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# First Report of Identification *Fusarium solani f. sp. cucurbitae* Race 1 and 2 the Causal Agent of Crown and Root Rot Disease of Watermelon in Iraq

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***Abstract* – *Fusarium solani* f. sp. *cucurbitae* (Fsc) the causal agent of the crown and root rot disease of watermelon, two races of this fungus were reported in this study race 1 and 2. Race 1 affects root, crown and fruit while race 2 affects fruit only, severe infection was observed in watermelon fields in some provinces in middle and south of Iraq, twelve fungal species were associated with diseased plants, Fsc was predominant and appeared in all samples with frequency ranged between 27.50 - 80.00%. Thirty isolates identified as Fsc according to their cultural and morphological characteristics. Molecular identification of these isolates by PCR technique using Species-Specific Primes concluded that nineteen isolates belong to race 1 and four isolates belong to race 2 while seven isolates were not amplified with primers of both races, and this is first report of Fsc race 1 and 2 the causal agent of crown and root rot disease of watermelon in Iraq.**

***Keywords* – *Fusarium solani* f. sp. *cucurbitae Race 1 and 2,* Crown and Root Rot of Watermelon, PCR Detection.**

## I. INTRODUCTION

Crown and root rot disease of watermelon (*Citrullus lanatus* (Thumb.) Matsum and Nakai) is one of the most prevalent disease causes economic losses of this crop worldwide [8]. Usually symptoms appears as a stunning and sudden wilt during plant growth stage, symptoms of the disease appears depending on the environmental conditions, plant age during infection, plant density and virulence of the pathogen population, typical symptoms appears as a rot in the crown and root cortex followed by yellowing old leaves and wilting whole shoot system during high temperature times of the day and eventually death the plant, also appears as a fruit rot, and stress can lead to high disease levels in the plants [22] [8] [18] [3] [14]. The causal agent of this disease is *Fusarium solani* f. sp. *cucurbitae* W.C. Snyder & H.N. Hansen (Fsc) teleomorph *Nectria haematococca* Berk. & Broome, which is one of the most important soil borne fungi. Two races have been identified in the world and designated as race 1 and 2 based on their tissue specificity, all of the cucurbits susceptible to this pathogen[27]. Fsc was reported to the first time on Squash (*Cucurbita pepo* L.) in1923 in South Africa [9] and then reported on several cucurbits include watermelon in various areas in the world such as USA [24] Italy [28] Newzealand [23] Japan [16] Spain [2] Tunis [6] and Iran[1]. Race 2 has been reported in limited areas in the world compared with race 1 which widespread in watermelon growing areas [2].

The two races are separated to the different mating populations according to their sexual compatibility, race 1 belong to mating population I while race 2 belong to V [25]. Fsc forms three types of asexual spores which are microconidia, macrocondia and chlamydospore , and these spores usually used in the morphological identification of the Fusarium species. The first molecular identification of the Fsc race 1 and 2 isolated from pumpkin has been reported by [20] used specific primers designed from the ITS region by PCR technique and the result shows spread race 1 more than race 2 in California, and [1] isolated Fsc race 1 from different provinces in Iran and found variation among these isolates by RAPD technique, so attributed that to the sexual stage of the pathogen and rule out the effect of geographical distribution in this variation. The objective of this study were to identify the fungi associated with the crown and rot root disease of watermelon, determine the predominant causal agent of this disease, confirm the races identification of *F. solani* f. sp. *cucurbitae* isolates collected in this study using phylogenetic analysis.

## II. MATERIALS AND METHODS

### *A. Isolation and Identification of the Pathogen*

Fifteen samples from several diseased and symptomatic watermelon plants were collected (Fig.1). These samples were obtained from some provinces in the middle and south of Iraq during June-August 2013. Samples were cut into 0.5-1cm and washed under running tap water for 30 minute, then surface sterilized in 1% sodium hypochlorite for 2 minute and cultured on Potato dextrose agar (PDA) supplemented with 200 mg\l Tetracycline and incubated at 25±1C° for 7 days, single spore technique was made for each isolate. Isolates were identified to the species level according to their cultural and morphological characteristics [11] [26] [10]. The isolation frequency and relative density of genera and species were calculated according to [15] as follows:

$$\text{Apperance } (\%) = \frac{\text{No. of samples of occurrence}}{\text{Total No. of samples}} \text{ X100}$$

$$\text{Frequency } (\%) = \frac{\text{No. of plant segments of species occurrence}}{\text{Total No. of segments used}} \text{ X100}$$

