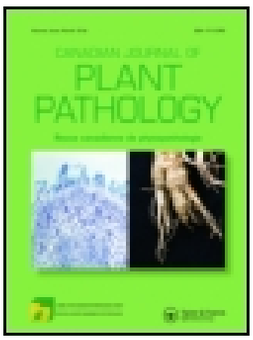

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Characterisation of *Fusarium* species infecting tomato in Mwea West Sub-county, Kirinyaga County, Kenya

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Abstract: *Fusarium* species cause various diseases of tomato, the most common being vascular wilt and cortical rots. A study was carried out to characterize *Fusarium* spp. found in the stems of wilted tomato plants from farms in Mwea West Sub-county, Kirinyaga County, Kenya. Isolates were collected from tomato stems showing vascular discolouration. One hundred and one isolates of *Fusarium* spp. were obtained and classified based on their pathogenicity on the tomato cultivar 'Money Maker'. The 10 most virulent isolates were identified by PCR amplification and sequencing of the TEF-1α gene as *Fusarium oxysporum* f. sp. *lycopersici, Fusarium oxysporum* and *Fusarium verticillioides*. *Fusarium oxysporum* f. sp. *lycopersici* causes vascular wilt in tomato, while *F. verticillioides* is known to infect maize, causing ear rot. The infection of tomato plants by *F. verticillioides* may reflect the rotation of tomato with maize in some fields, resulting in the accumulation of inoculum of this fungus and the subsequent infection of the tomato crop. The presence of vascular wilt caused by *F. oxysporum* and *F. verticillioides* suggests that strategies must be put in place to manage this disease. There is a need for surveillance by all agricultural stakeholders in order to check the progress of *F. verticillioides* that can infect tomato and other crops.

Keywords: *F. verticillioides*, *Fusarium oxysporum* f. sp. *lycopersici*, *Fusarium* spp, isolation, TEF-1α gene

Résumé: Les *Fusarium* causent diverses maladies chez la tomate, les plus courantes étant la flétrissure vasculaire et les pourritures du cortex. Une étude a été menée pour caractériser les espèces de *Fusarium* trouvées dans les tiges de plants de tomates flétris provenant de fermes du sous-comté de Mwea Ouest, dans le comté de Kirinyaga au Kenya. Des isolats ont été collectés sur des tiges de plants de tomate affichant de la décoloration vasculaire. En tout, nous avons obtenu 101 isolats de *Fusarium* spp. qui ont été classés en fonction de leur pathogénicité à l'égard du cultivar 'Money Maker'. Grâce à l'amplification par PCR et au séquençage du gène de la TEF-1α, les 10 isolats les plus virulents ont été identifiés en tant que *Fusarium oxysporum* f. sp. *lycopersici, Fusarium oxysporum* et *Fusarium verticillioides*. *Fusarium oxysporum* f. sp. *lycopersici* cause la flétrissure vasculaire chez la tomate, tandis que *F. verticillioides* infecte le maïs, causant la pourriture de l'épi. L'infection des plants de tomate par *F. verticillioides* peut signifier la rotation de la tomate avec le maïs dans certains champs, occasionnant l'accumulation d'inoculum de ce champignon et l'infection subséquente de la récolte de tomates. La flétrissure vasculaire causée par *F. oxysporum* et *F. verticillioides* suggère que des stratégies doivent être élaborées pour gérer cette maladie. Tous les intervenants du secteur agricole doivent être vigilants afin de freiner le progrès de *F. verticillioides* qui peut infecter la tomate et d'autres cultures.

