM of forward and reverse primers and 1.25 U of Taq DNA polymerase (Invitrogen, Brazil, Cat. No. 11615-050). The thermocycler was programmed for 5 min initial denaturation at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, 30 s annealing at the respective Tm (55°C for calmodulin and 60°C for EF-1α), 1 min extension at 72°C and a final extension at 72°C for 5 min. PCR products were separated by agarose gel electrophoresis (1% w/v in 0.5 × TAE) and visualized by ethidium bromide staining. PCR products were purified in a QIAcube workstation using the QIAquick PCR purification Kit (QIAGEN, Cat. No. 28106) and quantified with the Quant-it dsDNA HS Kit.

Sequencing and phylogenetic analysis

PCR products were sequenced in both directions with an ABI 3730xl sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were edited in CHROMAS Pro 1.6 (Technelysium Pty Ltd, South Brisbane, Qld, Australia) and compared with sequences in the NCBI (National Center for Biotechnology Information) using the BLAST-N software and the Megablast algorithm. MEGA 5.2 (Tamura et al. 2011) was used for alignment and phylogenetic analysis. All sequences were deposited in GenBank under the accession numbers KF640953–KF641168. Sequences were aligned together with 22 reference sequences from members of the FFSC and a sequence of *F. oxysporum* reported by O'Donnell et al. (2000), using the MUSCLE alignment program (Edgar 2004). Multiple alignments were subjected to a DNA substitution model analysis in MEGA, to select the model that best fits the data. Phylogenetic trees were constructed using the Kimura 2-parameter (K2P) model and the neighbour joining (NJ) method. Among-site rate variation was modelled by a gamma distribution (four categories). Tree topology support was assessed by 1000 bootstrap replicates. Concatenate alignments were made using DAMBE 5.2.56 (Xia 2013), and a phylogenetic tree was constructed from this data, as described above.

Pathogenicity assays

Rolled paper towel assay

The isolates *F. thapsinum* (F33 and F65), *F. andiyazi* (F106, F116 and F131), *F. nygamai* (CI62, DA15 and EM51) and *F. verticillioides* (F31, P03 and DA42) were tested. Commercial maize seeds (Garañón hybrid, Asgrow) were disinfected using a hydrothermal treatment to remove all possible accompanying mycobiota. Seeds were immersed in a Tween-20 solution (5 drops of Tween-20 per 100 ml of sterile distilled water) and sonicated for 5 min. The tween solution was then decanted, and seeds were immersed in a 0.75% sodium hypochlorite solution and placed in a thermo-bath at 52°C for 20 min. Finally, seeds were washed three times with sterile distilled water and allowed to air-dry in a laminar flow hood.

Fungal isolates were cultured on SNA medium with filter paper (Leslie and Summerell 2006), and SNA medium was added with streptomycin (0.05 g/ml) and neomycin (0.01 g/ml). Cultures were incubated at 25°C for 14 days. Mycelia and conidia were harvested by adding 10 ml of sterile distilled water and gently scraping the surface with a sterile Drigalski spatula. Conidial concentration was adjusted to 10^6 conidia/ml.

The rolled paper towel technique (Warham et al. 1996) was used with some modifications. Disinfected seeds were immersed for 5 min in the conidia suspension, placed on a 36 × 19.5 cm sterile paper towel, moistened with sterile distilled water, rolled and maintained in plastic bags. Three such rolls containing five seeds each were used as replicates for each isolate. The assays were conducted at 25 ± 2°C with a 14 h:10 h light/dark photoperiod for 14 days. Humidity was maintained on the paper towel rolls by minimal irrigation of sterile distilled water.

Disease symptoms were visually assessed using the severity scale reported by García-Espinoza (2009), in which 0 = healthy seedling without root rot; 1 = 0.1–2.0 cm of root rot; 2 = 2.1–5.0 cm of root rot; 3 ≥ 5.0 cm of root rot and 0.1–1.0 cm of rot in the base of the stalk; 4 ≥ 5.0 cm of root rot and 1.1–2.0 cm of rot in the base of the stalk; 5 = dead seedling. Data were converted to a disease index score using the formula reported by Asran and Buchenauer (2003). Plant height as well as shoot and root biomass (dry weight) were measured.