Fig. 1. Symptoms of crown and root rot disease of watermelon caused by *Fusariun solani Molecular identification of F. solani f. sp. cucurbitae race 1 and 2 by PCR technique*

### *B. Extract and purification of DNA*

Isolates of Fsc were grown on PDA and incubated at 25 ±1 c° for 7 days. Colony, mycelium and spores for each isolate was separately harvested by usning sterile glass rod and transferred into Eppendorf tubes 1.5 ml and then stored in -20 c° until its use in DNA extraction. DNA was extracted by two protocols, first is according to the method of [4] with simple modifications using grinding buffer (100 mM NaCl, 200 mM Sucrose, 100 mM Tris – HCL PH 9, 50 mM EDTA and 0.5% SDS), Potassium acetate, ethanol 100% and AE buffer (mM 10 Tris-HCL and (PH 9) mM 0.5 EDTA). Second protocol using standard kits of fungal DNA extraction (EZ-10 Spin Column Fungal Genomic DNA Mini-Preps Kit) produced by Bio Basic Inc. Canada, using universal digestion buffer, proteinase K, universal buffer PF, universal buffer BD, ethanol 100%, EZ-10 columns, universal PW solution, universal wash solution and TE buffer.

### *C. Primers preparation*

Two Species-specific primers has been used to identify *Fsc* race 1 and 2, produced by Bioneer company, Coria.(Table 1).

Primers diluted by adding sterile distilled water according to the producer recommendation for each primers separately to obtain the concentration 100 pmol/µl. Then kept at -20 C° until uses.

Table 1: Species-Specific primers used to identify Fsc race 1 and 2 .

| **Primer** | **Sequence (5’ → 3’)** | **pb** |
|---|---|---|
| Fsc 1 | F/ CGTGATTGGGACGGATGAGAGA | 22 |
| | R/ TGGCATCTTGGGCGGGGGGT | 20 |
| Fsc 2 | F/ ACGTGAGTGAGAGACATGACGG | 22 |
| | R/ TTCACAACAGACACTGACTCG | 21 |

(Hong et al. 2010)

### *D. Prepare the interactions of the PCR (Polymerase Chain Reaction).*

Used standard Kits of AccuPower ® PCR PreMix produced by Bioneer Company, Korea. Included 96 sterile Eppendorf tube 0.2 ml, each tube consist of the reaction mixture (Table 2). and Species - Specific Primers used to identify race1 and 2 of *Fsc* according to method of [13]. This technique depended on present DNA bands as a measure of presence specific races.

Table 2: Solutions used in the PCR interactions.

| Content | Concentration |
|---|---|
| Each: dNTP (dATP, dCTP, dGTP, dTTP) | 250 µM |
| KCL | 30 mM |
| MgCl2 | 1.5 mM |
| Toq DNA polymerase | 1 U |
| Tris-HCL (pH 9.0) | 10 Mm |

Added to each PreMix tube which contain the interactions mixture(Tab. 2) 1.5 µl of the primer strands and 3µl DNA of each sample individually/concentration of 25-50 ng/µl and 14 µl sterile distilled water, mixed by vortex, and prepared control treatment (Negative control) included the interactions mixture (Table 2) and 1.5 µl of the primer strands and 17 µl sterile distilled water except DNA to make sure that no DNA contamination in the solutions used in this reaction, and then placed the samples in a polymer thermal (ThermoCycler) produced by Analytic Jena, Germany, and program seated according to the method of [13], the program included Initial denaturation heating at a temperature of 94 C° for two minutes to separate the double strand of the DNA to single strand, and 30 cycle included; Denaturation at 94 C° for one minute to copy the DNA strand which will be a template for the subsequent reactions, and Annealing at a 55 C° for one minute to linked the primer with DNA template, and Extension cycle at 72 C° for two minutes to elongate the primer which linked with the DNA template, and then final Extension cycle at a 72 C° for 10 minutes. After the program finished, transferred the tubes from the ThermoCycler and kept at a temperature of 4 C° until uses.

