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Molecular identification of *Fusarium* spp. isolated from tomato plant in Iraq and China

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Abstract: This study was conducted to identify *Fusarium* spp. isolated from tomato plant in Iraq and China. A total of 12 isolates from Iraq (1-12) and four isolates from China (M1-M4) were used in this study. Based on Morphological characteristics (color, growth pattern, Macro and Micro-conidia shape) high differences between *Fusarium* isolates were found. Sensitivity test to the fungicide carbendazim revealed that one third of isolates(4/12) from Iraq had EC50 values over than 1000µg/ml indicated that many Iraqi isolates have developed resistance to carbendazim. Based on ITS sequences, *Fusarium* isolates were identified as follow, isolates 1, 3, 5, 6, 7, 10 were identified as *Fusarium oxysporum*, isolates 8, 11 as *F. solani*, isolates 12, M1 and M3 as *F. moniliforme,* isolates 2, M2 and M4 as *F. proliferatum*, *F. chlamydosporum* and *F. kyushuense*, respectively.

Key words: RAPD-PCR, *Fusarium* spp, tomato plant, Iraq, China.

Introduction

Tomato (*Lycopersicon esculentum* Mill) is one of the most important and widely cultivated vegetable crops in Basrah, Iraq (Matlobe *et al*., 1989).

Fusarium is one of the most important genera of plant pathogenic fungi with a record of devastating infections in various economically important plants such as tomato, eegplant, cucumber etc. (Messian *et al.,* 1991; Karkachi *et al*., 2010). *Fusarium oxysporum f.* sp. *lycopersici* the causal agent of tomato wilt was the common fungus associated with tomato roots, however several other species were isolated from wilted tomato plants (Al-halo, 1995).

The genus *Fusarium* has been identified to several species levels according to morphological and pathological characteristics (Di Pietro *et al*., 2003), unfortunately, identification of some *Fusarium* isolates are difficult base on morphological and pathological characteristics (AttitAllA, 2004).

Recently, molecular techniques have been widely introduced in the identification of pathogenic agents in plant pathology. These techniques include RAPD analysis, rRNA, ITS sequencing etc. (El-Fadly & El-Kazzaz 2008; Hirano & Arie, 2006). In present study, besides traditional morphological and pathological characterization, RAPD analysis and rRNA ITS sequencing were carried out

for identification of *Fusarium* isolates from Iraq and China.

Materials and Methods

The experiment of this study were conducted at College of Agriculture, University of Basrah, Iraq and the Department of plant pathology, College of plant Science and Technology, Huazhoung Agricultural University, Wuhan, China for identification of *Fusarium* spp. isolated from tomato plants.

Fusarium isolates

Twelve isolates numbered from 1 to 12 from Iraq and 4 isolates from China were used in this study.

Morphological characterization

All isolates were inoculated on Potato Dextrose Agar (PDA) medium and incubated in the dark for 4 days. The colony morphology, melanin formation, sporulatiom etc. were investigated. Identification of *Fusarium* spp. based on morphology were done according to Booth (1970).

Effect of Different pH levels

Isolates 1, 2, 3, 4, 9, 11 was inoculated on Potato agar medium whose pH adjusted to 3.0, 4.0, 4.5, 6.0, 6.5, 7.0, and 8, respectively. All these experiments were conducted in three replicates. Center of each plates were inoculated by 6 mm of mycelial discs obtained by cork borer from the periphery of a four-days-old colony of each isolate. Plates were incubated at a 25°C. observation on linear growth were recorded after seven days of inoculation.

Sensitivity of _Fusarium_ isolates to the carbendazim

The sensitivity of isolates to the fungicide carbendazim was examined by measuring radial growth of colony growth on fungicide amended PDA medium. PDA was amended with carbendazim at 0, 1, 2, 3, 4, 5µg/ml. The experiment was done by three replicates of each fungicide concentration and each isolate. For inoculations, mycelial discs (6 mm in diameter) of each isolate were placed upside down onto un amended and fungicide–amended PDA. Dishes were incubated at 25°C each colony in two perpendicular directions in three replicates. A linear regression analysis was performed to calculate the EC50 value.