Greenhouse pot assay

The same isolates as tested in the previous assay were evaluated under greenhouse conditions. Commercial maize seeds (Garañón hybrid) were disinfected as

described above. Three mycelial plugs (7 mm diameter) from a 7-day-old acidified PDA culture were transferred to 100 g of sterile cracked maize hydrated with 40 ml of sterile distilled water and incubated at 25°C for 14 days. The maize-fungus mix was then added to 3-kg plastic pots containing an autoclaved vermiculite:soil (1 : 1) mixture. This soil mixture was moistened and then autoclaved three times at 121°C for 1 h at 1-day interval, and the inocula were maintained for 5 days in a greenhouse at 28 ± 2°C. The substrate mixture of the control treatment was added with 100 g of sterile non-inoculated cracked maize. Inocula concentrations (UFC/g) were estimated at day six before sowing, using the Massive Stamping Drop Plate method (Corral-Lugo et al. 2012; Table S2) in Nash–Snyder agar plates. Three seeds were plated per pot, and triplicate treatments were set up in a completely randomized design. Pots were kept in a greenhouse at 28 ± 2°C with a natural photoperiod for 1 month, where they were watered weekly with half-strength Hoagland's nutrient solution (Millner and Kitt 1992). Visual disease symptoms on roots were assessed according to the severity scale reported by Soonthornpoct et al. (2000). Data were converted to a disease index score using the formula reported by Asran and Buchenauer (2003). Plant height and stalk thickness as well as shoot and root biomass (dry weight) were measured.

Microscopy

Root samples were taken from the greenhouse assay plants. The samples were washed in running water, and cut transversally and longitudinally with a scalpel blade. To visualize fungal tissue inside the maize plants, samples were stained with WGA, Alexa Fluor® 488 conjugate, which binds to the chitin residues of the fungal cell wall (Figueroa-López et al. 2014). Cut samples were incubated overnight at 4°C in 1 ml of 1× PBS buffer (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4) supplemented with WGA, Alexa Fluor® 488 conjugate (WGA, 1 ng/μl; Cat. No. W11261, Life Technologies, Eugene, OR, USA) followed by three washes with 1× PBS buffer (Figueroa-López et al. 2014).

For visualization, one tissue preparation was placed on a microscope slide, submerged in propidium iodide solution (PI; 4 ng/μl), and covered with a glass coverslip. To examine root tissue autofluorescence, root sections of non-inoculated plants were observed without any fluorophore (PI or WGA), while root sections stained with PI or WGA were used to verify the stain reaction and specificity of each fluorophore. Confocal microscopy was performed with an inverted laser scanning confocal microscope (Leica TCS SP5 X) using a white laser for 497 nm and 489 nm excitation wavelengths, with emission ranges of 502–548 nm for WGA (green fluorescence) and 598–706 nm for PI (red fluorescence), respectively.

Statistical analysis

Data from pathogenicity assays and morphological variables were subjected to analysis of variance (ANOVA) using the program SAS 9.0 (SAS Institute, Inc., Cary, NC, USA). Duncan's test was used for *post hoc* comparison of means ($P \leq 0.05$). Disease index percentages were arcsine-transformed prior to ANOVA. Normality of the data was corroborated with the Kolmogorov–Smirnov test and a quantile–quantile plot (qq plot).

Correlations between severity and morphological variables were performed by calculating Pearson's coefficients and their probabilities.

Results

Molecular identification of *Fusarium* isolates

Partial sequences of calmodulin and EF-1α genes allowed the identification of all isolates. Four species were identified: *F. verticillioides*, *F. nygamai*, *F. andiyazi* and *F. thapsinum* (Table S1). Similarities to DNA sequences in the GenBank database ranged from 97–100% for calmodulin, and from 99–100% for EF-1α. Of 108 isolates, 79 were identified as *F. verticillioides*, 23 as *F. nygamai*, 4 as *F. andiyazi* and 2 as *F. thapsinum*. Nine isolates previously identified by ITS rDNA sequencing as *F. verticillioides* were determined to belong to other species of the FFSC (asterisks in Table S1).

Isolates with identical gene sequences were analyzed as a single sequence in the phylogenetic analysis. Thus, only 33 sequences for each gene representing the entire set of data were used. One distance tree was generated for each gene (Figs 1 and 2). The distance tree derived from the partial sequences of the calmodulin gene (Fig. 1) reveals that nineteen isolates group with a *F. verticillioides* reference sequence, two with *F. thapsinum* and ten with *F. nygamai*; two isolates (F131 and F133) group in a separate cluster between *F. verticillioides* and *F. thapsinum*. The distance tree based on partial sequences of the EF-1α gene is consistent with the one derived from the calmodulin gene. The EF-1α gene enabled the identification of isolates F106, F116, F131 and F133 as *F. andiyazi* (Fig. 2; 96.7% bootstrap value). This was

