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ISOLATION AND MOLECULAR IDENTIFICATION OF PHYTOPHTHORA INFESTANS (MONT.) DE BARY THE CAUSAL AGENTS OF POTATO LATE BLIGHT

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Abstract

Potato (Solanum tubersum L.) is an important crop in Iraq. Samples of potato plants infected with late blight were collected from potato fields in Nineveh Governorate, northern Iraq, during the autumn season of 2020. The causal agent being isolated and identified initially according to its morphological features utilizing hyphal and sporangial structures. According to cultural and morphological features, the pathogen was identified from the phenotypic diagnosis as Phytophthora infestans and the pathogen was confirmed by Koch's hypotheses. This was confirmed by the molecular identification test, methodology confirmed it. Molecular identification pathogen was performed via implementing transcribed internal spacer (ITS) conserved ribosomal region of DNA. All sequences as ITS proved homologous to isolates of P. infestans in database of GenBank at similarity % of 99. At Gen-Bank, Iraqi isolate was assigned as Accession No. : MZ675523.1. Up to our best knowledge, this is 1st molecular record of P. infestans on potato in Iraq.

Keywords: Late Blight Potato, Oospores, PCR.

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Introduction

The potato, *Solanum tuberosum* L., which belongs to the Solanaceae family, is one of the most important and most widely used vegetable crops, as it tops the list of tuber crops (Hassan, 1999, Khan *et al.*, 1995). As the daily food constitutes more than 75-90% of the world's food (Santamaria and Elia, 1997) and it ranks fourth as a major crop after wheat, corn and rice (Bowen, 2003). Potatoes are vegetables rich in nutrients that are easy to digest and represent in the body, ranging from The percentage of dry matter in its tubers ranges from 15-29 % starch constitutes 10-24% and proteins from 1-2% and the proportion of mineral salts reaches 1%, consisting mainly of potassium salts 70% phosphorous salts, magnesium and calcium (Kawakami et al., 2005) . It is believed that the original homeland of the potato is South America (Chile, Peru and Mexico), from which its cultivation moved to Europe and the world (Porras, 2010). Currently, China and India are the largest producers of potatoes in the world, followed by the Russian Federation (FAOSTAT, 2021). Potatoes in Iraq are grown in two seasons spring and autumn, and their cultivation is concentrated in the governorates (Baghdad, Nineveh and Anbar). Iraq's production of potatoes for the spring and autumn seasons was estimated at 3,392 thousand tons for the year 2019, including it (114.6) thousand tons in Nineveh Governorate and (674.8) thousand tons for the year 2020, including (132,2) thousand tons in Nineveh Governorate and (466,1) thousand tons for the year 2021, including (194.99) thousand Tons in Nineveh Governorate (Annual Crop Production Report, 2020, 2021, 2022).

The potato is the world's first non-cereal food commodity, with a production of 325 million tons. Some of the inherent qualities give the potato a competitive advantage over the leading food crops. It is able to produce more protein and carbohydrates per unit area of grain than all the crops grown in all regions of the world, These diseases include late blight and bacterial wilt, which are the most economical diseases with a 100% loss of yield (Tadesse et al., 2021) Late blight caused by *Phytophthora infestans* (Mont.) de Bary .is one of the most dangerous pathogens that leads not only to direct crop losses but also to farmers incurring huge financial expenditures for disease control and preventive measures. (Majeed et al., 2017a). Late blight has been known since 1745 on potatoes and recorded in 1741 on tomatoes and mushrooms. It follows the kingdom of the fungi-like organisms chromista and the division Oomycota and describes the Oomycetes (Al-Taae, 2017). It appears that the source of this disease is central Mexico (Zimnoch-Guzowska et al., 2003). Potato late blight is one of the most devastating plant diseases in potato and tomato producing areas in the world (Olanya et al., 2012) and globally, the losses resulting from this disease have been estimated to be more than $3.2 billion (Havertkort et al., 2008). Trout et al. (1997) stated that a rapid and accurate method for detection of late blight was necessary in which the rDNA of four *P. infestans* isolates representing the four genotypes US1, US6, US7 and US8 was amplified using polymerase chain reaction (PCR). and internal universal primers (ITS) 4 and(ITS) 5. The nucleotide sequence was studied by PCR using an automated sequencer. The sequences were matched with published sequences from 5 other *Phytophthora* species. Polymerase chain reaction (PCR) amplification of *P. infestans* protein using the primers INF FW2 and INF REV yielded 613 base pairs, PCR assay was used to examine the long-term survival of sexual (sporangia and mycelium) for *P. infestans* in Buried leaves in a replicated experiment under natural field conditions. Oospores were continuously detected using PCR assay for up to 24 months after burial in soil, while asexual as mycelium and sporozoites were only detected for 12 months after burial. Mycelium and sporozoites were shown to be unviable using a baiting assay, while leaves containing Oospores remained viable for up to 24 months after burial (Hussain et al., 2005). The goal of the current research was identifying the causative crown rot agent disease noticed in strawberry in Nineveh governorate, according to mycological features, and pathogenicity test, and identify it using spacer as internal transcribed (ITS) conserved ribosomal region of DNA utilizing primers ITS4 and ITS1

Materials and methods

An isolate of *Phytophthora infestans* (Mont.) de Bary :Isolation was obtained from potato fields infected with late blight disease in Nineveh province, northern of Iraq, during the autumn season of 2020.