Extraction of genomic DNA

Extraction of DNA was done according to a plant genomic DNA kit. Each fungal isolate was grown in 40 ml of potato dextrose broth for 3 days at 25°C on a 120 rpm orbital shaker. Mycelium was removed from the liquid culture by vacuum pump and residual water was absorbed using filter paper. After frozen in liquid nitrogen, mycelium mats ground into fine powder using mortar and pestle. Genomic DNA was isolated using a plant genomic kit. The quality of DNA was estimated by electrophoresis on 1.0% agarose gels (0.35 mg in 35 ml0.5xTBE).

Polymorphic analysis of PCR with a single primer

Three RAPD primers OPA- 02,OPB-20, OPF-05, and two satellite primers ,M13,CNS1 (table 1) were used to amplify polymorphic bands from used isolates. The total reaction volume was 25 µL including 1.5 mM MgCl2,100 µM each dNTP, 200 nM oligonucleotide primers, 1 U Taq DNA polymerase, 2 ng template DNA, 2.5 µL PCR buffer (10mM Tris-HCl, pH: 8.3, 50 mM KCl). Primers were from Operon Technologies Inc. Primers were selected for a preliminary screen of several kits. Amplifications were performed in a DNA themocycler. For PCR amplifications, the thermocycler was programmed at 94 C for 3

min for initial denaturation, followed by 35 cycles of 1 min at 94°C, 1 min at 36°C and 3min at 72°C ,and final extension step was performed at 72°C for 5 min .at the end of amplification separation of PCR products were performed on 1.0% agarose gels in 0.5 TBE buffer at 100 V for 1 h and visualized under ultraviolet light (Vakalounnakis & Fragkiadakis, 1998; Balogun, 2007; Cai *et al.,* 2003).

The absence or presence of –polymorphic bands was recorded as "1" or "0" in each isolate, respectively. The resulted polymorphic data were entered to construct a phenogram using UPGMA algorithm in the SAHN program in the software package NTSYS-pc 2.1 (Department of Ecology and Evolution, State university of New York). Finally a phylogenetic tree was established by using the tree plot program of the software Package NTSYS-pc2.1.

ITS sequencing

Ribosomal sequence were amplified and sequenced by using the universal primar pair ITS1/ITS4 (table 1). PCR volume were 50 µl consist of 5 µl 10x buffer provided by the manufacture, 3µl genomic DNA, 2µl of each primer (10µg/ml), 4µ dNTP, 0.5 µl of Taq DNA polymerase. Amplifications were performed in a thermal cycler. The amplification starts with initial denaturation at 94°C for 3 min ,35 cycles of 1min at 94°C ,1 min at 55°C and 3 min at 72°C, followed by a final extension step of 5 min at 72 C ,PCR products were confirmed on 1.0% agarose gels in 0.5 TBE buffer at 100 V for 1 h, remain PCR products were sent to sequence directly.

Table (1): Primers used in DNA isolate Amplication.

Primer	Sequence (5' →3')	Characterization
ITS1	TCCGTAGGTGAACCTGCGC	Amplification and sequencing ITS region
ITS4	TCCTCCGCTTATTGATATGC	
OPA-02	TGCCGAGCTG	RAPD primers for amplifying polymorphic bands
OPB-20	GGACCCTTAC	
OPF-05	CCGAATTCCC	
M13	GAGGGTGGCGGT	Satellite primers for amplifying polymorphic bands
CNS1	GAGACAAGCATATGACTACTG	

Results and Discussion

Morphological characteristics

Morphological characteristics of colonies were investigated for isolates grown on PDA for 7 days (Fig .1). Isolates 3 and 8 showed gray colonies but 8 had more aerial mycelia. Isolates 2, M3, M2 and M4 could produce melanin with different colors. Isolates 2 and M3 produced yellow to dark and pink melanin from center, while isolates M2 and M4 produced pink and pink to yellow melanin evenly. Among all of the tested isolates ,isolate M4 had the most aerial mycelia.