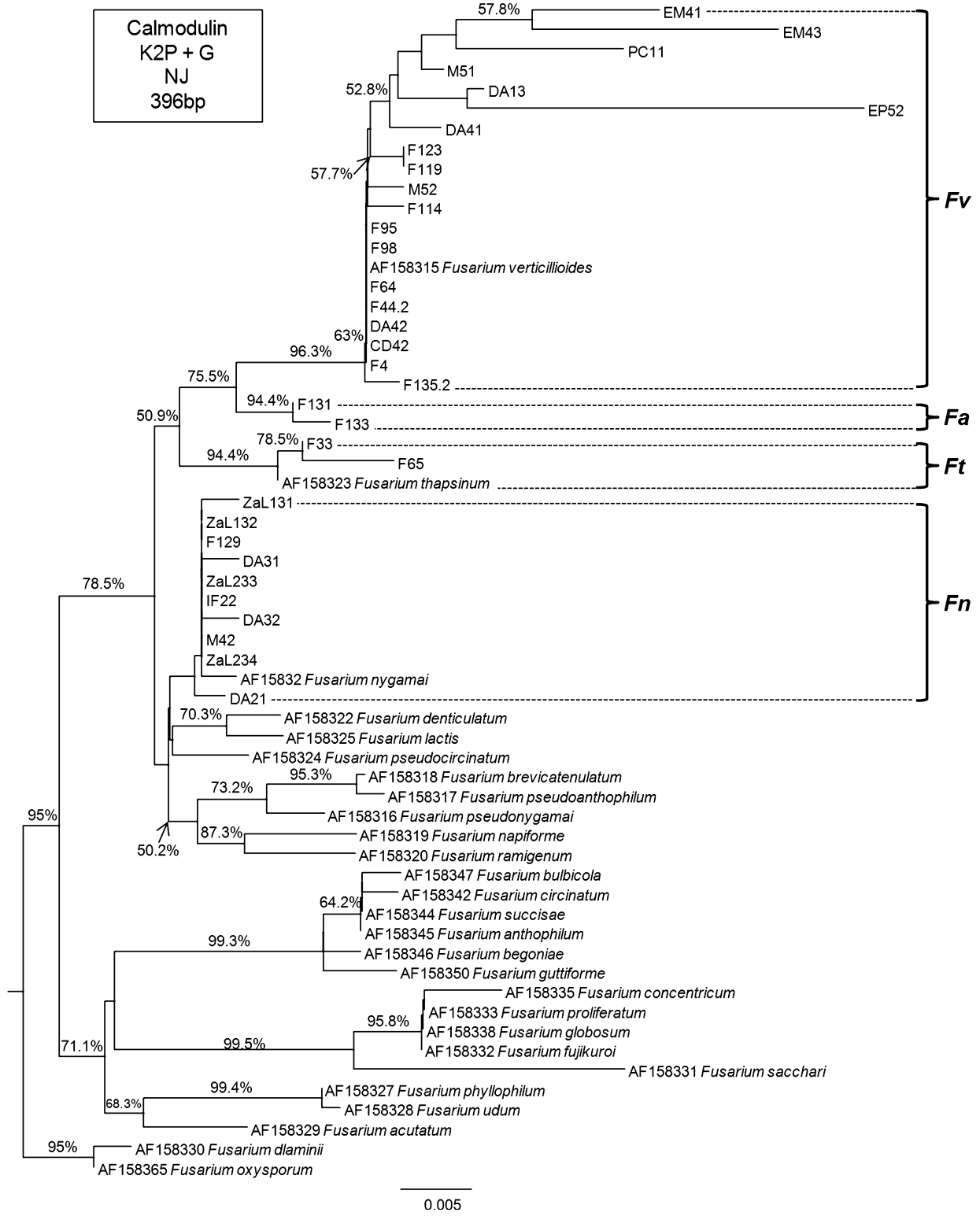

Fig. 1 Distance tree derived from partial sequences of the calmodulin gene. The tree was constructed with Mega 5.2 Beta (bootstrap = 1000), using the Kimura 2-parameter (K2P) substitution model with gamma distribution (+G). Bootstrap values are indicated as percentages. Thirty-three *Fusarium* isolates and 27 reference sequences (O'Donnell et al. 2000) were used to build the tree. NJ: neighbour joining.

