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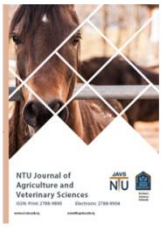

Fusariumoxysporum f. sp. *tuberosi* causing wilt disease on potato plants in Iraq.

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Fusariumoxysporum f. sp. *tuberosi*,

A B S T R A C T

Fusarium species are causing postharvest stem-end rot and dry rot throughout the potato growing season. At the current study, the causative wilt agent was identified in potatoes which were grown in Nineveh province, northern of Iraq, in autumn season of 2020. The disease manifests through symptoms like partial yellowing between the veins on the leaves of potatoes or overall wilting of the plant. Fungal species isolated from the infected potato plants were subsequently grown on potato dextrose agar, resulting in developing aerial mycelia appeared as white along violet-dark pigmentation. The fungus was identified from the phenotypic diagnosis as *Fusariumoxysporum*, and the pathogen was confirmed by Koch's hypotheses as well as affirmed through molecular test identification, where methodology proved it. Molecular based pathogen identification was carried out through an application of conserved DNA ribosomal region of internal transcribed spacer (ITS). Moreover, the complete verified ITS sequences are homologous to the isolates of *Fusariumoxysporum* in GenBank database having 99% similarity. At Gen-Bank, isolated one in Iraq was given #: MH859948.1 being an Accession. The best to the available knowledge, such is *Fusariumoxysporum* f. sp. *tuberosi* 1st molecular Potato record in Iraq.

Introduction

Solanaceae is the family potato (*Solanumtubersum* L.) is belonging to; it is considered as one of the mostly important and worldwide grown crops and has a vital function in nutrition of human. Potato is the 4th mostly essential food crop following rice, wheat, and corn in terms of human consumption (Muthoni and Nyamongo,2009). potato production exceeded 376 million tons globally, harvested from an area of 19.25 million hectares (Fawstat,2018). In 2019, potato was grown in Iraq on a land of 4.56 thousand donums to produce a sum of 392.3 thousand tons of potato for the spring and autumn crops (Central Bureau of Statistics,2020). Potatoes as staple food is utilized directly or being managed as industrial starch, ingredients of food or into other food products. Also, for the subsequent season, potatoes as seed tubers are re-utilized for growing the crop(Aktaruzzamam et al,2014). Potato crop cultivation is threatened by several fungal diseases including vascular wilt caused by *Fusariumoxysporum*, followed by other wilt pathogens *Verticilliumdahliae*, *V. alboatrum* and *V. tricorpus*(Jabnoum-Khiareddine et al 2005 and Daami-Remadi et al ,2011).Fusarium genus contains a complex species which has numerous clonal lineages(Michielse and Rep,2009). Thus, this is distributed on a worldwide basis and comprise a responsibility for powerful wilts as vascular, and capable of causing various rots of plant structures for instance, corms, bulbs, seedlings, tubers, cobs, roots and stalks of a widespread range of plants(Michielse and Rep,2009 and Enya et al,2008). Furthermore, species of Fusarium are causing postharvest stem-end rot and dry rot throughout the potato growing season (Bayona et al,2011 and Pont ,1976).Growth of *F. oxysporum* for infection is preferred by conditions of dry soil and 15º C is soil optimum temperature (MacMillan and Meckstroth , 1925). In the last couple years, Fusarium potatoes wilt has turned to be prevalent in all cultivation areas and has been often connected to symptoms of early death causing large losses of yield up to 30-50% and reduced quality of tubers(Daami-Remadi et al ,2011and Kerkeni et al,2013 and Ommati et al ,2013). The Fusarium wilt disease occurrence caused by *F. oxysporum* in potato fields in some regions of the world has reached 15-70%(Gachango et al ,2012). Moreover, the fungus *F.oxysporum*f. sp. *tuberose* causing dry rot on stem and end rot the tubers as the vascular potato wilts (Shrivastava , 1970 and Daami-RemadiMejda and Mahjoub,2004).