Isolation and Diagnosis: Samples of potato leaves that showed symptoms of blight were collected by visits to some affected potato fields in Mosul city and kept in clean nylon bags (polyethylene) and transferred to the laboratory of the Plant Protection Department - College of Agriculture and Forestry - University of Mosul. It was washed with distilled water for 25 minutes to remove dust and plankton, then the leaves were taken and cut into small pieces about 3-4 cm in length. Those parts were superficially sterilized with 1% sodium hypochlorite solution for 3 minutes, then washed with distilled water to get rid of the sterile material and dried between the folds of the sterile filter paper, then Placed between potato slices in a Petri dish, After the potato tubers were washed well with water, and they were superficially disinfected by soaking them in a solution of 1% sodium hypochlorite at a concentration for two minutes, then washed with distilled water and dried well, the dishes were incubated at a temperature of 16 ° C for 7 days until the appearance of fungal gametes on the top of the potato slices. After the appearance of the fungal growths on the potato slices, they were transferred to petri dishes with a diameter of 8.5 cm containing the electoral rye medium (Rye A), which was previously sterilized in an autoclave at a temperature of 121 °C and a pressure of 1.5 kg. cm2 for 20 minutes, the dishes were incubated At a temperature of 16 ˚ C for 10 days and kept in the dark, then the isolates are purified.The method of isolation was carried out according to Sobkowiak and Śliwka (2017):-

The middle elective rye (Rye A) is made from rye grains with agar according to Caten and Jinks (1968), which consists of the following:

1- 60 grams of rye grains (must be untreated with fungicides, stored well and free of rotting).

2- 20 grams of sucrose or glucose.

3- 15 grams of agar.

The method of work includes the following:-

1- Soak rye grains in distilled water so that the water covers them to a height of 2 cm and put at room temperature for 36 hours.

2- Water is poured, distilled water is added to the grains, and they are placed in a water bath for 3 hours at a temperature of 50 °C (the supernatant is kept).

3- The mixture is filtered using a sieve and we get rid of the grains.

4- The filtered liquid is mixed with the supernatant in paragraph 2 and mixed with glucose or sucrose and agar and adjusted to a volume of 1 liter.

5- It is placed in the autoclave at 120 ° C for a period of 20 minutes.

6- The middle of the incubated rye with an autoclave is cooled to 45°C, then 3 ml of the antibiotic Rifamycin and 1 ml of the antifungal Pimaricin are added.

Pathogenicity tests: The pathogenicity test was conducted according to the method of Sobkowiak and Śliwka (2017), which includes the following:

1- Placing the mycelium of the fungus *P. infestans* grown on the rye agar environment between two 1 cm slices of potato tubers. The two tuber slices are not completely separated from each other, but leave 20% of the surface uncut to ensure close contact of the slices with the moist environment for the development, reproduction and spread of the mycelium.

2- The slides are incubated for a week at 16° C and a relatively high air humidity (80 - 100%) until thick mycelium appears on the upper surface of the top slide.

3- Sporangia is collected from the mycelium using a brush and washed with deionized water and a counting slide is used to measure the concentration.

4- This vaccine is placed for 2 hours at a temperature of 7 ° C and then 5 hours at room temperature in order to increase the liberation of sporophytes from the sporophyte bag. During use, the vaccine is constantly mixed to prevent sedimentation of the mixture.

Genomic DNA extraction and PCR amplification: Pure cultures of *P. infestans* were grown in rye medium (Rye A) for 12 days at 25–28°C in the dark environmental. Mycelia were harvested by filtration through filter paper (Whatman No. 1). The harvested mycelia were used immediately for DNA extraction using Fungal/Bacterial/ Yeast Quick- DNA MiniPrep™, Catalog No. D6005 according to the USA Zymo Research procedures Protocol blew:

For optimal performance, add beta-mercaptoethanol (user supplied) to the Fungal/Bacterial DNA Binding Buffer to a final dilution of 0.5%(v/v) *i.e.*, 500 µl per 100 ml. then complete the fungal DNA extraction according to the recommendation from the manufactured company. Electrophoresis has been done to determine DNA fragment after the its extraction used the agarose gel at 1.5% according to Sambrook *et al* .(Sambrook, et. al.,1989).