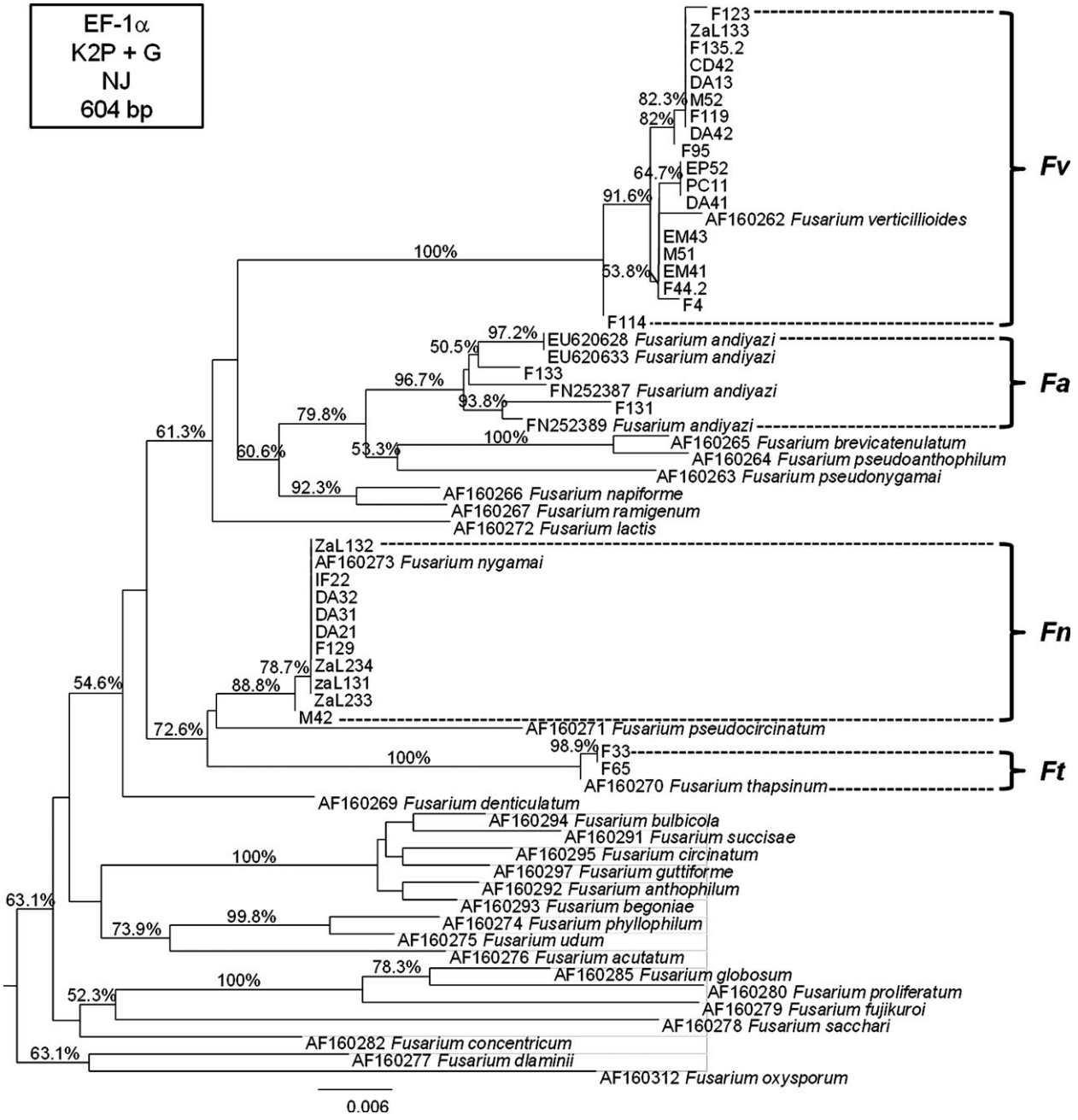

Fig. 2 Distance tree derived from partial sequences of the EF-1α gene. The tree was constructed with Mega 5.2 Beta (bootstrap = 1000), using a Kimura 2-parameter (K2P) substitution model with gamma distribution (+G). Bootstrap values are indicated as percentages. Thirty-three *Fusarium* isolates and 31 reference sequences (O'Donnell et al. 2000; Petrovic et al. 2009; Wulff et al. 2010) were used to build the tree. NJ: neighbour joining.

not possible with the calmodulin gene, as the phylogeny of *F. andiyazi* is based primarily on EF-1α (Kvas et al. 2009; Dal Prà et al. 2010; Wulff et al. 2010). Concatenate analysis (calmodulin + EF-1α) did not improve the bootstrap support of the tree (data not shown).

This molecular identification allowed us to analyze *Fusarium* species present in different maize tissues. The predominant species in seeds was *F. verticillioides* (89%; 63 isolates), followed by *F. andiyazi* (5%; four isolates), *F. thapsinum* (3%; two isolates) and *F. nygamai* (3%; two isolates; Fig. S1a). On roots, 54% of the isolates were identified as *F. nygamai* (20 isolates) and 46% were *F. verticillioides* (17 isolates; Fig. S1b). Only one isolate from stalk was identified as *F. nygamai*.

We detected mixed infections of *F. verticillioides* and *F. thapsinum* in maize seeds. In roots, *F. verticillioides* and *F. nygamai* were observed to co-infect in roots, with the latter being more frequent than the former (Fig. S2).

