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INVESTIGATION AND MOLECULAR IDENTIFICATION OF CUCUMBER DAMPING-OFF FUNGI UNDER GREENHOUSE CONDITION

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Abstract

A disease survey was done from the cucumber greenhouses in four locations (Qushtapa, Tandura, Daraban, and Choman) of Erbil province from July to October (2021) were obtained; the highest disease incidence was recorded in Daraban 57.44%, Tandura 45.76%, Qushtapa 21.88%, and the lowest disease incidence were recorded in Choman 18.3%. Different damping off fungi Isolated from soil, seeds, roots, and stem samples of cucumber plants. In all samples were isolated, *Pythium aphanidermatum*, *Globiporangium ultimum*, *Phytophthora parasitica*, *Phytophthora melonis*, *Fusarium solani*, and *Rhizoctonia solani* and identified confirmed molecularly, in all isolates *Rhizoctonia solani* is the most virulent fungus with 110 isolates, *Fusarium equiseti* 99 isolates, *Globiporangium ultimum* 90 isolates, *Fusarium solani* 88 isolates, *Pythium aphanidermatum* 63 isolates, *Phytophthora melonis* 61 isolates, *and the lowest isolates was Phytophthora parasitica* 46 isolates. *Also isolated important bio-agent Trichoderma citrinoviride*. Five isolated fungi were the first recorded in Iraq that isolated in cucumber seedlings caused damping-off, there are *Globiporangium ultimum* (Accession Numbers OP235917), *Fusarium equiseti* (Accession Numbers ON665765), *Phytophthora parasitica* (Accession Numbers ON665761), *Phytophthora melonis* (Accession Numbers ON665763), and *Trichoderma citrinoviride* (Accession Numbers ON665764).

Keywords: Cucumber, Damping-off fungi, Molecular Identification.

التحري عن الفطريات المسببة لمرض الموت المفاجئ للخيار تحت ظروف البيت البلاستيكي والتشخيص الجزيئي لها

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الخلاصة

اجريت دراسة مسحية لمرض الموت المفاجئ على الخيار بظروف البيوت البلاستيكية لأربع مواقع شملت (قوشتبة وداربن وتندورة وجومان) ضمن محافظة اربيل للفترة من تموز ولغاية تشرين الاول 2021. سجلت اعلى نسبة اصابة في منطقة داربن اذ بلغت 44.57% ثم تندورة 45.76% وقوشتبة 21.88% وأقل نسبة اصابة سجلت في منطقة جومان والتي بلغت 18.3 %. تم عزل عدة انواع من الفطريات من عينات التربة والبذور والجذور والسيقان لنباتات الخيار اذ تم عزل كل من: *Phytophthora Pythium aphanidermatum, Globiporangium ultimum, parasitica, Phytophthora melonis, Fusarium solani,* and *Rhizoctonia solani* وتم تأكيد التشخيص المظهري عن طريق التشخيص الجزيئي. أكثر الفطريات المعزولة تكرارا كانت*Rhizoctonia solani* وشملت 110 عزلة، و*Fusarium equiseti* 99 عزلة و*Globiporangium ultimum* 90 عزلة، و *Fusarium solani* 88 عزلة *Pythium aphanidermatum* 63 عزلة و*Phytophthora melonis* 61 عزلة واقل الفطريات عزلا كانت *Phytophthora parasitica* 46 عزلة، ايضا تم عزل فطر التضاد الاحيائي *Trichoderma citrinoviride*. خمسة من الفطريات المعزولة من بادرات الخيار والمشخص جزيئيا مع رقم الانضمام العالمي لكل منها في NCBI تسجل لأول مرة في العراق وهي: *Globiporangium ultimum* (Accession Numbers OP235917), *Fusarium equiseti* (Accession Numbers ON665765) ,*Phytophthora parasitica* (Accession Numbers ON665761), *Phytophthora melonis* (Accession Numbers ON665763), and *Trichoderma citrinoviride* (Accession Numbers ON665764).