Materials and Methods

Isolation and Diagnosis

Isolation was obtained from potato fields infected with wilt disease in Nineveh province, northern of Iraq, during the spring season of 2019. Potato samples were collected with symptoms of wilting through visits to the affected potato fields. They were stored in clean bags of plastic and conveyed to the Department of Plant Protection, "laboratories of Agriculture and Forestry College" – University of Mosul. In order to remove soil, samples were washed with running water for 30 minutes, then parts of the root system were taken with the crown area. cut into small parts with a length of approximately 4-5 mm. These parts were surface sterilized with NaClO at 1% concentration for 3 min and then washing was done with sterilized DW to remove the excess of the substance. The sterilization was dried between filter paper sterile folds, and the parts were distributed on 9 cm diameter Petri dishes comprising media of dextrose PDA and potato which was previously sterilized in the autoclave at 1.5 kg / cm^2 pressure and 121 ° C temperature for twenty min, after sterilization termination period, and the pressure fell to zero. The flasks were left to reduce the temperature of the mediumand the antibiotic Amoxicillin was added to it at a 250 ml / liter concentration for preventing bacterial growth. Incubation for the plates was done at 25 ° C for five days, and then the isolates were purified test of Pathogenicity

Pathogenicity test of *F. oxysporum*f. sp. *tuberosi* .

A pathogen's pathogenicity was carried out utilizing method of suspension spores using plastic pots (25 cm) in diameter containing soil after sterilization with formalin 37% at a concentration of 5%. The tubers were surface sterilized with 10% NaClO solution then planted in post after contaminating the soil with the pathogenic fungus 1x 10^7 spore's.ml^{-1}. one tuber per pots at a depth of 10 cm. The same process was carried out using water in the control and then directly planted. Symptoms were monitored and the infected plants were re-isolated to confirm the pathogenicity of the fungus and to apply Koch's hypotheses.

Extraction of Genomic DNA and Amplification of PCR

Single spore isolation of *F. oxysporum* was grown at 25–28°C in broth of dextrose potato (PDB) in dark for 10 days. Mycelia harvesting was performed through filtration using Whitman filter paper 1. Further, the mycelia being harvested were utilized instantaneously for the extraction of DNA while utilizing Fungal/Bacterial/ DNA MiniPrep™ Yeast, Catalog ‹D6005. This was accomplished conferring to the protocol factory methods provided underneath:

1. Wet weight of 50 – 100 mg (bacterial or fungal cells1) that has been re-suspended was added to water of 200 of or tissue addition of 200 mg to Lysis Tube (which is of 0.1 mm & 0.5 mm) in form of as ZR Bashing Bead™ or isotonic buffer (e.g., PBS). Addition of 750 µl as LysisSolutionto tube # 2 was done.

2. Bead beater securing as fitted was done along a tube holder of 2 ml having association and process at a speed as maximum for ≥ five min.
3. In a microcentrifuge, Centrifugation was done for the Lysis Tube of ZR BashingBead™ at 10,000 x *g* for one min.
4. The 400 µl of supernatant was transferred to a Zymo-Spin™ IV Spin Filter (Orange Top) was carried out in collection tubes, after that centrifugation was performed at 7,000 x *g* for 1 min.
5. Further, 1,200 µl Fungal/Bacterial Binding Buffer of DNA was incorporated to the filtrate in collection tubes from step # 4.
6. Mixture of 800 µl was transferred from step # 5 to a Zymo-Spin™ IIC Column 3in all collection tubes and then centrifugation was performed at 10,000 x *g* for one min.
7. Flow discarding was done from tube of collection and repeating to step # 6.
8. To the column of Zymo-Spin™ IIC, DNApre-wash buffer addition of 200 µl was done in a new tube of collection and centrifugation for one min at 10,000 x *g*.
9. To the column of Zymo-Spin™ IIC, Fungal/Bacterial DNA Wash Buffer addition of 500 µlandcentrifugtion for one min at 10,000 x *g*.
10. After moving the Zymo-SpinTM IIC column to a clean 1.5 ml microcentrifuge tube, the column matrix was immediately infused with 100 µl (at least 35 µl) of DNA elution buffer. Therefore, a 10,000 x g centrifugation for 30 seconds was used to elute the DNA.