Mots clés: *Fusarium* spp, *Fusarium oxysporum* f. sp. *lycopersici*, *F. verticillioides*, isolement, gène de la TEF-1α

Introduction

Fusarium species are the causal agents of various tomato diseases, including vascular wilt and cortical rots. *Fusarium oxysporum* is an abundant, fungal species complex with numerous morphologically indistinguishable plant pathogenic and non-pathogenic strains. Pathogenic *F. oxysporum*

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strains have been grouped into *formae speciales* based on their host plants (Van der Does et al. 2008). More than 120 *formae speciales* have been described (Van der Does et al. 2008; Lievens et al. 2008). *Fusarium oxysporum* f. sp. *lycopersici* (FOL) Snyder & Hansen belongs to the *F. oxysporum* species group complex and is a soilborne pathogen that causes vascular wilt in tomato. *Fusarium verticillioides* (Sacc.) Nirenberg (*Syn. F. moniliforme* Sheld.) mainly infects cereal crops such as maize and wheat. It is the main fungal agent that causes ear and kernel rot of maize (*Zea mays* L.) worldwide (Arias et al., 2012). The Mwea area in Kirinyaga County, Kenya, is a region where large volumes of tomatoes are grown (Wanjohi et al. 2018). Water has been harnessed from the rivers Ragati, Rwamuthumbi, Thiba, Rupangazi, and Nyamidi into irrigation canals for the cultivation of tomatoes and other crops (Mugo 2012). One of the diseases that cause major losses in yields of tomato in this area is vascular wilt caused by *F. oxysporum* f. sp. *lycopersici* (Mugo 2012).

The identification of fungi based on morphological criteria is difficult and can be erroneous (Saikia & Kadoo 2010). There are many new molecular techniques, such as analysis of gene sequences, used for the identification of fungal pathogens (Paplomatas 2004). Sequence-based characterization relies on obtaining sequences of specific gene regions and comparing them with reference sequences in public data bases (Saikia & Kadoo 2010). The translation elongation factor 1-alpha gene (TEF-1α) DNA sequence is preferred as a genetic marker in the identification of *Fusarium* spp. because it is able to discern up to the species level, at least for most of species (Gazis et al. 2011).

This study was conducted to isolate and identify *Fusarium* species causing vascular browning in infected tomato plants in Mwea West Sub-county, Kirinyaga County, Kenya, based on morphological and molecular characteristics, and pathogenicity tests.

Materials and methods

Sample collection and isolation of Fusarium spp. from tomato stems

A survey was conducted in October 2014 in Mwea West Sub-county, Kirinyaga County, Kenya. Fungal isolation was performed on diseased tomato stems showing symptoms of wilting and vascular discolouration collected from farms around Baricho, located at longitude 37°14 ‘27”E and latitude 0°33′ 7”S, Kagio (37°15′ 12”E, 0°37′ 27”S), Kangai (37°15′ 12”E, 0°37′ 18”S), and Kandongu (latitude, 37°17′ 38” E, latitude 0°39′ 45”S). There were 225 stem pieces obtained from 119 farms. The stem pieces from each diseased plant were thoroughly washed in tap water and then aseptically cut into 1-cm long pieces. The stem pieces were surface-sterilized in 2% sodium hypochlorite solution for 30 sec, then rinsed with three changes of sterile distilled water and then dried using sterile serviettes. The stem segments were aseptically placed on Potato Dextrose Agar (PDA) in Petri-dishes; five segments from a sampled stem were placed in the same Petri dish and this was done in duplicate. The samples were incubated on a laboratory bench at room temperature (24 C ± 2) for 5 days under illumination with fluorescent lights (Phillips TLD 30 w/08-BLB) suspended 40 cm above the bench and a 12-hr photoperiod (Leslie & Summerell 2006).

Fungal colonies with white to pink mycelia that are characteristic of *Fusarium* spp. were subcultured on PDA at room temperature. After one week, the isolates were cultured on SNA (Spezieller Nahrstoffarmer Agar) to allow for sporulation of the fungus (Burgess et al. 1994). After 14 days, a microscopic examination of the cultures was conducted. A portion of the mycelium was picked with a needle, placed on a slide and stained using lactophenol in cotton blue. This was then observed under a compound microscope at X10, X40 objective, and X100 under an oil emersion lens. Macroconidia, microconidia, phialides and chlamydospores were important characteristics in the identification of the isolates (Nelson et al. 1983). Single-spore cultures of the isolates were made and preserved on PDA slants at 4 C for molecular identification.