كلمات مفتاحية: الخيار، فطريات مرض الموت المفاجئ، التشخيص الجزيئي.

Introduction

Cucumber has been grown in Western Asia for at least 3,000 years and was most obviously brought to other regions of Europe by the Romans (17). Cucumber is a major commercial crop for people in Iraq, and it is commonly planted in the early spring and middle of summer, depends on irrigation, and from tropical to temperate climates (19). Cucumber, like many other cucurbit cultivars, is susceptible to a variety of fungal, fungal-like organisms, viral, and bacterial diseases that cause significant economic losses. Damping-off of young seedlings caused by *Pythium* spp., *Globisporangium* spp., *Fusarium* spp., *Rhizoctonia* spp., *Phytophthora* spp. and *Phoma* spp. Damping off diseases causes both pre and post-emergence damping off. Symptoms Pre or post emergence, seedlings may be attacked, resulting in gaps and uneven stands. Young plants begin to wilt and fall off. Usually a few days before the seedling dies, water-soaked sores appear on stems at the soil surface, the results on the seeds fail to emergence and seedlings results sagging and then become dying (8 and 13). Disease development after seedlings emerges from the soil surface. The infection causes a lesion to grow on the collar region, giving the area a pinched look-like. Young seedlings develop a rot at the crown; later, the tissue becomes soft and constricted and the plants wilt and fall over. The infection point at the stem becomes hard and thin, and such symptoms are usually referred to as "wire stem" appearance at the stem's base. Infection usually takes place at the ground level or through the roots (18).

Aims of the study are Survey of cucumber damping-off disease under greenhouse conditions in Erbil province, to isolation and molecular Identification of different pathogenic fungi which cause cucumber damping-off.

Materials and Methods

Survey and sample collection: The survey approach for the evaluation of cucumber damping-off disease was performed in four locations in Erbil province in green houses: South (Qushtapa), West (Tandura), North (Daraban), and Northeast (Choman). From July to October 2021. Samples were collected randomly in each location to measure disease incidence using the following formula (16).

$$\text{Disease incidence } \% = \frac{\text{No. of infected seedlings}}{\text{Total No. of seedlings}} X\ 100$$

Table 1 Geographical distribution of cucumber seedlings under greenhouses from infected samples were collected.

Site visited in Erbil province	No. of field visited	No. of cucumber greenhouses visited	No. of sample collected
Qushtapa	4	22	80
Tandura	3	34	120
Daraban	6	65	180
Choman	1	3	35
Total	14	124	415

Sample collections: Collecting cucumber seedlings which infected by some soil-borne pathogens, placing whole seedlings in plastic bags to avoid drying out, Samples were gathered at randomly throughout each field. Each sample was Labeled with essential knowledge: (name of location, variety of cucumber, origin or company producing of cucumber seeds, date of collection, and date of planting), samples were put in a cooler box then transported to the lab of higher education and research in plant protection Dept./ college of Agricultural Engineering Sciences; Salahaddin University- Erbil. To avoid dryness, samples have been placed in the refrigerator until isolation.

Isolation of fungi: Isolation from infested soil sample: Soil samples (0.5 kg and 10 cm dept) near the diseased seedlings were collected in the cucumber greenhouses fields. Serial dilution method according to (11) was used for isolation fungi from the soil. (1 g) of soil samples was diluted for Four concentration (1/10), (1/100), (1/1000), and (1/10000), 1ml of tube (1/10000 PPM) dispersed on the potato dextrose agar PDA plates and left it open until it dry in sterile condition. Petri dishes were incubated at 25 ± 2 C° until the colony becomes observed. All isolates were further purified by hyphal tip method on PDA (12).

