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Research Article

Characterization of *Fusarium* species associated with apple decline in Tunisian nurseries

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Abstract

Considerable losses of apple trees seedling were observed in many nurseries in Tunisia. From November 2012 to December 2013, surveys were conducted in 10 apples nurseries, and samples of roots were collected from apple seedlings showing decline symptoms. The isolation and the morphological and molecular identification (using ITS1 and ITS4) of obtained isolates revealed the presence of *Fusarium oxysporum* (111 isolates), *Fusarium solani* (55 isolates), *Fusarium equiseti* (4 isolates) and one isolate of *F. proliferatum. F. oxysporum* and *F. solani* were isolated from roots from all the surveyed nurseries. *F. equiseti* were isolated from roots from nurseries in Zaghouan 1 and Sbiba. *F. proliferatum* was recovered from roots in Zaghouan 1 nursery. The highest percent of isolation was found in Chbika 1 and Chbika for *F. oxysporum* (100%) and from Manzel nour for *F. solani* (40%). However, the lowest isolation percent was found in Oued mliz nursery. This investigation showed that there is no relationship between the *Fusarium* isolation percent and the plant vigors. The pathogenicity of *Fusarium oxysporum* and *Fusarium solani* was evaluated using Golden delicious apple variety grafted onto the MM106 rootstock planted in inoculated soil and revealed that some isolates of these two *Fusarium* species were virulent.

Keywords: Surveys, *Fusarium*, apple, decline, nurseries, characterization, pathogenicity.

Introduction

Apple decline is a soil-borne disease, reported in apple-growing areas throughout the world, such as Europe (Savory, 1966; Manici et al., 2003), North America (Willet et al., 1994), South-Africa (Tewoldemedhin et al., 2011a) and Australia (Dullahide et al., 1994). Symptoms of this disease include a general growth reduction, roots browning, and stunting of apple shoots (Savory, 1966; Hoestra, 1968; Caruso et al., 1988).

In economic terms, apple decline disease has significant consequences due to tree replacement costs incurred on sites exhibiting severe decline symptoms (Mazzola, 1998).

Apple decline can be associated to abiotic factors like phytotoxins (Benson et al., 1976), nutrient imbalance (Sadowski et al., 1988), poor soil conditions (Mai and Abawi, 1981) and also to biotic factors such as fungi and Oomycetes, acting individually or together.

Many researchers reported that apple decline is mainly associated with biotic factors because it can be controlled by fumigation, sterilization or pasteurization of the soil (Mai and Abawi, 1981; Slykhuis and Li, 1985). These factors include also phytopathogenic actinomycetes (Sewell and Roberts, 1985; Westcott et al., 1987), bacteria (Utkhede et al., 1992; Dullahide et al., 1994), *Fusarium oxysporum* fungi (Sewell, 1981; Mazzola, 1997) and nematodes (*Pratylenchus*) (Hoestra, 1968).

Several fungi were mentioned as the main biological causal agents of apple seedling decline (Dullahide et al., 1994; Mazzola, 1998, 1999). Plant pathogenic fungi can accumulate in apple rhizosphere and roots within 1–2 years after orchard establishment (Mazzola, 1999). The fungal pathogens are not lethal. They live saprophytically in the soil. The increase of their populations is in relation to the continuous return of the same crop, but it can be strongly affected by competition with other microorganisms (van Bruggen et al., 2006). The pathogenic effect of these microorganisms is mediated by the physiological and nutritional state of the crop (Lockwood, 1988). *Fusarium* species are widely distributed and many species inhabit soil ecosystems where they are rhizosphere and/or endophytic plant colonizers (Sutton et al., 1998). *Fusarium* spp. is frequently isolated from infected roots of apple trees in many apple-growing areas throughout the world (Dullahide et al., 1994; Mazzola, 1998). Although *Fusarium* (mainly) is frequently associated with apple decline, its role as a pathogen of apple is controversial. Most studies were unable to demonstrate that *Fusarium* isolates are pathogenic (Dullahide et al., 1994; Mazzola, 1998; Manici et al., 2003; Tewoldemedhin et al., 2011a). However, *F. tricinctum* and some isolates of *F. solani* and *F. avenaceum* have been shown to be pathogenic, with the latter two species having low virulence (Dullahide et al., 1994; Manici et al., 2003; Tewoldemedhin et al., 2011a).

The aims of this investigation were to (i) characterize the *Fusarium* species associated with the decline of apple in Tunisian nurseries, and (ii) determine the pathogenicity of the most predominant species of *Fusarium* in greenhouse experiment.

Materials and methods

Surveys and sample collection

From October to December, at a year interval, root samples of apple seedlings grafted onto the rootstock MM106 were collected from ten nurseries. Three of these nurseries were located in Kairouan area, three in Zaghouan area, one in Monastir area, one in Kasserine area and two in Jendouba and Beja areas (Figure 1). From each variety found, five samples of roots per vigor were collected. The characteristics of vigor of each sample of apple seedlings were noted in table 1.

Table 1. Characteristics of vigor index of apple seedlings

Vigor Index	Rootstock diameter (cm)	Scion height (cm)	Scion diameter (cm)
IV1	1 – 2	0- 25	0.3 – 0.5
IV2	2.5 – 3	26 – 50	1 – 1.5
IV3	3 – 3.5	51 – 75	2 – 2.5
IV4	4 – 5	>75	2.5 – 3

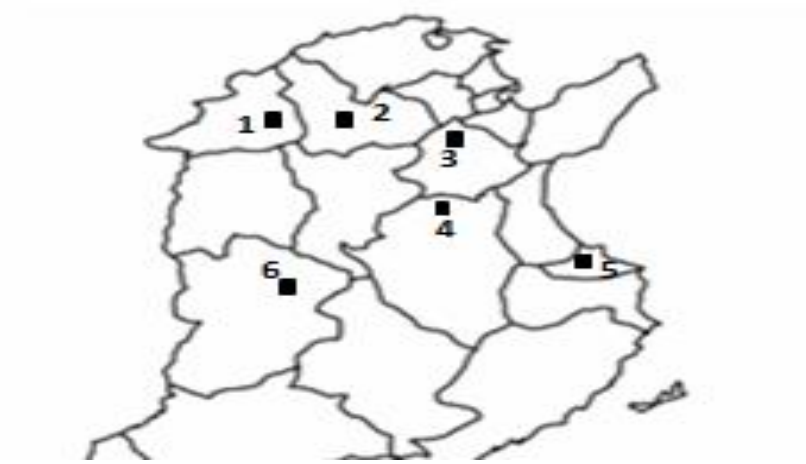

Figure 1. Apple growing areas investigated in Tunisia **(1:** Jendouba; **2:** Beja; **3:** Zaghouan; **4:** Kairouan; **5:** Monastir; **6:** Kasserine; (■ : indicates the apple nurseries location).