For initial denaturation and annealing, the condition being optimal has recognized following preforming numerous experiments for gaining such condition, the temperature has changed during the Gradient PCR operation for all samples to choose the best setting, and the DNA template concentration has changed from 1.5-2 µl, which are thought to be the two most important variables in primer annealing with complement.

DNA Agarose gel electrophoresis (EP)

In order to assess the pieces of DNA, EP was performed following the procedure of extraction. As well as to perceive outcomes of PCR interaction, where presence of standard DNA distinguished PCR interaction result's bundle size on the Agarose gel.

Agarose gel preparation

Based on (Sambrook et al ,1989),the agarose gel was prepared in 1.5% condensation via agarose melting of 1.5 g in formerly prepared TBE buffer of 100 ml. Agarose was boiled then kept to cool at (45-50 °C). In the pour plate, the gel was poured where the agarose support plate has been prepared following comb fixing to create holes that were holding the samples. Gently, the gel was poured to avoid air bubbles formation and kept for thirty min to cool. Of the agarose as solid, the comb was gently removed. In the EP's horizontal unit, the plate was fixed to a stand exemplified through the utilized tank in EP. Moreover, TBE buffer filled the tank while covering the gel surface.

Sample preparation

Following the mixing process, loading is processed to the gel pores. Three microliters of processor loading buffer (Intron/Korea) and five liters of fictitious DNA for EP (loading dye) were prepared. Moreover, the electricity current of 7 V\C^2 was exposed for a time period of 1-2 hour as long as the movement of tincture took place to the other side of gel. Thus, gel was further inspected through a UV source of 336 nm and after that gel was placed in pool containing a staining solution of 500 ml DW and 3µl Red safe Nucleic acid.

ITC Gene Detection through PCR Utilizing

The ITS gene detection procedure is carried out using amplification primers. Additionally, the ITS segment was amplified using the ITS1 F: 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS4 R: 5′ TCCTCCGCTTATTGATATGC-3′ forward and reverse primers. IDT (Integrated DNA Technologies Company, Canada) provided these primer sets. Similar to that, PCR amplification was performed in a 25 µl total volume that contained 1.5 µl DNA, 5 µl Taq PCR PreMix (Intron, Korea), and 1µl of every primer (10 pmol). After that addition of DW into a 25 µl tube of a total volume. Thus, the cycling conditions as thermal were performed as following: Denaturation for three minutes at 94 °C, after that 35 cycles for 45s whereas, at 52°C, 94 °C, for 1 minute along with 72 °C for one minute. However, the final step incubation was done at 72 °C for seven minutes with the help of a thermal Cycler (named as: Gene Amp, PCR system 9700; Applied Bio-system). Hence, PCR products were isolated from 1.5% agarose gel EP, further these were projected via UV exposure at 302 nm while following the process of red stain staining (Intron Korea).

Table 1. *F. oxysporum* isolate's RNA gene polymorphism

	Gene: 18S ribosomal RNA gene				
No.	Substitution type	Location	Nucleotide	Sequence ID with compare	Source	Identities
1	Transvertion	174	C\A	ID: MH859948.1	*Fusariumoxysporum* f. sp. *tuberosi*	99%
	Transvertion	176	T\A			

Sequencing and Sequence Alignment

Via 2% agarose gel EP, the products of PCR were separated and envisioned through UV exposure at (302 nm) following red stain staining or ethidium bromide.

Gene sequencing was done online biotechnology lab through (NICEM) website (http://nicem.snu.ac.kr/main/?en_skin=index.html) while machine used was Applied Biosystem DNA

sequencer 3730XL). Furthermore, the homology search was completed with the usage of program (BLAST) available online at (NCBI) website (http:// www.ncbi.nlm.nih.gov) as well as BioEdit program.