Pathogenicity tests

The isolates were tested for their pathogenicity on the tomato cultivar, ‘Money Maker’. This is an heirloom variety that does not have disease resistance, unlike newer hybrid varieties. Twenty-one-day old seedlings were inoculated by the standard root dip method (Reis et al. 2005). The seedlings were uprooted, shaken to remove adhering soil particles and washed carefully under running water. Two centimetres of the root system from the apex was cut with sterile scissors and then dipped in a spore suspension (approximately 10^7 conidia/mL) of each test isolate for 10 to 15 min. In the control treatment, plants were dipped in sterile distilled water for 10 to 15 min. The seedlings were planted in polythene sleeves (5 × 7 × 150 cm) containing a mixture of soil and sand in a ratio of (3:1), at density of one seedling per bag. The test for each isolate was replicated four times in a completely randomized design under greenhouse conditions. Plants were watered daily for the first three weeks, and every other day for the rest

of the experiment. Disease severity was assessed 45 days after transplanting/inoculation using a modified wilting severity scale of 1–4 (Tanwar et al. 2013), where: 1 = no symptoms, 2 = '1 to 25%' wilting, 3 = '26 to 49%' wilting, and 4 = wilting ≥ 50%. The percentage in this scale represents the extent of wilting of a single plant.

Molecular characterization of Fusarium spp. isolates

Ten isolates that were the most virulent in the pathogenicity test were selected for the molecular analysis. DNA was extracted from each isolate according to a procedure adapted from Green & Sambrook (2012).

The isolated DNA products were amplified by PCR. Amplification of the translation elongation factor 1-alpha (TEF-1α) gene was performed using the following primers: ef1 (5′-ATG GGT AAGGA (A/G) GAC AAG AC-3′) and ef2 (5′-GGA (G/A) GT ACC AGTG/C) AT CAT GTT-3′) (O'Donnell et al. 1998). The volume of the PCR mix was 25 µL and consisted of 2x Eppendorf Master Mix [Taq DNA polymerase 0.125 µL (1.25 U), 30 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$; 0.1% Igepal-CA630; 0.2 mM dNTP], 0.6 µM of each primer, and 2 µL of fungal DNA (Mohammed et al. 2016).

A Gene Amp 9700 thermocycler (Applied Biosystems, Waltham, MA, USA) was used for PCR amplifications under the following conditions: initial denaturation at 94 C for 85 sec followed by 35 cycles of denaturation at 95 C for 35 sec, annealing at 59 C for 55 sec and extension at 72 C for 90 sec, followed by a final extension for 10 min at 72 C. PCR products were examined by electrophoresis in a 1.5% agarose gel in TBE buffer (Mohammed et al. 2016). The purified PCR products were sent to Inqaba Biotechnical Industries (Pretoria, South Africa) for sequencing. The protocol for sequencing of the PCR products was a modified version of the protocol described in Applied Biosystem's ABI PRISM© BigDye™ Terminator Cycle Sequencing Ready Reaction Kit manual.

The trace files generated by the DNA analyser sequencer from Applied Biosystems were imported into CLC main workbench v. 6.8.1 for a quality check, editing, and assembly. Following this procedure, all the trace files were trimmed to remove low-quality bases at the extreme ends of the chromatograms using the default settings of the software.

For each sample, trimmed sequences for the reverse and forward orientations were assembled into contigs against the *Fusarium oxysporum* f. sp. *lycopersici* TEF1-alpha gene as the reference for scaffolding using the default assembling algorithm. Corrections of bases on the resultant contigs were done manually to ensure the correct base calling for the subsequent consensus sequences. These ambiguities and disagreements were resolved by referring to the chromatogram files of the respective reads in that consensus sequence and independent of the reference sequence. Clean scaffolds were saved as consensus files in FASTA (FAST-ALL) formats. The sequences were blasted individually for similarity in the Fusarium-ID isolate database (http://isolate.fusariumdb.org) and Fusarium Multilocus Sequence Typing (MLST) database [(http://www.cbs.knaw.nl/Fusarium) (Geiser et al. 2004)].