Isolation of fungi from apple roots

A total of 270 of roots samples were collected from surveyed nurseries. These roots were washed under tap water to remove adhering soil and cut aseptically into small pieces of 3 to 5 mm in diameter, followed by dipping in sodium hypochlorite solution (3%) for 3 min. Then these pieces were rinsed twice with sterile distilled water and air dried in a laminar flow hood. Small root segments were placed in Petri plates (90mm) contained potato dextrose agar medium (PDA) amended with 100 µg ml^{-1} of streptomycin, and incubated in darkness at 25°C. Developed colonies were transferred to PDA plates and purified by single-sporing using Water Agar medium (2%).

Morphological and molecular characterization of isolates

Obtained isolates were identified after one week of incubation at 25°C, based on morphological criteria as described by Leslie and Summerell (2006).

Then, one representative isolate from *F. solani* and one isolate from *F. oxysporum* selected randomly and 5 isolates of *Fusarium* spp. were used for the molecular characterization.

Extraction of genomic DNA of each isolate was made according to the protocol of Möller et al (1992). The ITS region was amplified with universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Ristaino et al. 1998). PCR was performed in a 50 µl of volume reaction (2 µl of DNA (50 ng/µl), 0.5 µl of Taq polymerase (5U/µl), 3 µl of $MgCl_2$ (1.25mM), 5 µl of PCR buffer (10x), 5 µl of dNTP (1.25mM), 5µl of each of 5 µM forward (ITS1) and reverse (ITS4) primers and 24.5 µl of sterile distilled water. The PCR product was analyzed by electrophoresis in agarose gel (1%), purified and sequenced at Biotools society (Monastir, Tunisia). Obtained sequences where analyzed by BLAST (Basic Local Alignment Search Tool) and compared with sequences of *Fusarium* spp. from the GenBank (Table 2). Then, the sequences were deposited in the GenBank.
The percent of isolation of each *Fusarium* species was evaluated with vigor index groups found in each variety and in each nursery.

Table 2**.** *Fusarium* species selected from GenBank and included in this study

Species	Strain number	Origin	Host
Fusarium equiseti	HM008677	China	*Gerbera jamesonii*
	KU377478	Venezuela	*Theobroma cacao*
	FJ467356	China	-
	FJ441009	China	-
	KT192259	China	*Sophora japonica*
	KT192255	China	*Sophora japonica*
	KT211523	Brazil	*Cassava root*
	AB425996	Japan	wheat
	FJ467369	China	-
	KP067237	Colombia	-
	KY318493	South Africa	-
Fusarium solani	KP784419	Colombia	*Stevia rebaudiana*
Fusarium oxysporum	MG491207	Malaysia	mushroom
Fusarium proliferatum	FJ040179	China	Rice

Pathogenicity test

The species isolated from all nurseries with high percents were used for the pathogenicity tests: 26 isolates of *Fusarium oxysporum* and 21 isolates of *Fusarium solani* were selected from different nurseries randomly regardless of the vigor. They tested on 18-months-old apple seedlings (variety Golden delicious grafted onto MM106 rootstock). These plants were grown in a greenhouse, in plastic pots (23 cm diameter x 23 cm deep) filled up with 50% sterilized soil, 25 % sterilized peat and 25% sand.

The inoculum of each *Fusarium* species was prepared according to Strauss and Labuschagne (1995) methodology, which consists to inoculate sterile wheat seed twice by 10 mycelia discs from 2-wk-old *Fusarium* cultures grown on PDA. Then, inoculated wheat seed were incubated at 25°C for 2 weeks and shaken every two day to ensure thorough seed colonization. Then, the potting mix was inoculated using 1% (v/v) of prepared inoculums. The pathogenicity of each isolate was tested using three repetitions. Experiment was conducted according to a completely randomized design.

After six months of inoculation, apple plants were uprooted and washed to eliminate the adhering potting mix. Plant height and roots fresh weights were recorded. Disease severity was estimated based on the disease severity of plant aerial part (This parameter is rated onto 0-5 scale, where: **0**=no obvious symptoms; **1**=moderate discoloration of plant leaves (≤25%); **2**= moderate discoloration and falling leaves (≤50%); **3**= moderate discoloration of plant collar, stem and leaves (≤75%); **4**= extensive discoloration of plant collar and stem with falling leaves (>75%); and **5**= dead plant). However, the density of root rot was rated onto a 0–5 scale (**0**=no obvious symptoms; **1**=moderate discoloration of root tissue; **2**=moderate discoloration of tissue with some lesion; **3**=extensive discoloration of tissue; **4**= extensive discoloration of tissue with girdling lesions; and **5**= dead plant) (Tewoldemedhin et al., 2011a). Pathogen re-isolations were performed from roots of inoculated plants to confirm Koch postulate.

Statistical analysis

The results were subjected to a one-way analysis of variance (ANOVA) using Statistical Package for the Social Sciences software (SPSS), version 20.0. Means separations of the values were carried out using the Student–Newman–Keuls (S-N-K) test to identify significant differences at $P \leq 0.05$.

Results

Diagnosis and analysis of surveyed nurseries

The survey conducted during this study showed the presence of apple seedlings decline. These symptoms include drying and browning of the apical part of the scion and/or browning of the collar of rootstocks, and rot and browning of the roots (Figure 2). In all nurseries, apple seedlings were grafted onto the rootstock MM106 except in Jendouba nursery were grafted onto the rootstock MM106 with apercent of 50% and onto the rootstock MM111 (50%). The rotation of crops for 4 years was used in all nurseries (Table 3).