Pathogenicity assays

Rolled paper towel assay

All isolates were able to infect maize and produce characteristic fusariosis symptoms (root rot, as well as wilting, stalk thinning, and reduced aerial and root growth). Different levels of aggressiveness were observed between species and between isolates of the same species (Fig. 3a). Isolates FvDA42 and FvP03 of *F. verticillioides* and FnCI62 of *F. nygamai* registered the highest severities of all isolates tested (90.6, 77.3 and 88%, respectively; Fig. 3a). These isolates also reduced the height of the seedlings in 35 to 50% (Fig. 3b). In addition, a negative correlation between severity and seedling height ($r = -0.83$, $P = 0.0008$) was observed. All strains reduced shoot and root biomass and showed significant differences with respect to the control, though no differences were observed between isolates (Fig. 3c and d). No correlation was observed between severity and shoot ($r = -0.51$, $P = 0.09$) or root biomass ($r = -0.44$, $P = 0.14$).

Greenhouse pot assay

As in the previous assay, all isolates were able to infect maize plants. Different levels of aggressiveness were observed between *F. thapsinum* and the three other species (Fig. 3e). Severity of *F. verticillioides*, *F. nygamai* and *F. andiyazi* was high, and no differences

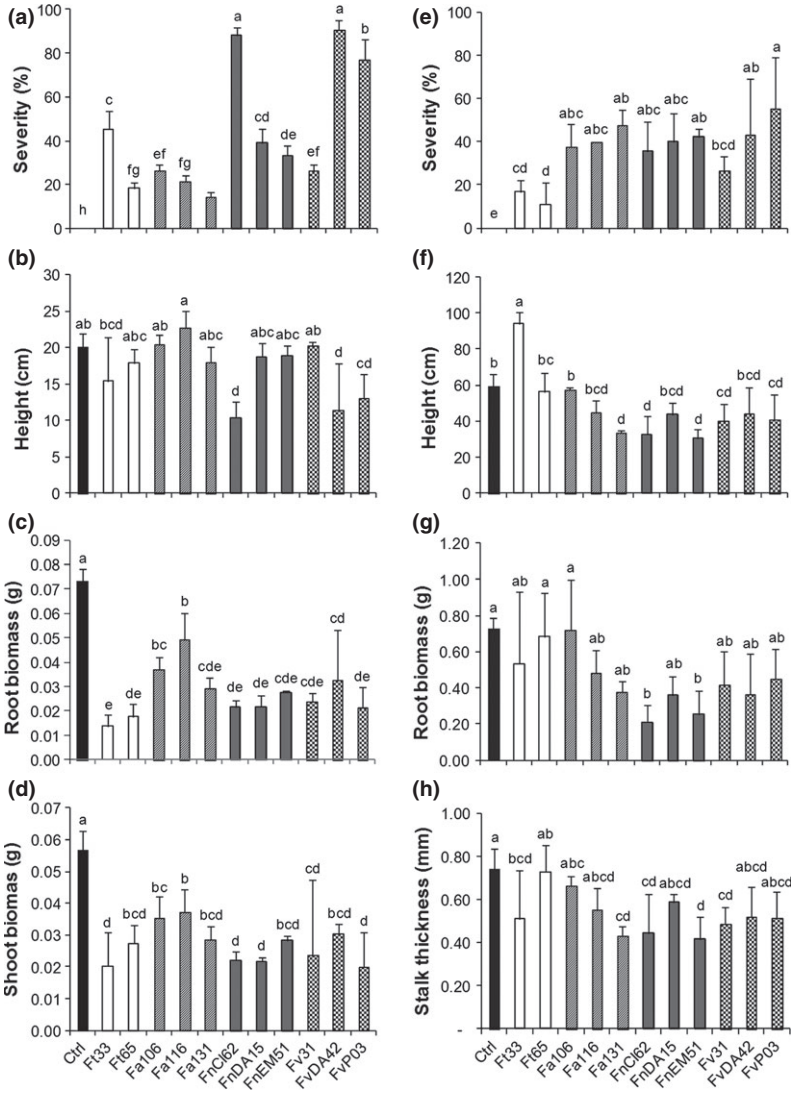

Fig. 3 Severity (a and e), height (b and f), root biomass (c and g), shoot biomass (d) and stalk thickness (h) of maize plants infected with different isolates of several *Fusarium* species. Data were collected in either a rolled paper towel assay (left panels, a–d) or a greenhouse pot assay (right panels, e–g). Different letters above the bars indicate significant differences ($P \leq 0.05$) between treatments according to Duncan's test. ■ Ctrl = Control; □ *F. thapsinum* isolates; ▨ *F. andiyazi* isolates; ▩ *F. nygamai* isolates; ▩ *F. verticillioides* isolates.