Results

Macroscopic and microscopic characteristics of isolates of Fusarium *spp. from diseased tomato stems*

One-hundred and one isolates of *Fusarium* were recovered from 101 of the 225 sampled tomato plants. Growth on PDA produced cream-coloured to yellowish and white aerial mycelia, which in some cases became pink and later purple in colour (Table 1). The isolates showed variation in the pigments produced in media, which varied from no pigmentation (creamy white) to purple pigmentation. The microscopic features that were important in identification included septation and the shapes of the microconidia and macroconidia, and the structure of the chlamydospores (Nelson et al. 1983). Masses of conidiophores on sporodochia were produced in culture. The macroconidia were produced on sporodochia or from monophialides on hyphae, appearing at the tip of the phialide one at a time. The macroconidia in some cases were abundant while in other cases were few or even absent. They were slightly curved (sickle cell shape), and had 3 to 5 septations (Table 1). Microconidia accumulated at the tip of the conidiogenous cells, the phialides. They were borne on short monophialides arising from aerial mycelia. All isolates produced microconidia, which were oval or kidney shaped, mainly single celled with a few having two cells. The resting spores, chlamydospores, were produced in cultures that were over two weeks old. Some isolates did not produce chlamydospores (Table 1).

Pathogenicity tests results

The symptoms on infected tomato plants appeared as yellowing of the lower leaves and in later stages, drooping of the leaves. In severe infections, there was browning of the vascular bundles and wilting of the plant. Of the 101 isolates, four were found to be virulent, 15

Table 1. Morphological characteristics of *Fusarium* spp. isolated from diseased tomato stems collected from Mwea West Sub-county, Kenya.

Culture no.	Species identified	Colour of mycelium	Colour of reverse	Micro-conidia and number of septations	Macro-conidia and number of septations	Presence and position of chlamydospores
F1	*F. verticillioides*	White	cream	0	3 septate	None
F11	*F. verticillioides*	Cream	Cream	0	None	None
F41	*F. o.* f. sp. *lycopersici*	White- pink	Purple	0	3 septate	None
F66	*F. o.* f. sp. *lycopersici*	White	Cream	0	3 septate	Terminal and solitary
F68	*F. oxysporum*	White	Cream	0	3 septate	None
F84	*F. verticillioides*	Orange	Orange	0	3 septate	None
F98	*F. verticillioides*	White-pink	Cream	0	3 septate	None
F99	*F. oxysporum*	White	Cream	0	4 septate	None
F100	*F. oxysporum*	Purple	Purple	1	3 septate	Terminal and solitary
F101	*F. oxysporum*	White- pink	Purple	1	3 septate	None

moderately virulent, 67 weakly virulent, while 15 were avirulent. The four virulent isolates produced total discolouration of the vascular bundles on the tomato 'Money Maker'. The 15 moderately virulent isolates caused a faint discolouration of the vascular bundles, while the 67 weakly virulent isolates caused wilt symptoms and yellowing of the leaves but no vascular discolouration. The four highly virulent, 15 moderately, and 67 weakly virulent isolates were re-isolated from test plants and were re-inoculated again onto tomato 'Money Maker', and symptoms similar to those of the previously infected plants were observed.

Molecular analysis of isolates

The TEF-1α gene primers EfI and Ef2 primers amplified fragments of 570 to 710 bp for all isolates. The identity of the isolates was determined by comparing their TEF 1α sequence with those in Fusarium-ID (http://isolate.fusariumdb.org) and MLST (Mutilocus Sequence Typing) (http://www.cbs.knaw.nl/Fusarium) database (Aoki et al. 2014). The BLAST analysis of the TEF1-α gene sequence data identified the closest matches (99–100% similarity) of the isolates in the Fusarium-ID and MLST databases. The reference descriptions in the Fusarium-ID and MLST databases that were the closest match for each of the isolates are shown in Table 2. Both the Fusarium-ID and MLST databases included collections from the Northern Region Research Laboratory (NRRL) databases in some of the reference descriptions, and these are also included in Table 2.