### *E. Replication result assay*

Investigated presence of the DNA bands by exposing the agarose gel to the UV light at the wavelength 320 nm in the UV Transilluminator device produced by Cleaver,UK. and documented the figures by Jell Documentation System produced by the same company. Estimated molecular size and weight by measuring DNA bands migration compare to the DNA Ladder.

## III. RESULTS AND DISCUSSION

### *A. Isolation and identification of the pathogen*

The results showed the existence of 12 species of fungi associated with the roots and crown of watermelon (Table 3). *F. solani* was predominant in all samples with 100% and 50.04% percentage of Appearance and frequency respectively, this result agreed with many studies reported spread this pathogen in various countries worldwide such as South Africa, Australia, Canada, United States, Japan and Tunisia [18]. Fsc forms white to cream color mycelium on the PDA (Fig. 2). Microscopic examination was showed three types of spores, microconidia were oval, renal and spindle shape, some divided by transverse septum to two equal cells, produced from long monophilides growth laterally on the aerial mycelium. Macroconidia were spindle to cylindrical shape asymmetric heterogeneous in their dimensions, divided to 1-7 cells by transverse septa.

Table 3. Fungus associated with crown and root rot disease

| Fungus name | Frequency (%) | Appearance (%) | *No. of sample of species occurrence (%) |
|---|---|---|---|
| *Alternaria alternata* (Fres.)Keissler | 21.72 | 46.66 | 5-9, 12,13 |
| *Aspergillus flavus* Link ex Gray | 25.00 | 13.33 | 12,13 |
| *Aspergillus niger* Van Tieghem | 11.66 | 26.66 | 1,8,14,15 |
| *Cladosporium cladosporioides* (Fresen.) G.A. de Vries | 1.85 | 13.33 | 5,6 |
| *Curvularia lunata* (Wakker) Boedijn | 1.25 | 26.66 | 1-3,15 |
| *Drechslera halodes* (Drechsler) Subram. & B. L. Jain | 7.83 | 40.00 | 7-9,12-14 |
| *Fusarium oxysporum Schlesht.* | 2.63 | 26.66 | 1,3,7,8 |
| *Fusarium solani (Mart.) Sacc.* | 50.04 | 100.00 | 1-15 |
| *Macrophomina phaseolina (Tassi) Goid* | 9.12 | 60.00 | 1-5,7-10 |
| *Pencillium* sp. | 8.33 | 13.33 | 12,13 |
| *Phoma pinodella* (L.K. Jones) Morgan | 1.55 | 26.66 | 6-9 |
| *Ulocladium atrum* Preuss | 1.71 | 26.66 | 6-9 |

* 1= Babylon-Muhanawia-1, 2= Babylon- Muhanawia -2, 3= Babylon-Al. Muhanawia -3, 4= Baghdad-Abu.Ghraib, 5= Anbar-Husaiba-1, 6= Anbar-Husaiba-2, 7= Theqar-Rifai-1, 8= Theqar-Rifai-2, 9= Theqar-Rifai-3, 10= Diyala-Khanaqin-1, 11= Diyala-Khanaqin-2, 12= Babylon-Musayib-1, 13= Babylon-Musayib-2, 14= Baghdad-Taji, 15= Karbalaa-Hindiyah.

Chlamydospore which produced as a single cell or pairs on the lateral small branch or formed in the middle of the hypha. These results agreed with many researchers [5] [17] [10]. Farther more the results were showed associated number of fungi such as *A. alternata*, *Aspergillus flavus*, *Aspergillus niger*, *Cladosporium cladosporioides*, *Curvularia lunata*, *Drechslera halodes*, *Fusarium oxysporum*, *M. phaseolina* , *Pencillium sp.*, *Phoma pinodella*, *Ulocladium atrum*, with % of Appearance and frequency ranged between13.33 - 60.00% and 1.55 – 25.00% respectively.