Isolation from infected seeds and seedlings: Fungi were isolated from collected samples of seeds, roots, and hypocotyl. Initially, seeds and seedlings were rinsed with tap water to remove soil residue. The infected seeds submerged in Ethanol 70% in petri dishes (9 cm) for 3 min. and then transferred to petri dishes contain sterile distilled water (SDW) for 3 minutes to get rid ethanol residue, samples were dried on sterile filter paper, place them onto Potato Dextrose Agar (PDA) medium each 5 pieces per petri-dishes. The root and stems was cut into small pieces about 2 mm and afterward submerged in Ethanol 70% in petri dishes (9 cm) for 3 min. and then transferred to petri dishes contain sterile distilled water (SDW) for 3 minutes to get rid ethanol residue, samples were dried on sterile filter paper, place them onto PDA medium each 5 pieces per dishes. The plates were maintained for 72 h in an incubator at 25 ± 2 C° and were examined daily. Finally, Procedures for isolation were carried out following the approach described by (7).

Identification of fungal isolations: Traditional methods: Microscopic slide was made for fungal growths to identify of isolated fungi by compound microscope at 40X according to

the classification Keyes (6 and 15). Fresh fungal growths were sub-cultured to a new plate to maintain fungal growth. Traditional method (microscopic identification) was performed by (14). To identification fungal isolated under compound microscope by detecting and measuring some distinguishing fungal parts like hyphae, spores, conidia, conidiophores, sporangium, and all other fungal units.

Molecular identifications: DNA Extraction: Genomic DNA was isolated from eight individuals pure culture fungi which detected by traditional method significant causal agents of cucumber damping off disease, samples was extracted from 20-30 mg of each specimens by using Jena Bioscience plant and fungi DNA preparation Kit (Jena Bioscience GmbH .07749 Jena Germany), According to (2).

Polymerase Chain Reaction (PCR) Amplification 5.8S Ribosomal RNA (rRNA): PCR amplification for 5.8S rRNA partial gene was done in 50 µl of reaction mixture containing; 25 µl of 2x Taq DNA Polymerase Master Mix (AMPLIQON A/S Stenhuggervej 22), 2 µl of ITS1 primer (5'- TCCGTAGGTGAACCTGCGG -3'), 2 µl ITS4 primer (5'-TCCTCCGCTTATTGATATGC-3'), 17 µl DNase free water and 4 µl DNA template by Bioresearch PTC-200 Gradient thermocycler. Temperature profile included step one is an initial denaturation at 95 C° for 5 min, step two followed by 35 cycles of a denaturation at 95 C° for 1 min, a primer annealing at 55 C° for 1 min, an extension at 72 C° for 1 min and final step is an extra extension at 72 C° for 5 min.

Sequencing of DNA: The fungi sample of PCR product 5.8S rRNA partial gene have sequenced by ABI Prism Terminator Sequencing Kit (Applied Biosystem) at macro Gene Company of Korea. Chromatograms of 5.8S rRNA were edited and base calls checked using Finch TV program software.

Sequence alignment: The 5.8S rRNA gene sequence were applied to Basic Local Alignment Search Tool (BLAST) is a searching tool that applies the sequence alignment method (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and is available at the NCBI (National Center for Biotechnology Information) website to comparing and alignment laboratory or query sequence with other biological sequence to find out more similarity with fungi species.

Results and Discussion

Cucumber damping-off disease survey: The survey of cucumber damping-off disease showed that the highest disease incidence was recorded in Daraban (57.44%), Tandura (45.76%), Qushtapa (21.88%), and also the lowest disease incidence was recorded in Choman (18.30%) figure 1.