between isolates were observed, except for isolate Fv31, which caused less damage (Fig. 3e). Indeed, this was similar to the damage caused by *F. thapsinum* isolates (Fig. 3e). Plant height was reduced by infection with *F. verticillioides* Fv31 and FvP03, *F. nygamai* FnEM51 and FnCI62, and *F. andiyazi* Fa131 (Fig. 3f). Infection with *F. thapsinum* Ft33 significantly increased height in plants (Fig. 3f) and reduced stalk thickness (Fig. 3h). A low yet significant negative correlation was observed between plant height and severity ($r = -0.60$, $P = 0.03$). *F. nygamai* FnCI62 and FnEM51 were the only isolates that significantly reduced root biomass (Fig. 3g). A negative correlation between root biomass and severity was detected ($r = -0.61$, $P = 0.035$). No significant differences in shoot biomass were observed (data not shown). Stalk thickness was affected by infection with *F. andiyazi* Fa131, *F. nygamai* FnCI62 and FnEM51, and *F. verticillioides* Fv31 (Fig. 3h), and it correlated with severity ($r = -0.64$, $P = 0.02$).

Microscopy

Fungal infection in maize plants was visually confirmed by confocal microscopy, using the plants from the greenhouse pot assay. A faint red autofluorescence was weakly observed in the maize roots (Fig. S3a). Propidium iodide completely stained cell walls of root tissues (Fig. S3b) and was able to stain cell nuclei in necrotic roots as well (data not shown). WGA staining exhibited an affinity with plant root tissues. We also observed a uniform pattern of green fluorescence delimiting cell walls of cortical cells, the endodermis, pericycle, xylem and pith (Fig. S3c). Epidermal and other cells of the vascular tissue did not exhibit WGA staining (Fig. S3c).

Fungal colonization was demonstrated by identifying hyphal structures that were intra- or intercellularly stained with WGA. At the end of the greenhouse experiment (30 days post-inoculation), roots of non-inoculated plants (control) were colonized and displayed low levels of green fluorescence in the intracellular space of the epidermis and adjacent cortical cells (arrow in Fig. 4a and asterisk in Fig. 4b). This is commonly observed in these types of experiments (Oren et al. 2003; Wu et al. 2011).

Colonization of *F. thapsinum* and *F. andiyazi* isolates was limited to the epidermis and cortical cells (Fig. 4c, d, e and f). Plants inoculated with *F. verticillioides* and *F. nygamai* isolates exhibited a robust colonization pattern throughout the entire root sections, from the epidermis to the pith (Fig. 4g, h, i and j). In all cases, hyphae were observed in the intercellular (arrows in Fig. 4d, f, h and j) and intracellular spaces (asterisks