Figure 2. Symptoms of browning of the apical part of the stem (a) and root rot (b and c) of seedlings of apple variety 'Golden delicious' grafted onto the rootstock ‘MM106’ cultivated in Tunisian nurseries."

Isolation, morphological and molecular characterization of *Fusarium* species

The results of the morphological and molecular identification revealed the presence of 111 isolates of *Fusarium oxysporum* (Figure 3), 55 isolates of *Fusarium solani* (Figure 4), 4 isolates of *Fusarium equiseti* and one isolate of *F. proliferatum.* The origin of all isolates of *F. equiseti, F. proliferatum* and *only the isolates of F. oxysporum* and *F. solani* used in the pathogenicity tests were placed in the table 4. Isolation percent of each species is shown on Table 5. The predominant species were *F. solani* (32.16%) and *F. oxysporum* (64.91 %). *F. equiseti* was isolated from roots from nurseries in Zaghouan1 (1.17 %) and Sbiba (1.17 %). *F. proliferatum* was recovered from roots from Zaghouan 1 (0.58 %) (Table 5). The lowest isolation percent was found in Oued mliz stoolbed nursery, with only 10 % of *F. oxysporum* isolates found in the MM106 rootstock. The isolation from MM111 rootstock roots from Oued-mliz stoolbed didn’t give any isolate of *Fusarium* spp.

Table 3. Characteristics of samples collected from surveyed nurseries.

Nurseries	Location	Rootstocks	Varieties	Vigor index	Samples number	Age (Months)	Years	Previous crop
Manzelnour	Monastir	MM106	Anna	IV3	5	18	2013	Fallow
			Lorka	IV3	5			
Zaghouan1	Zaghouan	MM106	Anna	IV2, IV3, IV4	15	9	2013	Fallow
Zaghouan2	Zaghouan	MM106	-		5	9	2013	Fallow
			Starkrimson	IV1, IV3	10	18		
			Anna	IV2, IV3, IV4	15	18		
Zaghouan3	Zaghouan	MM106	Starkrimson	IV1, IV2, IV4	15	10	2013	Fallow
			Royal Gala	IV2, IV4	10	10		
			Chahla	IV3, IV4	10	10		
			Anna	IV2, IV3, IV4	15	10		
Chebika2	Kairouan	MM106	Anna	IV1, IV2, IV4	15	18	2012	Fallow
Chebika1	Kairouan	MM106	Anna	IV1, IV2, IV3, IV4	20	10	2012	Fallow
Chebika3	Kairouan	MM106	Royal Gala	IV1	5	10	2013	Fallow
			Starkrimson	IV1	5	10		
			Richared	IV1, IV2, IV3	15	10		
			Redcheif	IV1	5	10		
			Block feild	IV1	5	10		
			stoolbed	IV3	5	12		
Sbiba	Kasserine	MM106	Royal Gala	IV2, IV4	10	9	2013	Fallow
			Richared	IV2, IV4	10	9		
			Golden delicious	IV1, IV3	10	9		
			Starkrimson	IV2, IV4	10	9		
Oued mliz	Jendouba	MM106	-		20	24	2013	Fallow
		MM111	-		20	24		
Beja	Beja	MM106	-		10	18	2012	Fallow

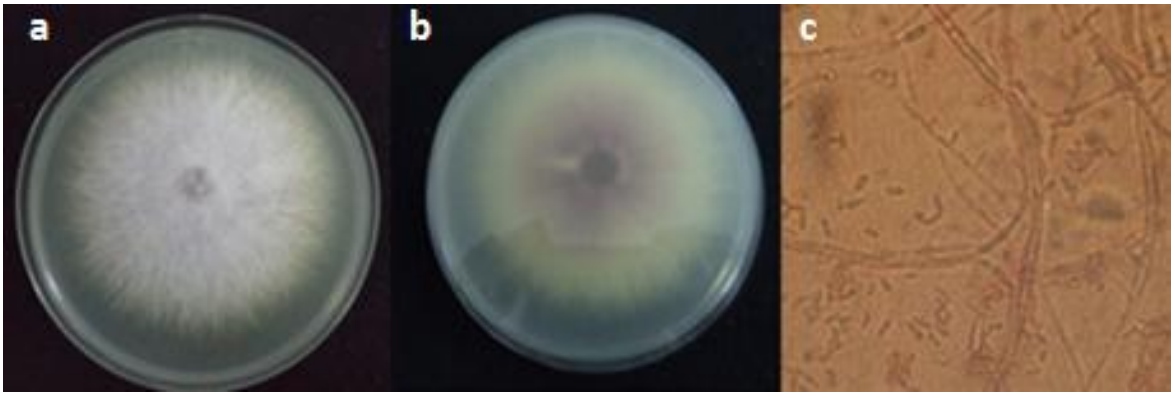

Figure 3. Morphological characteristics of *Fusarium oxysporum:* mycelium colony on PDA medium (**a**, **b**), microconidia and short phyalides (**c**)

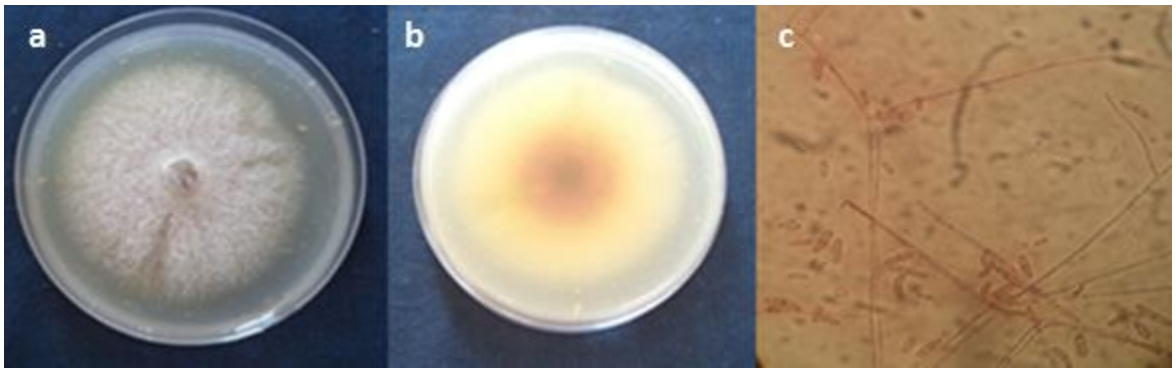

Figure 4. Morphological characteristics of *Fusarium solani:* mycelium colony on PDA medium (**a**, **b**), long phyalides and microconidia (**c**).