All of the isolates tested could produce two kinds of conidia, Maro-conidia and micro-conidia, Micro-conidia were similar ,oval spores with one cell. As shown in Fig. 2, macro-conidia from isolates 10, 2, 11 and 4 were similar, straight or light curve with relative round or blunt ends, but that from isolate 9 showed as curve spores with sharp ends. All of the macro-conidia multi celled spores. According to Booth (1970) isolates 1, 5, and 10 were identified as *F. oxysporum* while isolates 8 and 11 identified as *F. solani* and the rest was identified as *Fusarium* spp.

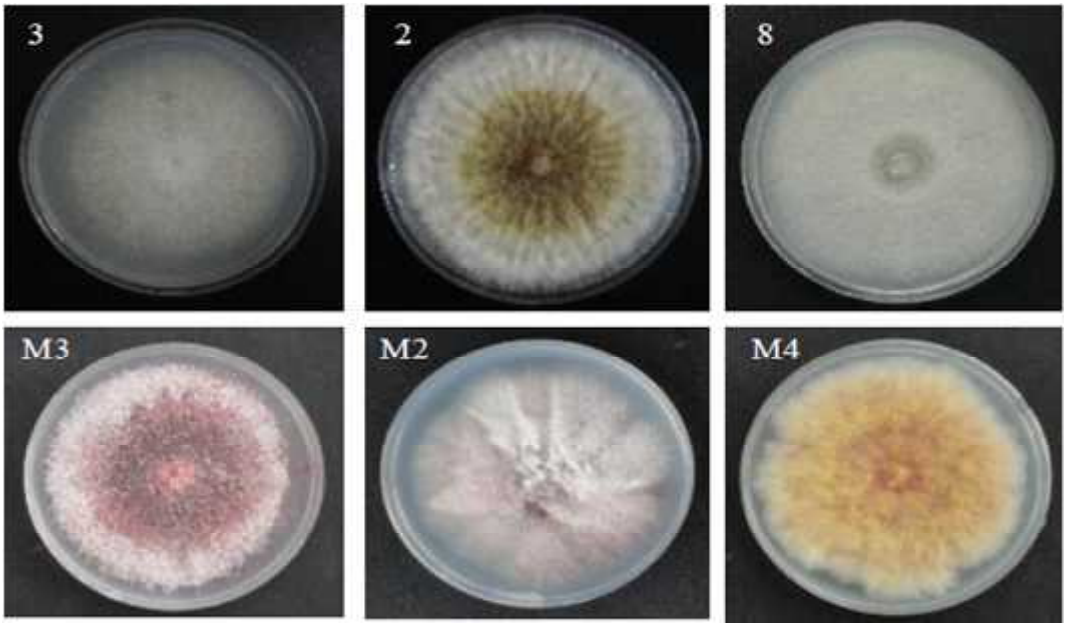

Fig. (1): Colony morphology of *Fusarium* isolates ,3, 2, and 8 from Iraq, M3, M2, and M4 from China.