Only two of the highly virulent isolates closely matched isolates of *F. oxysporum* f. sp. *lycopersici*; F41 and F66 in the MLST database, although in the Fusarium-ID database they were simply classified as *F. oxysporum* (Table 2). The eight other isolates were identified as *F. oxysporum* and *F. verticillioides* in the MLST database. A phylogenetic tree was constructed to show the relationships between the *Fusarium* spp., which indicated they could be grouped into four groups (Fig. 1).

Discussion

Microscopic characteristics such as the presence of oval to kidney shaped, non-septate microconidia, macroconidia with a slight curvature, 3–5 septations and a pointed apical cell confirm that the isolates were of *Fusarium* spp. according to descriptions by Nelson et al. (1983) and Leslie & Summerell (2006). The variability in the macroscopic, microscopic, and pathogenicity tests may be indicative of the fact that the isolates are different physiologically.

Identification by molecular means did not correspond with the morphological characterization. Isolates of *F. verticillioides* were identified by molecular means but morphologically, they resembled *F. oxysporum*. On PDA agar, isolates of *F. oxysporum* are indistinguishable from those of *F. verticillioides*. Leslie & Summerell (2006) described isolates of *F. oxysporum* as having white to pale violet mycelia producing dark magenta pigment on PDA, while those *F. verticillioides* have white mycelia and produces violet pigments in PDA. The two species have similarities in their macroconidia and microconidia. The macroconidia of the two species have 3–5 septations while microconidia are non-septate or have only one septation. Chlamydospore characteristics are distinctive in the two species, in that *F. verticillioides* does not form chlamydospores, although it has swollen cells in the hyphae that can easily be mistaken as chlamydospores. However, formation of chlamydospores is not

Table 2. Isolates identified using the TEF1α maker gene in a search of the Fusarium ID and MLST databases; the closest matching reference isolate (based % similarity) is listed and the accession number is indicated.

Isolate	Fusarium-ID Reference isolate	Fusarium MLST Reference isolate	Similarity	Accession number
F1	FD_01856_EF1α [*Fusarium* sp.] NRRL 43608	*F. verticillioides*, NRRL 20960 CBS 119285	100	MH791037
F11	FD_01856_EF-1 α [Fusarium sp.]	*F. moniliforme*, (NRRL 43608; CBS 130180	100	MH791038
F41	FD_01199_EF-1 α [*F. oxysporum*] (NRRL 26037)	*F. oxysporum* f.sp. *lycopersici* N8; elongation Factor 1- α (NRRL 34936; CBS12368)	100	MH587166
F66	FD_01199_EF-1 α [*F. oxysporum*] (NRRL 26037)	*F. oxysporum* f.sp. *lycopersici* N8; elongation Factor 1- α (NRRL 34936; CBS12368)	99	MH879137
F68	FD_00786_EF-1 α [*F. oxysporum*] (NRRL 38592)	*F. oxysporum* species complex 191 (NRRL 36356)	100	MH837647
F84	FD_01856_EF1α [*Fusarium* sp.]	*F. moniliforme*, (NRRL 43608; CBS 1301800)	99	MH910615
F98	FD_01856_EF-1α [*Fusarium* sp.]	*F. moniliforme*, (CBS 130180	100	MH931254
F99	FD_00733_EF-1 α [*Fusarium* sp.] (NRRL 458881)	*F. oxysporum* species complex (NRRL 20433)	99	MH837646
F100	FD_00789_EF1α [*F. oxysporum*) (NRRL38595)	*F. oxysporum* species complex 191, (NRRL 38593)	99	Mk972462
F101	FD_01215_EF1 α [*F. oxysporum*] (NRRL25356)	*F. oxysporum* (NRRL 36356; CBS 21.49)	99	Mk894442

The nucleotide sequence data reported are available in the GenBank databases under the listed accession numbers. CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands. Northern Region Research Laboratory cultures (NRRL).