Preparation of sample : Three µl of the processor loading buffer (Intron/Korea) has been mixed with 5 µl of the supposed DNA to be electrophoresis (loading dye). An Electric current of 7 v\c2 has been exposed for 1-2 h till the tincture has reached to the other side of the gel. The gel has been tested by a UV light at 336 nm after used the 3µl of Red safe Nucleic acid staining solution and 500 ml from distilled water.

Detection of Gene *ITS* Using PCR: Detection of ***ITS*** gene was conducted by using primers for amplification. A fragment of ***ITS*** was amplified using a forward primer (***ITS1*** F: 5′TCCGTAGGTGAACCTGCGG -3′) and a reverse primer (***ITS4*** R: 5′ TCCTCCGCTTATTGATATGC-3′) (Supplied by IDT, Integrated DNA Technologies company, Canada). The PCR amplification was performed in a total volume of 25µl containing 1.5µl DNA, 5 µl Taq PCR PreMix (Intron, Korea), 1µl of each primer (10 pmol) then distilled water was added into tube to a total volume of 25µl. The thermal cycling conditions were done as follows: Denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 45s, 52°C for 1 min and 72 °C for 1min with final incubation at 72 °C for 7 min using a thermal Cycler (Gene Amp, PCR system 9700; Applied Biosystem). The PCR products were separated by 1.5% agarose gel electrophoresis and visualized by exposure to UV light at 302 nm after staining with the red stain (Intron Korea).

Sequencing and Sequence Alignment: The PCR products were separated on a 2% agarose gel electrophoresis and visualized by exposure to UV light at 302 nm after staining with the red stain. Sequencing of gene was performed by national instrumentation center for environmental management (nicem) online at (http://nicem. Snu. ac. kr/main/? En-skin=index.html), biotechnology lab, machine is DNA sequencer 3730XL, Applied Biosystem), Homology search was conducted using Basic Local Alignment Search Tool (BLAST) program which is available at the National Center Biotechnology Information (NCBI) online at (http:// www. Ncbi .nlm .nih .gov) and BioEdit program.

Results and Discussion

Fungal isolation: The phenotypic form of *P. infestans* is a white colony ,The mycelium is undivided and the sporophyte of the sporanhia was lemon-shaped with terminal nipple and borne on branching stalks, which is a specific group of growths. and dimensions of 21-38 at 12-23 µm (Hartman and Huang, 1995).

Fungal pathogenicity results: The first symptoms of the disease appeared on the potato plant 15 days after inoculation with the fungus The infection appeared on the top or edges of the leaves in the form of unspecified dead spots and then expanded to the entire surface of the leaf and then turned black, and symptoms appeared on the upper surface of the leaves more clearly than on the lower surface, and under the appropriate conditions of low temperatures and high humidity appeared on the lower surface The leaves have white or gray fuzz, which is the sporangia and sporangia of the causative fungus. Then re-isolation from the infected leaves to prove Koch's hypotheses where it was confirmed that the cause of late blight is the fungus *P. infestans*.

Sequencing and Sequence Alignment: To confirm the morphological identification, the internal transcribed spacer (ITS) region of the Fusarium isolate was amplified with universal primers ITS1 and ITS4. The isolate was partially diagnosed after conformity with the copies of the gene bank at National Center Biotechnology Information (NCBI). The genes were gave 99% (diagnostic accuracy) match with isolation MZ675523.1 and the Query cover of the sequence was at 99% Error Value 0.0.

Table(1) Represent Type of Polymorphism of 18srRNA Gene from *Phytophthora infestans* Isolate.

Gene: 18S ribosomal RNA gene						
N	**Type of substitution**	**Location**	**Nucleotid**	**Sequence ID with compare**	**Source**	**Identities**
1	**Transvertion**	**340**	**T\A**	**ID: LS479174.1**	***Phytophthora infestans***	**99%**

Phytophthora infestans genomic DNA sequence contains 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, strain Fuz38

Sequence ID: LS479174.1Length: 914Number of Matches: 1

Range 1: 283 to 407GenBankGraphicsNext MatchPrevious Match

Score	**Expect**	**Identities**	**Gaps**	**Strand**
222 bits(245)	**3e-57**	**124/125(99%)**	**0/125(0%)**	**Plus/Plus**

Query 1
AACTAGATAACAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCAGC 60

Sbjct 283 .....**T**.. 342

Query 61
GAACTGCGATACGTAATGCGAATTGCAGAATTCAGTGAGTCATCGAAATTTTGAACGCAT 120

Sbjct 343 402

Query 121 ATTGC 125

Sbjct 403 407

Fig. 1. Sense flanking sequencing of partial ITS gene in comparison to gene standard of LS479174.1, from Gene Bank. Sample query represents; Subject represent of database of (NCBI)