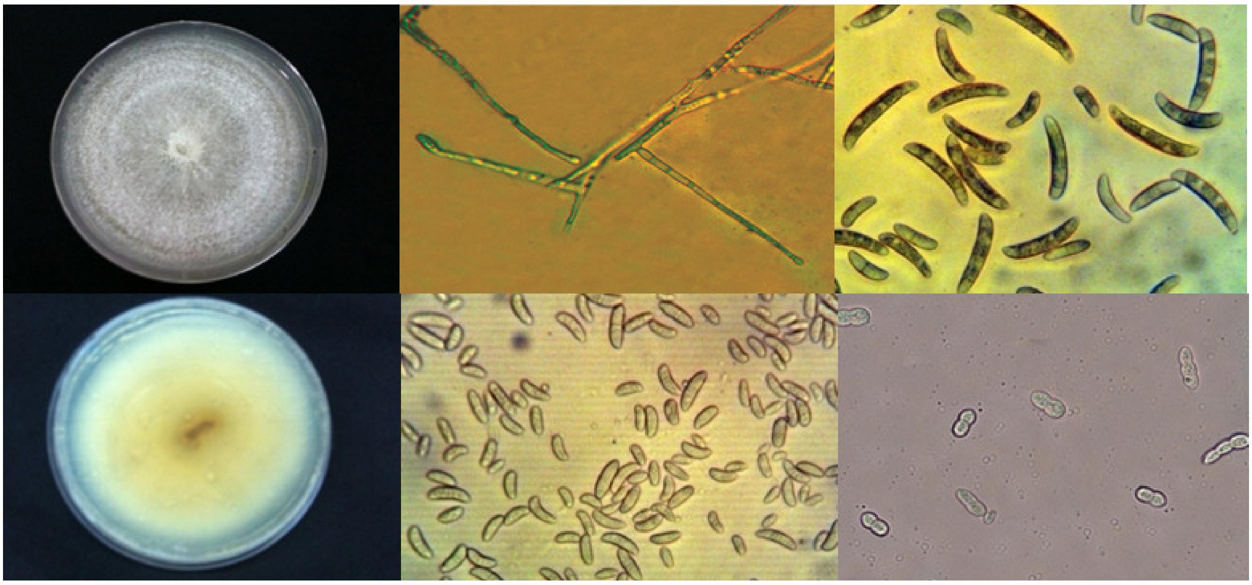

Fig 2. Cultural and microscopic characteristics of F. solani the causal agent of the crown and root rot disease of watermelon. (A, B) Growth of the fungal colony on the PDA (C) Long monophialides conidiospher X10 (D) Microconidia X40 ( E) Macroconidia X40 (F) Chlamydospore X40

### *B. Molecular identification of F. solani f. sp. cucurbitae race 1 and 2 by PCR technique.*

The results were showed that 19 isolates of Fsc amplified positively with primer of race 1 (Fsc1) of 580 bp fragment (table 4), while no band was observed with the primer of race 2 (Fsc2) (Fig. 3 a,b,c), Four isolates were amplified positively with primer of race 2 (Fsc2) of 580pb fragment compared to the DNA Ladder, and did not show any reaction with the primer of the race 1 (Fsc1) (Fig. 3 d,e). These results were agreed with that found by [13], as shown their study spread both races of Fsc in Korea depending on the molecular identification.This result agreed also with [1] they found spread Fsc race 1 in three provinces in Iran. [7] found that the incidence of race 1 is larger in the watermelon fields in Tunisia and spread widely, compared to race 2 which spread lower but it exists in the north, middle and southern of Tunisia. The results showed presence of 7 isolates did not show interaction with the primers of race 1 and 2 even after several repeats were A3FS-7 , T4FS-2 , A3FS-1 , Z2FS-6 , Z2FS-1 , G7FS-2 and G7FS-1 (Fig. 3 a-e), despite the morphology identification showed that these isolates belong to *F. solani* based on the taxonomic keys [5] [10] and this may attributed to variation in the fungus genes [1] or may represent a new race unidentified in the world. The adoption of phylogene in identifying races of the fungus may abbreviate the time required for identification and increased the accuracy of the results compared to the morphology identification which is often difficult and complex [20] [1] [13]. The first molecular identification of Fsc races 1 and 2 by using PCR technique was made by [20] the study concluded that race 1 is spread more than race 2 on pumpkin in California, researchers adopted previously on the mating population or pathogenicity test in identification specie and races of Fsc [19] [12] [5], and this is first report of identification *F. solani* f. sp. *cucurbitae* race 1 and 2 in Iraq