RESULTS

Isolation and Diagnosis

On PDA, the colonies of fungi were isolated and comprised of aerial mycelia eing white in which afterword formed pigments as dark violet which are *F. oxysporum*characteristic (Figure 1, D). *F. oxysporum*has 3 conidia types: microconidia, macroconidia, and chlamydospores. In contrast, other *Fusarium*spp. have just 2 conidia types: chlamydospora and macroconidia. Shapes of microconidia, macroconidia, chlamydospores and conidiophores (Figure 1, A-C) represent the exclusive *F. oxysporum* characteristics.

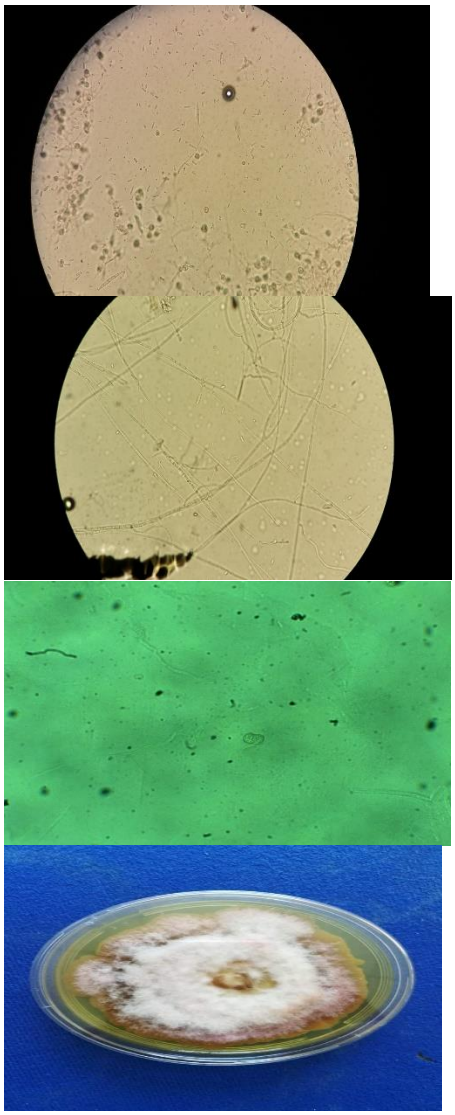

Fig 1. A, Conidiophores; B, Macroconidia and microconidia; C, Chlamydospores; D, 1-week-old *F. oxysporum* colony growing on agar media of potato dextrose; E, Disease-symptoms on potato plant showing yellowing between the veins; F, Potato which was inoculated with *F. oxysporum* established symptoms of stem-end rot following incubation of seven 7 weeks.

The conidiophors and monophialialides were the morphological traits of the isolate of *F. oxysporum* that was under study. Microconidia are ellipsoidal to slightly curved or cylindrically straight, with recorded dimensions of 1.9 11.2 µm, 2.3 3.3 µm, and Macroconidia Shape Fusiform, slightly curved, with 2–5 points on both ends, measuring 27.7–35.6(µm) in length and 3.4–3.3(µm) in width. Globose-ellipsoidal, in pairs or singly, are the chlamydospores' shape. Length: 7.219.3 µm, width: 6.729.5 µm. This trait is consistent with what Booth stated(Booth,1971).

Pathogenicity test

The first symptoms of the disease appeared on the potato plant 33 days following fungus inoculation in the form of yellowing between the veins of the lower leaves of the plant. After that, the lower leaves wither and dry completely and appear attached to the stem. Other symptoms associated with wilting may appear, such as stunting and leaf curl. Then it was re-isolated from infected plant to prove the hypotheses of Koch, where it was confirmed that the cause of the wilt disease was the fungus *Fusariumoxysporum*(Fig 1, E).

Sequencing and Sequence Alignment

For confirming the identification morphologicaly, the *F. oxysporum* (ITS) isolate region was amplified with ITS primers being universal. The isolate was diagnosed partially following conformism with the gene bank copies present at (NCBI) genes which provide the (diagnostic accuracy of) 99% match with that of isolation: thus,

the MH859948.1 Query sequence cover was observed to be 99% (Figure 1).