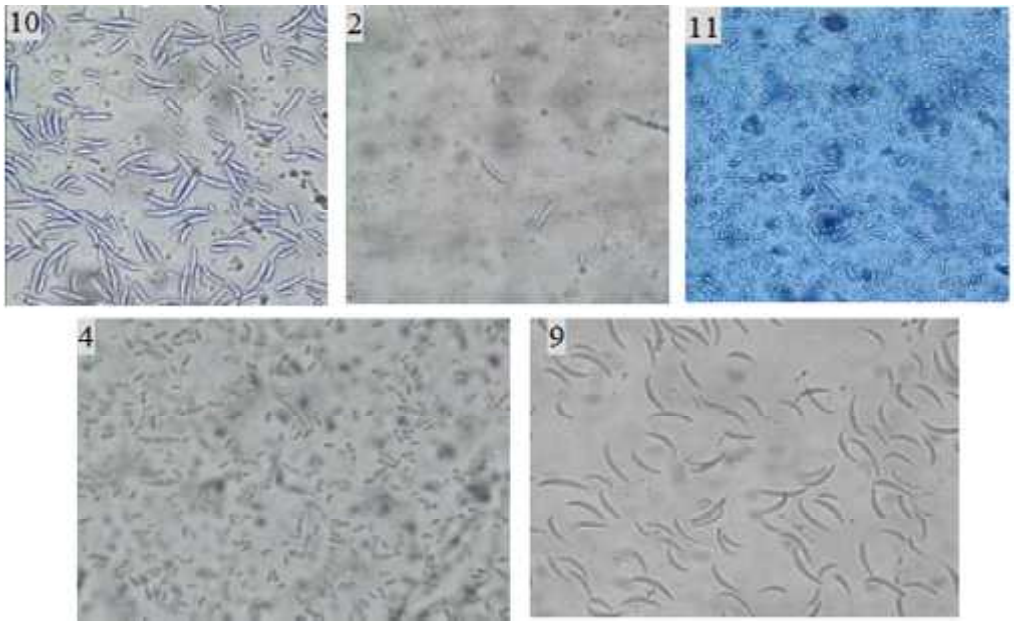

Fig. (2): Macro and Micro-Conidia shapes of *Fusarium* isolates.

Effect of different pH levels

Growing the isolates was investigated at all the pH levels (table 2). The fungal growth was maximum at pH 7.0 days of inoculation and it was decreasing according to the decrease or increase of the pH values and with the minimum of growth at pH 3 in tested isolates. This Result was in agreement with Ramantthan *et al.* (2010).

Table (2): Effect of different pH levels on the growth of some *Fusarium* isolates.

Colony diameter (cM)								pH / isolates
mean	8	7	6.5	6	4.5	4	3	
5.2	5.5	8.0	7.1	6.0	5.0	4.0	1.0*	4
5.7	6.0	8.3	7.3	5.9	5.5	4.5	3.0	11
5.3	5.6	8.0	7.0	5.5	5.0	4.3	2.1	1
5.6	5.7	8.8	7.5	6.0	5.0	4.6	2.0	2
5.5	5.5	8.7	7.7	6.0	5.3	4.6	2.3	9
	5.6	8.4	7.3	5.8	5.1	4.4	2.0	mean

***Each number is mean of three replicate./ L.S.D at 0.01 for :- pH=0.09, isolate=0.1,interaction=0.2.**

Sensitivity to fungicide Carbendazim

The sensitivity of isolates to fungicide carbendazim was assayed on PDA based on the mycelia growth inhibition (Table 3). Results showed that one third of isolates (4/12) from Iraq had EC50 values over than 1000 µg/ml, which indicated that many Iraqi isolates have developed resistance to carbendazim. For isolate 9, the EC50 was 98.8 µg/ml, indicating that it was a middle resistant isolate. In practice, the benzimidazole fungicides including Carbendazim should not be used to control *Fusarim* diseases on tomato anymore. Other fungicides with different actions mode might be used to replace benzimidazole fungicide immediately. In Chinese isolates, one isolate (M2) also was confirmed to be resistant isolate. Previous study indicated that *Fusarium* spp. was differed in their sensitivity to benzimedazol fungicides (Tort *et al.*, 2004; Iqbal *et al.*, 2010).

Polymorphic analysis of PCR with a single primer

Based on the preliminary experiments, three RAPD primers and two micro-satellite primers were used to amplify PCR products. Five primers produced 24 polymorphic bands. The RAPD amplification pattern of primers OPF-05 is shown in Fig3 as an example. Based on genetic identity calculated by software NTsys 2.1, a phylogenetic tree of used isolates was generated (Fig 4). Three groups could be divided at the 0.6 coefficient level. Group 1 contains Iraqi isolates, group 2 was isolates from both Iraq and China, group 3 contain 2 Chinese isolates. RAPD–PCR technique was used to identification of *Fusarium* spp. in previous study (El-Kazzaz *et al*., 2008; El-Fadly & El-Kazzas, 2008; Hiranon & Arie, 2006).