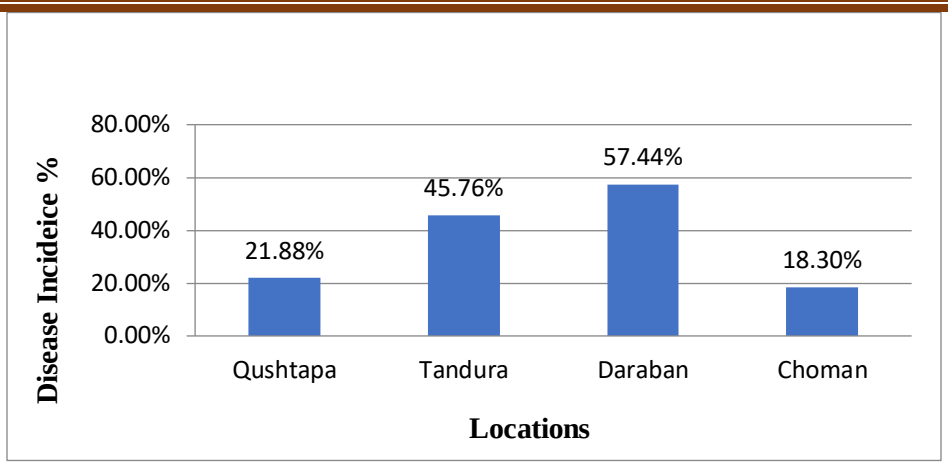

Figure 1 Disease incidence of cucumber damping-off disease in four locations in Erbil province.

Isolation and identification of cucumber damping off fungi: Traditional identification: Isolated fungi from collected samples were identified by traditional methods (Microscopicaly) according to the classification Keyes (6 and 15) showed that different fungi were isolated in the four locations as shown in table 2. And the percentage of isolated fungi showed in table 3. Then compared isolated damping off fungi in all locations table 4.

Table 2 Isolated fungi from different locations in Erbil province.

Fungal isolates	Qushtapa	Tandura	Daraban	Choman
Globisporangium ultimum	10	6	68	6
Pythium aphanidermatum	13	4	39	7
Fusarium solani	29	37	7	15
Fusarium equseti	16	19	4	60
Phytophthora parasitica	20	26	0	0
Phytophthora melonis	14	32	15	0
Rhizoctonia solani	58	35	13	4
***Phoma* spp.**	7	12	5	0
Trichoderma citrinoviride	3	2	0	2
***Trichoderma* spp.**	1	0	3	0
***Aspergillus* spp.**	13	23	23	5
***Pencillium* spp.**	4	14	8	0
***Rhizopus* spp.**	6	7	10	5
***Alternaria* spp.**	2	3	0	0
Total isolates	196	220	195	104

Table 3 Isolated percentage of fungi from soil, seed, root, and stem of cucumber.

Fungal isolates	Qushtapa	Tandura	Daraban	Choman
Globisporangium ultimum	5.1%	2.73%	34.87%	5.77%
Pythium aphanidermatum	6.63%	1.8%	20%	6.73%
Fusarium solani	14.795%	16.8%	3.59%	14.42%
Fusarium equseti	8.163%	8.63%	2.05%	57.7%
Phytophthora parasitica	10.2%	11.81%	0%	0%
Phytophthora melonis	7.14%	14.54%	7.69%	0%
Rhizoctonia solani	29.59%	15.9%	6.66%	3.84%
Trichoderma citrinoviride	1.53%	0.91%	0%	1.92%

Table 4 Isolated damping off fungi in all locations.

Isolated fungi	No. of isolate
Globisporangium ultimum	90
Pythium aphanidermatum	63
Fusarium solani	88
Fusarium equseti	99
Phytophthora parasitica	46
Phytophthora melonis	61
Rhizoctonia solani	110
Trichoderma citrinoviride	7

(4) surveyed for damping off diseases in Sulaimania-Kurdistan of cucumber greenhouses from Feb-May 2014, collected samples from 200 cucumber greenhouses from 21 locations, 12 different fungal and bacterial were isolated those are *Rizoctonia solani*, *Fusarium solani* and *Pythium aphanidermatum*, and also recorded the highest disease incidence 23.7%, the overall mean incidence of damping-off disease reached to 6.82%.