Table 4. Fusarium isolates used in PCR

| No. | Isolate code | Primer | | Race | No. | Isolate code | Positive PCR | | Race |
|---|---|---|---|---|---|---|---|---|---|
| | | Fsc 1 | Fsc 2 | | | | Fsc 1 | Fsc 2 | |
| 1 | D5FS-7 | + | - | 1 | 16 | B1FS-1 | + | - | 1 |
| 2 | D5FS-2 | + | - | 1 | 17 | T4FS-15 | + | - | 1 |
| 3 | A3FS-7 | - | - | | 18 | Z2FS-6 | - | - | |
| 4 | K8FS-1 | + | - | 1 | 19 | T4FS-13 | + | - | 1 |
| 5 | B1FS-4 | + | - | 1 | 20 | T4FS-16 | + | - | 1 |
| 6 | T4FS-4 | + | - | 1 | 21 | T4FS-11 | + | - | 1 |
| 7 | D5FS-5 | + | - | 1 | 22 | T4FS-10 | + | - | 1 |
| 8 | T4FS-14 | + | - | 1 | 23 | A3FS-2 | - | + | 2 |
| 9 | T4FS-2 | - | - | | 24 | B6FS-6 | + | - | 1 |
| 10 | D5FS-3 | + | - | 1 | 25 | B6FS-2 | - | + | 2 |
| 11 | T4FS-6 | + | - | 1 | 26 | Z2FS-1 | - | - | |
| 12 | A3FS-1 | - | - | | 27 | G7FS-2 | - | - | |
| 13 | K8FS-3 | + | - | 1 | 28 | B6FS-9 | - | + | 2 |
| 14 | T4FS-8 | + | - | 1 | 29 | B1FS-3 | - | + | 2 |
| 15 | T4FS-12 | + | - | 1 | 30 | G7FS-1 | - | - | |

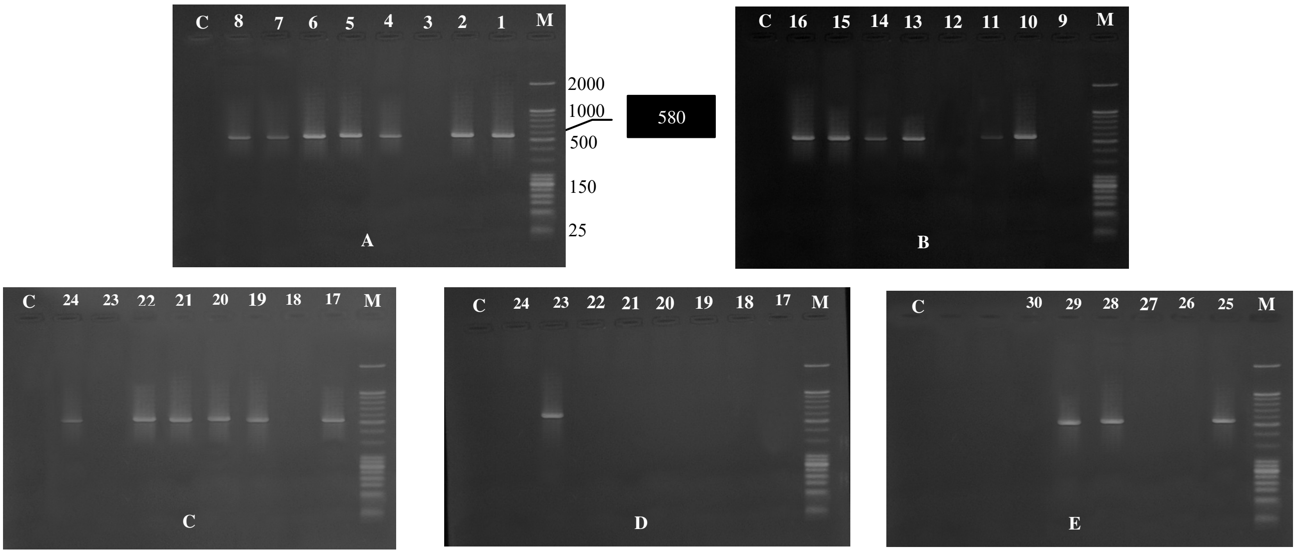

Fig.3. Demonstrate the specificity of the primers on Agarose gel. A,B,C, PCR amplification of 580 bp fragment with primer of race 1. D,E, PCR amplification of 580 bp fragment with primer of race 2. Lane M (2000 pb) DNA ladder. Lane 1-30, isolates of *F. solani* f. sp. *cucurbitae*. Isolate no. 3,9,12,18,26,27 and 30 did not amplified with primers of race 1 and 2.

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