Results of the morphological identification were confirmed by the molecular analysis of rDNA ITS sequences of the representative isolates of *F. oxysporum* and *F. solani*. The rDNA ITS sequences were 497 base pairs for *F. oxysporum* and 532 base pairs for *F. solani* (Figure 5).
A BLAST search of the rDNA ITS sequence of *Fusarium oxysporum* gave 100% of similarity with ITS sequences of the isolate of *F. oxysporum* from GenBank MG491207 (Lambuk et al., 2017). A BLAST search of the rDNA ITS sequence of *Fusarium solani* gave 99% of similarity with ITS sequences of the isolate of *F. solani* from GenBank KP784419 (Alvarez and Latorre 2015). The molecular analysis of rDNA ITS sequences of the other five isolates of *Fusarium* spp., gave two *Fusarium* species: four isolates of *Fusarium equiseti*, and one isolate of *F. proliferatum* . The average of rDNA ITS sequences of *F. equiseti* was 505 base pairs (Figure 5).

Table 4. Origin of *Fusarium oxysporum* and *F. solani* isolates used in the pathogenicity tests on golden delicious variety apple trees grafted on MM106 and the *F. equiseti* and *F. proliferatum* isolates

Isolates	Species	Nurseries	Location	Year	GenBank accession number
FC13	*F. oxysporum*	Chebika2	Kairouan	2012	
FC3	*F. oxysporum*	Chebika2	Kairouan	2012	
FC11	*F. oxysporum*	Chebika2	Kairouan	2012	
FC12	*F. oxysporum*	Chebika2	Kairouan	2012	
FC13-1	*F. oxysporum*	Chebika2	Kairouan	2012	
FC9	*F. oxysporum*	Chebika2	Kairouan	2012	
F95	*F. oxysporum*	Zaghouan2	Zaghouan	2013	
111	*F. oxysporum*	Sbiba	Kasserine	2013	
PO2	*F. oxysporum*	Chebika1	Kairouan	2012	
FWM1	*F. oxysporum*	Oued Mliz	Jendouba	2013	
FWM2	*F. oxysporum*	Oued Mliz	Jendouba	2013	
F164	*F. oxysporum*	Manzel nour	Monastir	2013	
F26	*F. oxysporum*	Chebika1	Kairouan	2012	MF993098
FO3	*F. oxysporum*	Chebika1	Kairouan	2012	
F105	*F. oxysporum*	Zaghouan2	Zaghouan	2013	
F35	*F. oxysporum*	Chebika1	Kairouan	2012	
F37	*F. oxysporum*	Chebika1	Kairouan	2012	
F224	*F. oxysporum*	Chebika3	Kairouan	2013	
F2	*F. oxysporum*	Chebika1	Kairouan	2012	
FMJ1	*F. oxysporum*	Beja	Beja	2012	
F40	*F. oxysporum*	Chebika1	Kairouan	2012	
F27	*F. oxysporum*	Chebika1	Kairouan	2012	
FO2	*F. oxysporum*	Chebika1	Kairouan	2012	
FO1	*F. oxysporum*	Chebika1	Kairouan	2012	
F212-1	*F. oxysporum*	Chebika3	Kairouan	2013	
F168	*F. oxysporum*	Manzel nour	Monastir	2013	
F178	*F. solani*	Sbiba	Kasserine	2013	
F180	*F. solani*	Sbiba	Kasserine	2013	
F28	*F. solani*	Chebika1	Kairouan	2012	
F169	*F. solani*	Manzel nour	Monastir	2013	
F48	*F. solani*	Chebika1	Kairouan	2012	
F212	*F. solani*	Chebika3	Kairouan	2013	
F104	*F. solani*	Zaghouan2	Zaghouan	2013	
F55	*F. solani*	Chebika3	Kairouan	2012	
F186	*F. solani*	Chebika3	Kairouan	2013	
S9	*F. solani*	Zaghouan1	Zaghouan	2013	
184	*F. solani*	Chebika3	Kairouan	2013	
F29	*F. solani*	Chebika1	Kairouan	2012	
F147	*F. solani*	Zaghouan1	Zaghouan	2013	

Continue

Isolates	Species	Nurseries	Location	Year	GenBank accession number
F30	*F. solani*	Chebika1	Kairouan	2012	
F8	*F. solani*	Chebika3	Kairouan	2012	
F73	*F. solani*	Zaghouan3	Zaghouan	2013	
F181	*F. solani*	Sbiba	Kasserine	2013	MF993095
28	*F. solani*	Zaghouan2	Zaghouan	2013	
F153	*F. solani*	Zaghouan2	Zaghouan	2013	
F22	*F. solani*	Chebika2	Kairouan	2012	
F182	*F. solani*	Sbiba	Kasserine	2013	
FE1	*F. equiseti*	Zaghouan1	Zaghouan	2013	MF993080
FE2	*F. equiseti*	Sbiba	Kasserine	2013	MF993082
FE3	*F. equiseti*	Sbiba	Kasserine	2013	MF993083
FE4	*F. equiseti*	Zaghouan	Zaghouan	2013	MF993084
FP	*F. profileratum*	Zaghouan1	Zaghouan	2013	MF993107

Table 5. Percent of *Fusarium* species isolation from collected rootstocks samples.