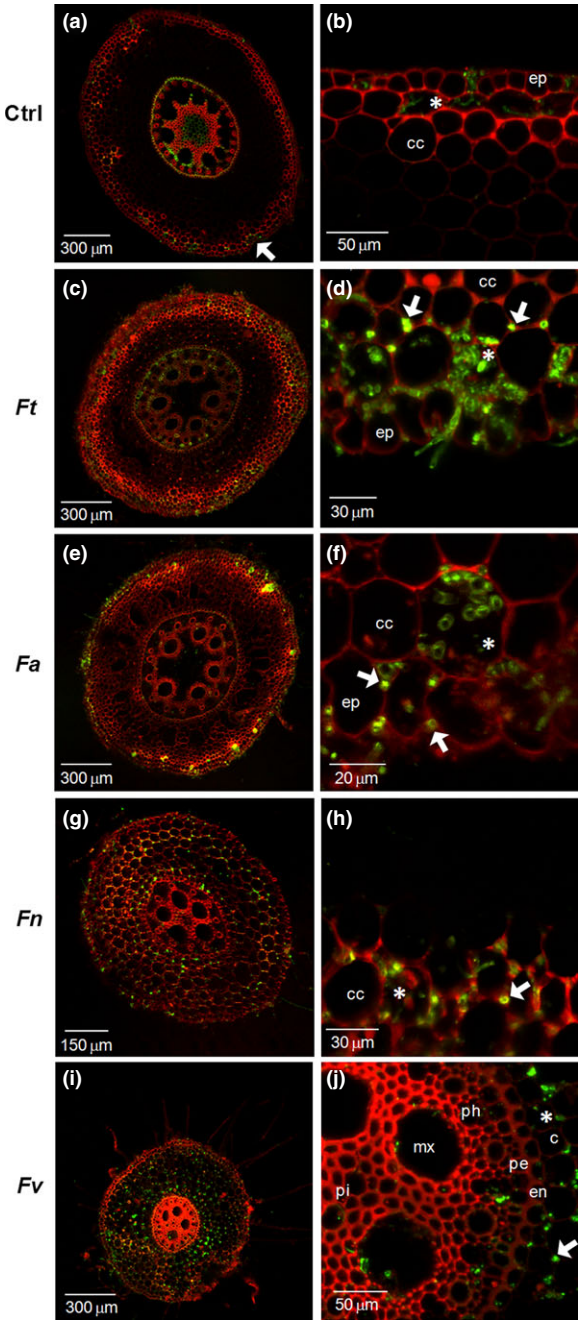

Fig. 4 Transversal sections of maize roots colonized by different *Fusarium* species. One-month-old maize seedlings were inoculated with the respective *Fusarium* species and grown under greenhouse conditions before excision. Root of a non-inoculated plant (a and b; Ctrl = control) showing low levels of fungal colonization in the epidermis and the adjacent cortical cells (b). Root colonization by *F. thapsinum* (c and d) and *F. andiyazi* (e and f) was observed in the epidermis and cortical cells. *F. nygamai* (g and h) and *F. verticillioides* (i and j) colonized all root tissues. (ep: epidermis; cc: cortical cells; en; endodermis; pe: pericycle; mx: metaxylem; ph: phloem; pi: pith). Arrow indicates hyphae detected in the intracellular spaces. *Indicates cells that have been completely invaded by fungal hyphae.

in Fig. 4d, f, h and j). Hyphae occupied most of the intracellular space of epidermal and cortical cells (asterisk in Fig. 4d and f) in a mosaic pattern, leaving some cells uninfected (Fig. 4d, f, and j).

Discussion

The study of maize infections caused by *Fusarium* species in Mexico has not been greatly represented in the literature, despite their significant impact on production and food safety. In our study, a collection of 108 *Fusarium* isolates from Sinaloa, obtained from different maize tissues and crop cycles, were identified using a phylogenetic approach with two protein-encoding nuclear genes that can resolve most of the species in the FFSC.

Regardless of the infected tissue, the isolates could be classified into four species: *F. verticillioides* (72.89%), followed by *F. nygamai* (21.49%), *F. andiyazi* (3.73%) and *F. thapsinum* (1.86%). These species have been previously associated with maize worldwide; although to the best of our knowledge, this is the first report to address the maize infectivity of *F. nygamai*, *F. andiyazi* and *F. thapsinum* in Mexico.

As previously reported, most of the identified isolates belong to *F. verticillioides*, which has been associated with maize ear and root rot in Mexico (Figueroa-Rivera et al. 2010; Reyes-Velázquez et al. 2011), as well as worldwide (Zainuddin et al. 2011; Covarelli et al. 2012; Madania et al. 2013). In our study, this species predominated on seeds and was less frequently isolated from maize roots, which is an observation corroborated by several other reports (Figueroa-Rivera et al. 2010; Reyes-Velázquez et al. 2011). The second most frequently isolated species in this study was *F. nygamai*, which was particularly represented in roots. So far, this species has only been isolated from maize ears in Malaysia (Zainuddin et al. 2011) and India (Chehri 2011) and is associated with ear rot. Two isolates were identified as *F. thapsinum*, which is a major pathogen of sorghum (Jurjevic et al. 2005; Leslie et al. 2005), although some isolates have been recovered from maize that are associated with ear rot (Klittich et al. 1997; Madania et al. 2013). Four seed isolates were identified as *F. andiyazi*; this species is also reported as a sorghum pathogen (Leslie et al. 2005), even though it was recently isolated from maize ears in Syria (Madania et al. 2013) and from rice plants in Malaysia (Wulff et al. 2010). Interestingly, none of the isolates were identified as either *F. proliferatum* or *F. subglutinans*. These two species have been widely reported along with *F. verticillioides*, especially on maize ears in different regions of Mexico (Figueroa-Rivera et al. 2010; Reyes-Velázquez et al. 2011) and worldwide (Chehri 2011; Zainuddin et al. 2011; Zhang et al. 2012; Stumpf et al. 2013).

Infection of maize ears by several species of *Fusarium* was previously reported by Munkvold et al. (1997) and Kedera et al. (1999). The present study confirms these observations and provides additional evidence of mixed infections on maize roots and ears, for members of the FFSC. These results imply that grain contamination during storage may result in the presence of a broad spectrum of mycotoxins (Leslie et al. 2005; Leslie and Summerell 2006). Monitoring mycotoxins may be required in the future, in locations where grain can be infected by a mix of *Fusarium* species.