	ACCESSION ID	Gene	Country	Source	Compatibility
1	MZ675523.1	ribosomal RNA gene	Iraq	Phytophthora infestans	100%
2	MW829630	ribosomal RNA gene	Nigeria	Phytophthora infestans	100%
3	LS479181	ribosomal RNA gene	China	Phytophthora infestans	100%
4	MN121470	ribosomal RNA gene	Argentina	Phytophthora infestans	100%
5	MK507866	ribosomal RNA gene	United Kingdom	Phytophthora infestans	100%
6	MF498864	ribosomal RNA gene	Pakistan	Phytophthora infestans	100%
7	KX090423	ribosomal RNA gene	Tunisia	Phytophthora infestans	100%
8	KU992300	ribosomal RNA gene	USA	Phytophthora infestans	100%

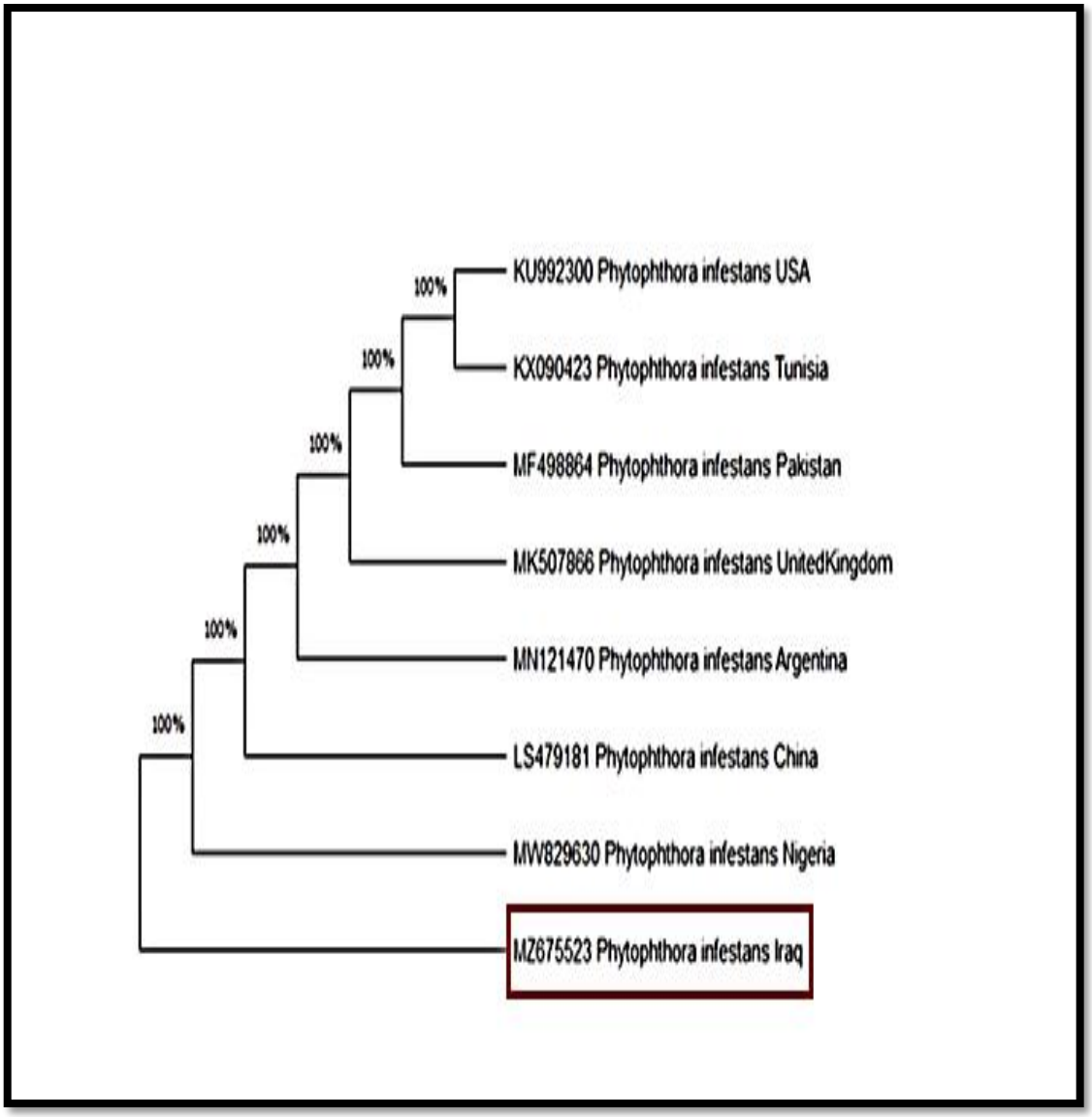

Fig. 2: Phylogenetic tree constructed by the neighbor-joining method showing the phylogenetic relationships of *P. infestans* compared with the reference sequences from gene bank.

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