Nurseries	Rootstocks	Samples number	Isolation percent (%)			
			F. oxysporum	***F. solani***	***F. equiseti***	***F. proliferatum***
Manzelnour	MM106	10	50.00	40.00	0.00	0.00
Zaghouan1	MM106	15	21.42	14.28	1.33	0.67
Zaghouan2	MM106	30	18.51	14.81	0.00	0.00
Zaghouan3	MM106	50	37.77	35.55	0.00	0.00
Chebika2	MM106	15	100.00	29.41	0.00	0.00
Chebika1	MM106	20	100.00	8.69	0.00	0.00
Chebika3	MM106	40	17.50	15.00	0.00	0.00
Sbiba	MM106	40	44.73	13.15	1.33	0.00
Oued mliz	MM106	20	10.00	0.00	0.00	0.00
	MM111	20	0.00	0.00	0.00	0.00
Beja	MM106	10	27.27	0.00	0.00	0.00

The ITS sequence of *Fusarium equiseti* shared 99 to 100% similarity with those of the same species available in GenBank. In fact, the BLAST alignment with ITS sequences of isolates from GenBank revealed 99% of similarity for the isolate FE1 (MF993080) with many *F. equiseti* isolates like HM008677, FJ441009 and FJ467356 and some *F. incarnatum* like MG543801 and MG543800. For FE2 (MF993082), 99% of similarity was shown with *F. equiseti* (KU377478 KT211523, KT192259, KT192255, AB425996) and *F. incarnatum* isolates (MG461658,

KX184815). The sequence of FE3 (MF993083) gave a similarity of 99% with *F. equiseti* isolates (FJ467369, FJ467356) and *F. incarnatum* KU204760. The isolate FE4 (MF993084) showed 98% of similarity with the *F. equiseti* isolates KY318493 and KP067237 and *F. verticillioides* isolates KX553874, KU204755.

A BLAST search of the rDNA ITS sequence of *F. proliferatum* isolate MF993107 gave 99% of similarity with ITS sequences of the GenBank *F. proliferatum* isolates (KU939073, KU939072, MG562501) and 99% with *Fusarium fujikuroi* isolates (KP998524 , KJ000430).

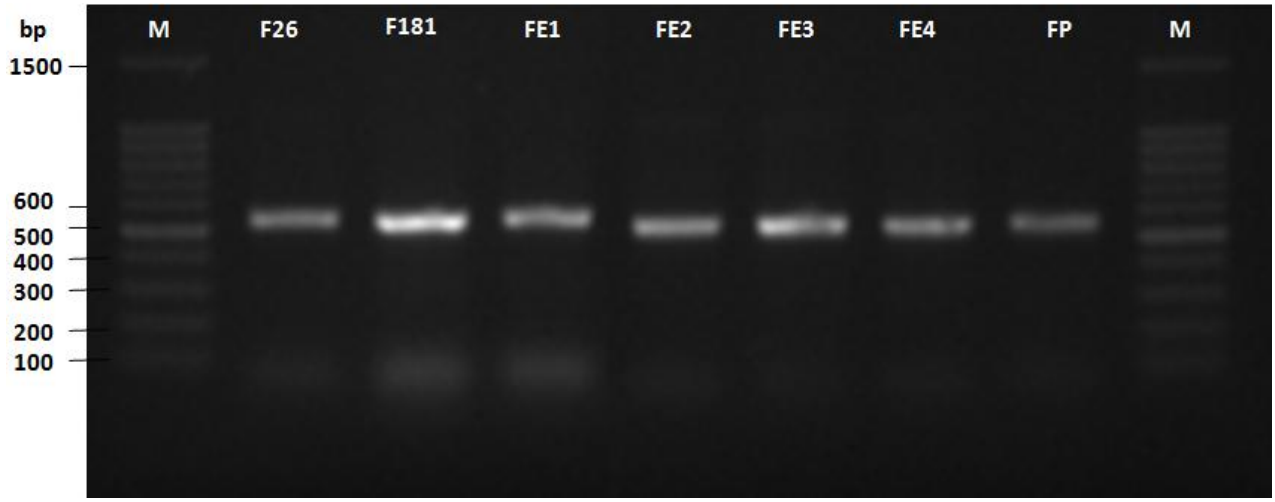

Figure 5. Agarose gel Electrophoresis of PCR products with primers ITS1 and ITS4 of *Fusarium oxysporum* (F26), *F. solani (*F181), *F. equiseti* isolates (FE1, FE2, FE3 and FE4) and *F. proliferatum* (FP), M: 100 pb DNA molecular marker.

Relationship between vigor index and percent of *Fusaruim* spp. isolation

Four apple seedling vigor indexes were found in surveyed nurseries. Statistical analysis did not show any significant difference between the isolation percent of each *Fusarium* species and the different apple plants vigor as demonstrated in table 6.

Table 6. Percent of *Fusarium* species isolation in each vigor index group in each apple variety.

Nurseries	**Rootstocks**	**Varieties**	***Fusarium* spp.**	**Vigor index**			
				IV1	**IV2**	**IV3**	**IV4**
Manzelnour	**MM106**	Anna	*F. oxysporum*	-	-	50.40±2.07	-
			F. solani	-	-	39.00±1.00	-
		Lorka	*F. oxysporum*	-	-	52.00±2.00	-
			F. solani	-	-	41.00±1.00	-
Zaghouan1	**MM106**	Anna	*F. oxysporum*	-	22.00±1.00	21.50±1.5	21.30±2.54
			F. solani	-	14.70±1.30	14.50±1.41	14.70±0.97
			F. equiseti	-	1.33±1.82	-	-
			F. proliferatum	-	-	0.67±1.49	-
Zaghouan2	**MM106**	Anna	*F. oxysporum*	-	18.60±1.95	19.00±1.82	18.25±1.5
			F. solani	-	14.25±0.96	15.00±2.16	14.00±1.63
		Starcrimson	*F. oxysporum*	17.25±1.71	-	-	18.00±1.63
			F. solani	15.20±4.76	-	-	14.80±4.32
Zaghouan3	**MM106**	Anna	*F. oxysporum*	-	37.20±4.97	36.90±5.00	37.60±4.83
			F. solani	-	34.80±3.35	35.40±1.14	34.40±1.67
		Royal gala	*F. oxysporum*	-	37.80±1.92	-	38.20±0.84
			F. solani	-	36.60±2.70	-	35.50±0.58
		Starkrimson	*F. oxysporum*	36.30±2.54	37.00±4.69	-	36.60±4.72
			F. solani	34.00±3.56	35.75±1.48	-	33.75±4.02
		Chahla	*F. oxysporum*	-	-	38.00±3.00	39.50±2.89
			F. solani	-	-	34.10±3.29	34.60±4.61