Table 2. Conformism ratio amongst copies particularly diagnosed and others copies at NCBI

Score	Expect	Identities	Gaps	Strand
872 bits (966)	0.0	486/488 (99%)	0/488 (0%)	Plus/Plus

The outcomes demonstrated the genetically association amongst *F. oxysporum*isolated in form of black prism) and are saved in Gen-Bank database globally. Moreover, figure 2 implies comparison amongst the local Iraqi *F. oxysporum*f.sp.*tuberosi*strain isolate with that of the *F. oxysporum*f.sp. *tuberose strain* documented in (NCBI) which have been isolated from various countries. Further, this exhibited a compatibility of 99% having an Iranian accession #: MH859948.1 as well as 99% with Poland accession # MW776326.1: Poznan and 99% with accession # MW704331.1from India, 99% # MW600442.1 ,MW533013.1and MW429367.1form China, Brazil: Parana and Mexico, respectively. The ITS sequence nucleotide of an Iraqi isolate was assigned a GenBank Accession # MW811385.1. In Iraq, such is the 1st potato wilt disease report caused by *F. oxysporum*.

Query 1 ACTCCCAAACCCCTGTGAACATACCACTTG TTGCCTCGGCGGATCAGCCCGCTCCCGGTA 60
Sbjct 51 110
Query 61 AAACGGGACGGCCCGCCAGAGGACCCCTA AACTCTGTTTCTATATGTAACTTCTGAGTaa 120
Sbjct 111 170
Query 121 aacaaaaaataaatcaaaaCTTTCAACAACGGATCTC TTGGTTCTGGCATCGATGAAGAA 180
Sbjct 171 **...C.T.**..... 230
Query 181 CGCAGCAAAATGCGATAAGTAATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGA 240
Sbjct 231 290
Query 241 ACGCACATTGCGCCCGCCAGTATTCTGGCG GGCATGCCTGTTCGAGCGTCATTTCAACCC 300
Sbjct 291 350
Query 301 TCAAGCACAGCTTGGTGTTGGGACTCGCGT TAATTCGCGTTCCTCAAATTGATTGGCGGT 360
Sbjct 351 410
Query 361 CACGTCGAGCTTCCATAGCGTAGTAGTAAA ACCCTCGTTACTGGTAATCGTCGCGGCCAC 420
Sbjct 411 470
Query 421 GCCGTTAAACCCCAACTTCTGAATGTTGAC CTCGGATCAGGTAGGAATACCCGCTGAACT 480
Sbjct 471 530
Query 481 TAAGCATA 488
Sbjct 531 538

Figure 1. Sense flanking of partial ITS gene sequencing in comparison to the gene MH859948.1standard from Gene Bank. Here, the sample query signifies; Subject signify (NCBI) database

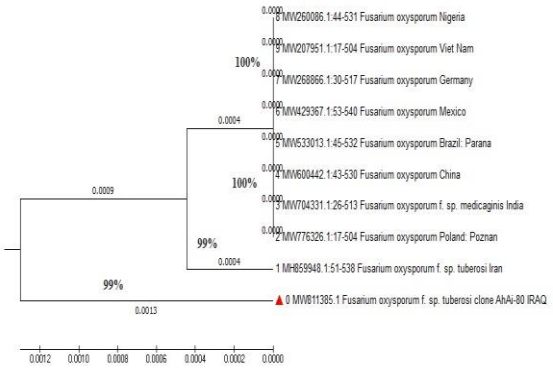

Fig. 2: Phylogenetic tree constructed through a neighbor-joining method presenting the phylogenetic relationships of *F. oxysporum*f.sp. *tuberosi* associating to the gene bank's reference sequences.

Conclusion

The results exhibited that the *F.oxysporum*f. sp. *tuberosi*was identified as an agent of causative Potato wilt in Iraq. According to our knowledge, the *Fusariumoxysporum*f. sp. *tuberosi*is is being = recorded for the first time of molecular Potato in Iraq.

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