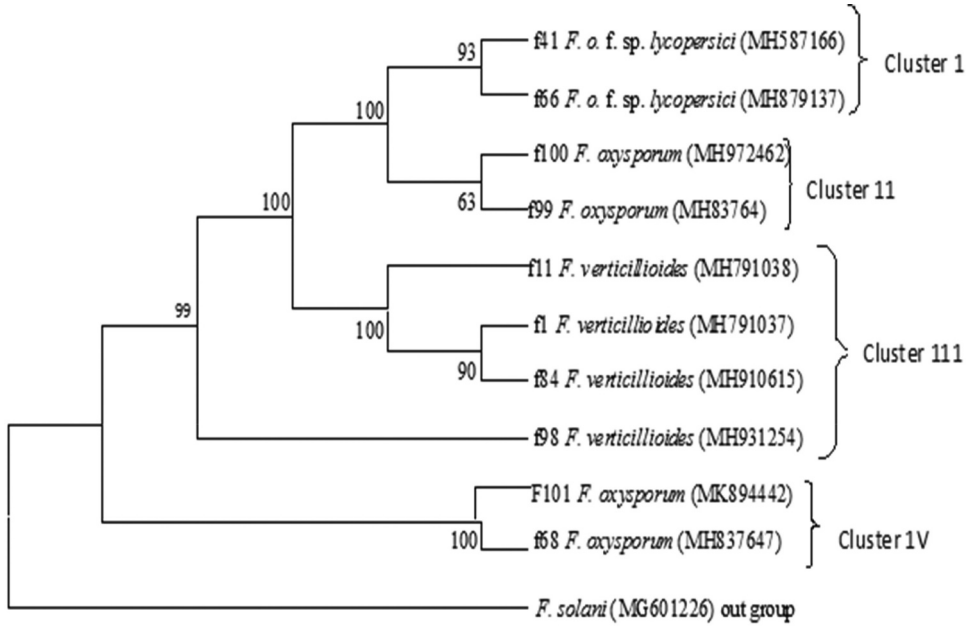

Fig. 1 Phylogenetic tree generated using by MEGA 7 software. Maximum Parsimony Analysis for the DNA sequence of the TEF 1α gene using 10 sequences of *Fusarium* spp. The numbers at the nodes represent bootstrap support values of 1 000 replicates. The sequences were submitted to GenBank and their accession numbers are indicated in parentheses.

a definitive characteristic in the identification of *F. oxysporum* as this depends on the culture conditions (Gagkaeva 2008).

The phylogenetic tree placed the isolates into four groups, with isolates of *F. oxysporum* and *F. verticillioides* grouped closely together. The closest matches of the isolates in the Fusarium ID and MLST databases was found to be with *Fusarium* spp. *F. oxysporum, F. oxysporum* f. sp. *lycopersici*, and *F. verticillioides*. Four of the 10 isolates identified by molecular methods were *F. verticillioides* (Sacc.) Nirenberg

(syn. *F. moniliforme* Sheld.). *Fusarium verticillioides* previously has been isolated from the roots of diseased tomato plants (Chehri 2016). Srivastava et al. (2010) reported that *F. verticillioides* infected cotton causing boll rots, while Hirata et al. (2001) isolated the fungus from rotting banana fruits imported to Japan from Mexico. In this study, *F. verticillioides* infected the tomato plants, resulting in vascular discoloration and wilting. The rotation of tomato with maize, which is a major crop grown in the study area, may account for the occurrence of this fungal pathogen on the former. The occurrence of vascular wilt means that strategies must be put in place to manage this disease. There is a need for surveillance by all agricultural stakeholders in order to check the progress of this pathogen in tomato and other crops.

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