Continue

Nurseries	**Rootstocks**	**Varieties**	***Fusarium* spp.**	**Vigor index**			
				IV1	**IV2**	**IV3**	**IV4**
Sbiba	**MM106**	Royal gala	*F. oxysporum*	-	43.00±3.08	-	42.70±4.18
			F. solani	-	13.60±4.16	-	14.20±3.27
		Richared	*F. oxysporum*	-	44.80±4.15	-	46.00±5.83
			F. solani	-	13.60±2.51	-	13.00±3.08
		Golden delicious	*F. oxysporum*	44.60±4.22	-	44.80±3.45	-
			F. solani	12.60±2.7	-	13.20±2.86	-
		Starcrimson	*F. oxysporum*	-	47.00±2.55	-	45.00±4.06
			F. solani	-	12.00±2.55	-	13.00±2.45
			F. equiseti	-	1.33±1.82	-	-
Chebika2		Anna	*F. oxysporum*	100.00±00.00	100.00±00.00	-	100.00±00.00
			F. solani	29.60±3.85	29.60±2.61	-	29.00±3.53
Chebika1		Anna	*F. oxysporum*	100.00±00.00	100.00±00.00	100.00±00.00	100.00±00.00
			F. solani	8.80±1.64	8.60±6.11	9.00±4.41	8.40±4.77
Chebika3		Richared	*F. oxysporum*	17.40±5.59	17.60±2.51	17.80±2.28	-
			F. solani	15.00±5.00	14.00±5.66	16.00±4.69	-
		Redcheif	*F. oxysporum*	17.60±3.71	-	-	-
			F. solani	15.40±11.22			
		stoolbed	*F. oxysporum*	-	-	18.60±8.50	-
			F. solani	-	-	15.40±9.40	-
		Royal Gala	*F. oxysporum*	17.60±5.32	-	-	-
			F. solani	15.00±6.12	-	-	-
		Starkrimson	*F. oxysporum*	16.40±3.65	-	-	-
			F. solani	14.00±6.52	-	-	-
		Block feild	*F. oxysporum*	17.20±4.15	-	-	-
			F. solani	15.20±5.02	-	-	-

(-) No plants in this group.

Pathogenicity tests

After six months of inoculation of apple seedlings with the 26 isolates of *Fusarium oxysporum*, results showed that the disease severity ranged between 2 and 4. A significant difference ($P \leq 0.05$) between the disease severity of the aerial part of control plants (not inoculated) and that of the seedlings inoculated by the majority of tested isolates. The highest virulent isolates were FMJ1, F164 and F26. The lowest virulent isolate was F212-1.

The root rot and browning index ranged from 0.80 to 4.2. The isolates F164 and F26 revealed to be more pathogenic than other tested isolates with root rot indexes 4.2 and 4, respectively. The lowest isolate was 111. The other isolates showed weakly virulents with low root browning indexes (<2.4) (Figure 6).The variance analysis of the root rot index showed a significant difference ($P \leq 0.05$).

All *F. oxysporum* isolates reduced the seedlings height while this reduction was very low and varied between 0.84% in case of the isolate F40 and 24.85% in case of the isolate F164. Statistical analysis did not give any significant difference.

The roots fresh weight was between 6.04 for the isolate FO3 and 32.51 for the isolate FO2. Only some isolates were reduced this parameter. This effect was not significant for all isolates except the isolate FO3 for which the percent of decrease reached 77.09% compared to the control one (Table 7).

Thus, these results showed the virulence only of some isolates of *F. oxysporum* like F164 and F26 on apple seedlings.

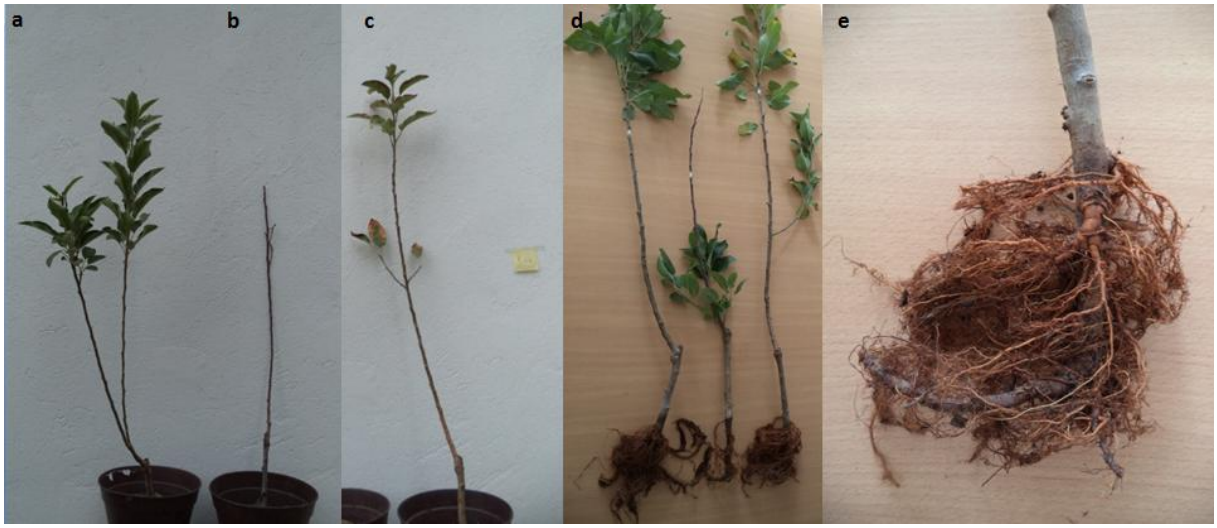

Figure 6. *Fusarium oxysporum* symptoms caused by isolates F164 (**b**, **c**), F26 (**d** and **e**) on golden variety apple seedlings grafted on MM106, recorded six months after inoculation, compared to the control (**a**).