Table (3): Sensitivity of different *Fusarium* isolates to fungici Carbendazim.

Isolate	EC_{50} (µg/ml)	Isolate	EC_{50} (µg/ml)	Isolate	EC_{50} (µg/ml)	Isolate	EC_{50} (µg/ml)
1	2.0	5	2.3	9	>1000	M1	2.0
2	2.0	6	2.2	10	2.0	M2	>1000
3	2.3	7	2.2	11	3.1	M3	2.4
4	3.1	8	2.3	12	1.5	M4	3.7

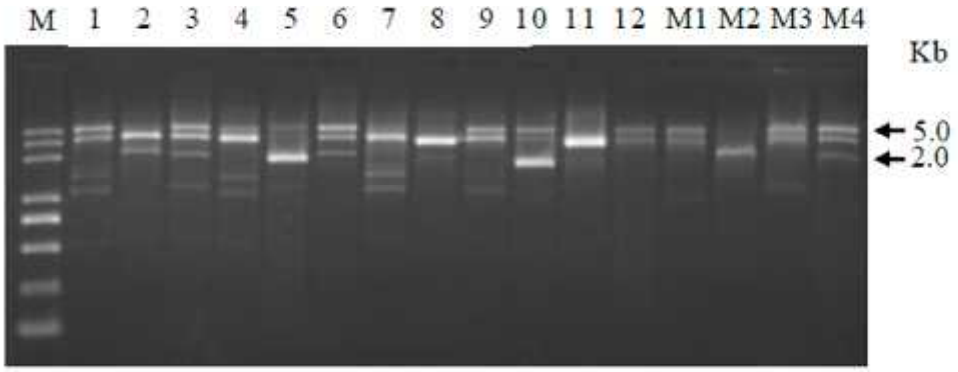

Fig. 3. Example of electrophoresis patterns of RAPD profiles from 16 *Fusarium* isolates with primer OPF-05. M = size marker, the largest to smallest bands are 5.0, 3.0, 2.0, 1.0, 0.75, 0.5, 0.35 and 0.1 kb in length. Lanes 1-16 represent isolates 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, M1, M2, M3 and M4, respectively.

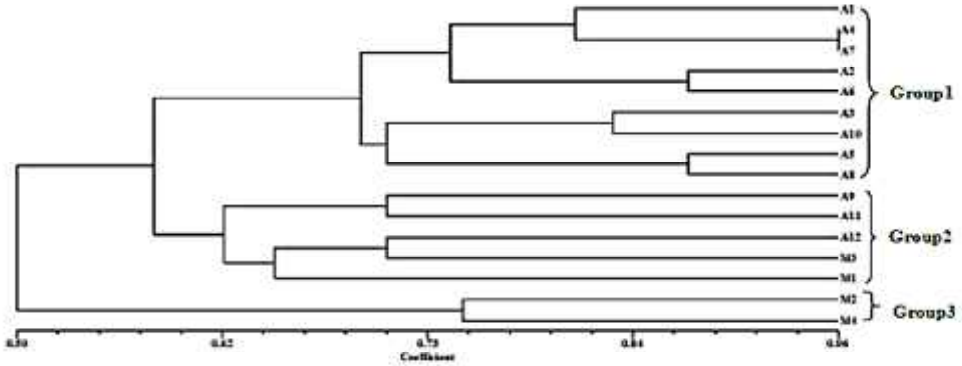

Fig. 4. Dendrogram from RAPD analysis of different isolates from Iraq and China. A1, …, A12 represent Iraq isolates 1, …, 12, respectively. M1, M2, M3, M4 isolates are from China.