Pathogenicity and aggressiveness of *F. verticillioides* isolates towards maize have been widely studied (Oren et al. 2003; Wu et al. 2011). In contrast, *F. nygamai*, *F. andiyazi* and *F. thapsinum* isolates have only been associated with ear rot, with little or no accompanying data regarding their pathogenicity on maize plants. Plants infected with *F. nygamai*, *F. andiyazi* and *F. thapsinum* developed the same symptomatology as plants infected by *F. verticillioides* (root rot, as well as wilting, stalk thinning, and reduced aerial and root growth), to different degrees. *Fusarium nygamai* proved to be as aggressive as *F. verticillioides*, and it reduced both shoot and root biomass. In our greenhouse assay, *F. andiyazi* isolates demonstrated the ability to cause severe damage under high inoculum conditions. *Fusarium thapsinum* caused minor damage in both assays, in agreement with Jardine and Leslie (1999).

The spatial infection patterns of the different *Fusarium* species were assessed by confocal microscopy. We found that labelling with WGA, Alexa Fluor 488 conjugate (WGA) was highly effective for visualizing mycelia on maize roots, without any processing of the samples. Even when WGA bound to maize tissues, it was possible to distinguish between the plant and fungus due to the pattern and localization of the fluorescence (Fig. S3d). The presence of arabinogalactan proteins in plant cell walls could account for the binding of WGA on maize roots, and varying degrees of organ-specific and tissue-specific expression of these proteins may explain the lack of WGA staining on epidermal cells and other cells (Showalter 2001).

One month after fungal inoculation, maize roots were colonized by a different *Fusarium* species. In all cases, hyphae moved mainly through the intercellular spaces and colonized some cells in which the entire intracellular space was filled in a mosaic pattern. This same behaviour has been previously reported for

F. verticillioides (Oren et al. 2003; Wu et al. 2011) and other maize pathogens like *Colletotrichum graminicola* (Sukno et al. 2008). *Fusarium nygamai* was also able to colonize the vascular tissue, unlike the other two species that limited their infection to the epidermis and the adjacent cortical cells. It has been established that the success of *F. verticillioides* at infecting maize is the result of a long period of co-evolution (Pamphile and Azevedo 2002; Bacon and Yates 2006). *F. nygamai* may also have experienced the same process, as it was confounded with *F. verticillioides* until the advent of molecular techniques that allowed their differentiation. On the other hand, the relatively poor colonization and aggressiveness of *F. andiyazi* and *F. thapsinum* towards maize may be due to differences in host preferences (Jardine and Leslie 1999; Leslie and Summerell 2006).

Maize root rot in northern Sinaloa, Mexico is caused by a group of fungi that belong to the FFSC. While our pathogenicity assays demonstrated the ability of all species to infect maize roots, our findings strongly suggest a differential distribution of the four identified species in maize plants, with *F. verticillioides* being the predominant species on maize ears (seed) and *F. nygamai* on maize roots. One question open for future research is the ability of these fungal isolates to produce mycotoxins. Further experiments should also focus on performing co-infection assays under controlled conditions for the different identified members of the FFSC, in order to confirm observations of mixed infections that are present in the field.

Acknowledgements

The authors are grateful to the Fundación Produce Sinaloa and the Secretaría de Investigación y Posgrado del Instituto Politécnico Nacional (SIP-IPN) for financial support. KYLM acknowledges the Consejo Nacional de Ciencia y Tecnología (CONACyT, México) and the SIP-IPN for Ph.D. fellowships. The authors thank Dr. Simoneta Negrete-Yankelevich for her critical suggestions regarding data analysis, María Fernanda Valenzuela-Perea for her help in setting up the greenhouse pot assay and Dr. Brandon Loveall of Improvence for English proofreading of the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Spatial distribution of *Fusarium* species in maize. Percentage of *Fusarium* species found on maize seed (a) and root (b). *Fv = F. verticillioides*; *Fn = F. nygamai*; *Fa = F. andiyazi*; *Ft = F. thapsinum*.

Figure S2. Proportion of species found in mixed infections on maize seeds and roots. Each pie chart represents a separate maize plant. The names of the isolates obtained from each plant are indicated next to the corresponding percentage in the pie chart.

Figure S3. Root sections of non-inoculated plants used to verify the staining reaction and specificity of fluorophores. a) Root without staining (autofluorescence); b) root stained with propidium iodide (4 ng/μl); c) Root stained with WGA, Alexa Fluor® 488 conjugate (1 ng/μl); d) Root stained with both fluorophores. (ep: epidermis; cc: cortical cells; en; endodermis; mx: metaxylem; px: protoxylem; pi: pith).

Table S1. Origin of *Fusarium* isolates.

Table S2. Inoculum concentrations of fungal isolates tested in the greenhouse assay. Concentrations were determined by the Massive Stamping Drop Plate method (Corral-Lugo et al. 2012).