Table 7. Disease severity, plants heights, and root fresh weights of apple seedlings after 6 months of inoculation by *Fusarium oxysporum.*

Codes	Roots fresh weights (g)	Heights of seedlings(cm)	Root rots	Disease severity
Control	26.37±4.72 $^{b-f}$	121.10±24.55^{a}	1.00±0.71^{a}	1.60±0.55^{a}
FC13	16.398±7.95^{a-f}	113.60±11.78^{a}	1.60±0.55^{ab}	3.20±0.45^{bcd}
F168	21.426±6.49^{a-f}	102.50±18.16^{a}	1.60±0.55^{ab}	2.80±0.45^{bcd}
PC3	19.59±2.63^{a-f}	107.32±8.97^{a}	2.00±0.00^{ab}	3.32±0.46^{cd}
FO1	13.78±10.39^{a-d}	99.50±11.80^{a}	1.40±0.55^{ab}	3.60±0.55^{cd}
F212-1	19.71±3.20^{a-f}	106.16±9.76^{a}	1.40±0.55^{ab}	2.00±0.00^{ab}
FO2	32.51±5.17^{f}	118.64±14.04^{a}	1.40±0.55^{ab}	3.00±0.00^{bcd}
F27	11.62±8.31^{abc}	107.00±3.37^{a}	1.40±0.55^{ab}	2.60±0.55^{bcd}
FC13-1	27.85±5.19^{c-f}	103.87±14.57^{a}	2.00±0.00^{ab}	3.00±1.00^{bcd}
F40	31.57±5.78^{ef}	120.08±2.84^{a}	2.00±0.00^{ab}	2.75±0.43^{bcd}
FMJ1	21.17±11.16^{a-f}	104.10±14.99^{a}	2.00±0.00^{ab}	4.00±0.00^{d}
F2	29.85±10.80^{d-f}	116.87±14.97^{a}	1.75±0.43^{ab}	3.00±0.00^{bcd}
F224	26.38±11.66^{b-f}	106.90±28.71^{a}	1.40±0.55^{ab}	3.60±0.55^{cd}
F37	19.24±4.39^{a-f}	101.87±15.35^{a}	1.50±0.50^{ab}	3.00±0.00^{bcd}
F35	14.95±9.77^{a-e}	92.70±0.27^{a}	2.60±0.55^{b}	3.60±0.55^{cd}
FC11	13.91±4.90^{a-d}	117.10±11.28^{a}	1.80±0.45^{ab}	2.80±0.45^{bcd}
F105	30.82±5.42^{ef}	110.60±5.21^{a}	2.00±0.00^{ab}	3.00±0.00^{bcd}
F95	28.51±5.61^{c-f}	107.40±18.85^{a}	2.60±0.89^{b}	3.40±0.55^{cd}
111	18.47±6.96^{a-f}	103.50±11.08^{a}	0.80±0.55^{b}	3.00±0.55^{abc}
Po2	24.37±7.02^{a-f}	107.61±7.01^{a}	1.80±0.55^{b}	3.09±0.45^{bcd}
FC9	15.28±4.83^{a-e}	107.74±9.50^{a}	2.80±0.84^{b}	2.60±0.55^{abc}
FWM1	11.63±1.80^{abc}	109.60±4.51^{a}	2.60±0.55^{b}	2.80±0.45^{bcd}
FC12	12.46±6.45^{abc}	108.20±8.20^{a}	2.80±0.45^{b}	2.60±0.55^{abc}
FWM2	15.19±2.26^{a-c}	101.00±5.29^{a}	1.80±0.84^{ab}	2.60±0.55^{abc}
F164	10.68±9.24^{ab}	91.00±17.93^{a}	4.20±1.10^{c}	4.00±1.00^{d}
F26	11.71±14.55^{abc}	97.80±20.63^{a}	4.00±1.00^{c}	4.00±1.00^{d}
FO3	6.04±4.88^{a}	96.40±9.80^{a}	2.40±1.52^{ab}	3.60±0.89^{cd}

(*) means ± standard error in the column followed by the same letter are not significantly different according to SNK test at P≤0.05.

Evaluation of the virulence of 21 isolates of *Fusarium solani*, six months after the inoculation of Golden variety apple trees grafted on MM106, showed that the disease severity of the aerial part index ranged between 2.2 and 4. A significant difference was registered (P≤0.05) between the disease severity of aerial parts of control seedlings and that of seedlings inoculated with some isolates tested. F212, S9, F181 and 28 appeared to be the most virulent with a disease severity index ranging between 3.75 and 4. The lowest virulent isolates were F178 and F180.

The root rot and browning index ranged from 1 to 3.80. The isolates F181, 28, F153 and F22 seem the most aggressive with a root rot index ranging between 3 and 4 (Figure 7). The lowest isolate was F104. The variance analysis of the severity of rot and browning of roots in apple seedlings revealed a significant difference.

The height of seedlings was between 85.9 cm for F29 and 125.5 for F30. Statistical analysis revealed no significant difference between seedlings height inoculated with different *F. solani* isolates and that of the control one.

The fresh weight of roots varied from 8.46 g for seedlings inoculated by F153 to 32.18 g for seedlings inoculated by F155. All isolates showed statistically comparable to the control (Table 8). Thus, these results showed the virulence of some isolates of *F. solani* like F181, 28 and F153 on apple seedlings.

Figure 7. Comparison between apple root seedling (MM106) inoculated with the isolates '28' of *Fusarium solani* (**a**) and that of the control (**b**).

Table 8. Disease severity, plants heights, and root fresh weights of apple seedlings after 6 months of inoculation by *Fusarium solani.*