Identification of *Fusarium* isolates based on ITS sequences

Based on ITS sequences including the internal transcribed spacer 1 and 2 the gene encoding the 5.8S small subunit of the ribosomal RNA was amplified and sequenced using the universal primer pair ITS1 and ITS4. Based on the ITS sequences, a phylogentic tree was conducted using the software package phylip 3.69. As shown in Fig. (5) 16 isolates could be grouped in 5 groups. Group 1 and group 2 only contain one isolates, respectively. Group 3 and group 4 include three isolates and group 5 has six isolate. Blast searches of the ITS sequences from individual group in GenBank showed that isolate M4 from group 1 had the highest similarity (99%) with *Fusarium kyushuense* (EF487532). Isolate M2 from group 2 showed the highest similarity (100%) with *Fusarium chlamydosporum* (Gu361930). Group 3 had two different kinds of isolates, isolate 2 had the highest similarity (100%)

with *Fusarium proliferatum* (GQ924905), but isolates 8,11 had the highest similarity (100%) with *Fusarium solani* (FJ426390), Isolates from group 4 showed highest similarity with *F. moniliforme* (GU257903), while *Fusarium* isolates from group 5 showed highest similarity with *Fusarium oxysporum* (Gu391929).

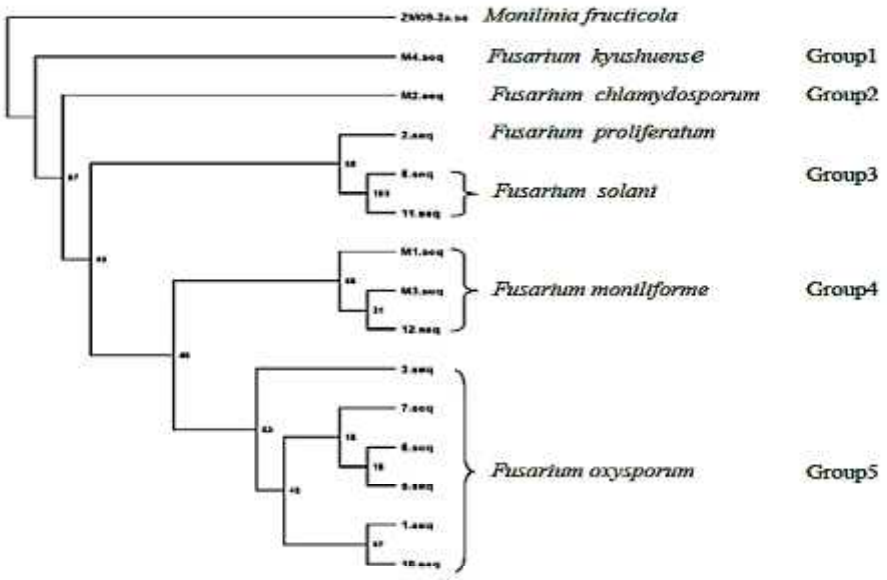

Fig. 5. Phylogenic tree based on the rRNA ITS sequences. Peach brown rot Monilinia fructicola isolate ZM09-2a was used as outgroup.

Therefore, the isolates 1, 3, 5, 6, 7, 10 were identified as *F . oxysporum*, isolates 8 and 11 as *F. solani*, isolates 12, M1 and M3 as *F. moniliforme*, isolate 2, M2 and M4 as *F. proliferatum, F. chlamydosporum and F. kyushuense,* respectively. RAPD-PCR based onDNA-Sequencing had been used to differentiate between species of many fungi and become very useful mean in fungi identification this technique was employed to differentiate between *Fusarium* spp. in previous studies (Roberts *et al.*,1995; Leong *et al.,* 2010).

Conclusions

The traditional methods based on morphological characteristics are time-consuming and may lead to misidentification among closely related species, therefor molecular identification based on ITS sequences were able to correctly identify *Fusarium* species. Sensitivity test to the fungicide carbendazim indicated that many *Fusarium* isolates were isolated from Iraq, and have developed resistance to carbendazim.

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