Codes	Roots fresh weights (g)	Heights of seedlings (cm)	Root rots	Disease severitys
Control	26.37±4.72^{a-d}	121.10±24.55^{ab}	1.00±0.71^{a}	1.60±0.55^{a}
F178	10.90±5.56^{ab}	110.70±12.28^{ab}	1.40±0.55^{a}	2.20±0.45^{ab}
F180	13.33±6.27^{abc}	94.87±20.12^{ab}	2.00±0.00^{abc}	2.20±0.45^{ab}
F28	19.31±4.27^{a-d}	109.32±10.61^{ab}	1.60±0.55^{ab}	3.00±0.71^{bc}
F169	17.30±2.30^{a-d}	87.75±19.11^{a}	1.25±0.43^{a}	2.75±0.43^{abc}
F48	21.81±2.32^{a-d}	102.00±7.90^{ab}	1.25±0.43^{a}	3.50±0.50^{bc}
F212	21.82±6.73^{a-d}	112.25±5.84^{ab}	2.00±0.00^{abc}	3.75±0.43^{c}
F104	16.96±10.17^{a-d}	91.30±25.90^{ab}	1.00±0.00^{a}	2.60±0.55^{abc}
F55	32.18±5.63^{d}	109.00±7.36^{ab}	2.20±0.45^{abc}	3.20±0.45^{bc}
F186	28.29±6.53^{bcd}	121.00±10.43^{ab}	1.20±0.84^{a}	3.20±0.45^{bc}
S9	20.35±11.59^{a-d}	108.92±25.97^{ab}	2.00±0.71^{abc}	3.80±0.45^{c}
184	16.15±2.99^{a-d}	118.00±8.03^{ab}	2.00±0.00^{abc}	3.40±0.55^{bc}
F29	18.25±5.65^{a-d}	125.50±2.37^{b}	1.80±0.45^{abc}	3.00±0.00^{bc}
F147	18.54±8.50 $^{a-d}$	105.80±13.12^{ab}	2.00±0.00^{abc}	3.00±0.00^{bc}
F30	8.46±2.31^{a}	85.90±2.56^{a}	1.60±0.55^{ab}	3.00±0.00^{bc}
F8	10.70±3.45^{ab}	116.30±4.84^{ab}	1.40±0.55^{a}	3.00±0.00^{bc}
F73	14.75±3.10^{a-d}	106.75±11.18^{ab}	1.80±0.45^{abc}	2.80±0.45^{abc}
F181	10.85±11.89^{ab}	100.20±21.35^{ab}	4.00±1.00^{d}	4.00±1.00^{c}
28	13.54±10.17^{abc}	107.20±13.74^{ab}	3.80±0.84^{d}	3.80±0.84^{c}
F153	8.61±9.60^{a}	107.70±17.43^{ab}	3.00±1.87^{bcd}	3.20±1.64^{bc}
F22	28.74±17.49^{bcd}	93.60±7.08^{ab}	3.20±1.10^{cd}	3.40±0.89^{bc}
F182	30.78±12.42^{cd}	111.40±21.13^{ab}	2.20±0.45^{abc}	2.60±0.55^{abc}

(*) means ± standard error in the column followed by the same letter are not significantly different according to SNK test at P≤0.05.

Discussion

The present investigation showed that *Fusarium oxysporum*, *F. solani*, *F. equiseti* and *F. proliferatum* are associated with apple decline seedlings in Tunisian nurseries. *Fusarium oxysporum* and *F. solani* were identified in all nurseries with high isolation percents. However, the two species *F. equiseti* and *F. proliferatum* were present just in two nurseries. These species were also isolated from apple orchards in different countries (Tewoldemedhin et al., 2011a; Mazzola, 1998; Manici et al., 2003).

The BLAST alignment of rDNA ITS sequences of *F. equiseti* and *F. proliferatum* isolates gave the same similarity percent with more than one *Fusarium* species from GenBank. This requires the use of other loci like the largest subunit of RNA polymerase (RPB1), the second largest subunit of RNA polymerase (RPB2) and the translation elongation factor 1 (EF-1), to identify these isolates (O'Donnell et al., 2010, 2013).

The test of 26 isolates of *F. oxysporum* showed that all isolates gave a significant disease severity index in the aerial seedling part. However, they didn't caused a significant root rot except the two isolates F164 and F26 that showed more pathogenic than others. Previous studies also showed that *Fusarium oxysporum* isolates, obtained from apple orchards, were nonpathogenic (Tewoldemedhin et al., 2011a; Mazzola, 1998; Manici et al., 2003). Thus, its phytotoxicity may be due either to an environmentally induced change towards a high production of fusaric acid, through enhancing phytotoxicity through additive effect with other occurring metabolites (Bacon et al., 1996; D'Mello et al., 1999), or to modifications in host susceptibility induced by biotic or abiotic stress (Fisher and Petrini, 1992).

The pathogenicity test of 21 isolates of *Fusarium solani* revealed that the isolates F181, 28, F153 and F22 are the most aggressive with a root rot index ranging between 3 and 4. In addition, the isolates F212, S9, F181 and 28 showed virulent with a disease severity index ranging between 3.75 and 4. These results are in agreement with previous studies defined that *F. solani* isolates were either not pathogenic or weakly virulent towards apple seedlings (Tewoldemedhin et al., 2011a; Mazzola, 1998; Manici et al., 2003). In addition, Manici et al. (2013) demonstrated that *Fusarium* spp. did not show any correlation with shoot length, suggesting an insignificant role in apple replant disease.

Concerning the seedlings growth, results of this study revealed that there is no significant reduction of height or root weight induced by the majority of *F. solani* and *F. oxysporum* isolates. In the same work, Mazzola (1998) revealed that the majority of *Fusarium* isolates evaluated in pathogenicity assays had only a marginal impact or no effect on growth of apple transplants and *Fusarium* spp. accounted for more than 30% of fungal isolates recovered from apparently healthy apple trees roots, but only 6% of those were exhibiting symptoms of apple decline (apple replant disease).Our results showed a low pathogenicity of *F. oxysporum* and *F. solani* on apple plants after six months of inoculation, confirming the secondary role of *Fusarium* spp. in apple decline (replant diseases) (Manici, 3003; May et al., 1994; Traquair, 1984).

For the pathogenicity test of *Fusarium equiseti* isolates, a previous study done in South-Africa showed that this specie was not pathogenic to apple seedlings (Tewoldemedhin et al., 2011a). Also, there is no study about the pathogenicity of *F. proliferatum* on apple seedlings. Most evidence inferred from previous studies has defined a complex of pathogens/parasites as causal agents of apple seedling decline (Mazzola and Manici, 2012). The primary agent was determinate as a complex of multiple fungal species of the genera *Cylindrocarpon*, *Rhizoctonia*, *Phytophthora* and *Pythium* (Tewoldemedhin et al., 2011a, b; Kelderer et al., 2012). In addition, disease severity due to the fungal complex often increases with the association of the nematode *Pratylenchus penetrans* (Mazzola, 1998).

For this reason, the identification and pathogenicity tests of the *Pythium* and *Phytophthora, Cylindrocarpon* and *Rhizoctonia* species associated to apple seedlings in Tunisian nurseries will be done in further study.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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