In Najaf-Iraq (3) isolate and identify three isolates of the pathogenic fungus *Pythium aphanidermatum*, which causes cucumber seedling damping-off. (9) was isolated *F. equseti* on tomato in Penjwen- Sulaimania-Iraq. (1) isolated *R. solani* in Al-Qadisyah – Iraq in the cucumber greenhouses. (5) studied of *R. solani* caused cucumber damping off in Basrah – Iraq. In 2016 (10) was studied on tomato damping off in Sulaimania – Iraq caused by *R. solani* and *F. solani.* The results showed that these fungi (*Globisporangium ultimum, Fusarium equseti, Phytophthora parasitica, Phytophthora meloni, and Trichoderma citroviride*) was isolated and molecular identification in Iraq on cucumber seedlings for the first time figure 2 and 3.

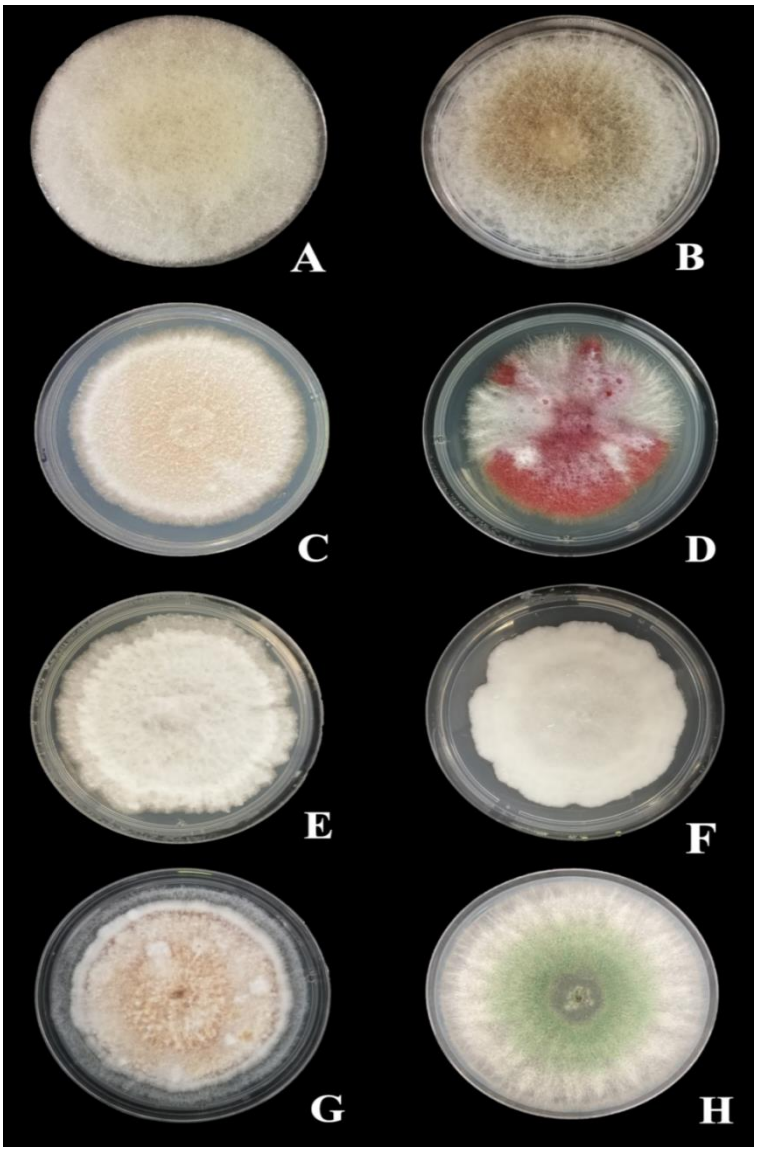

Figure 2 colony view on PDA of fungal isolates; (A) *Globisporangium ultimum*, (B) *Pythium aphanidermatum,* (C) *Fusarium solani* (D) *Fusarium equseti,* (E) *Phytophthora parasitica*, (F) *Phytophthora melonis,* (G) *Rhizoctonia solani,* and (H) *Trichoderma citroviride.*

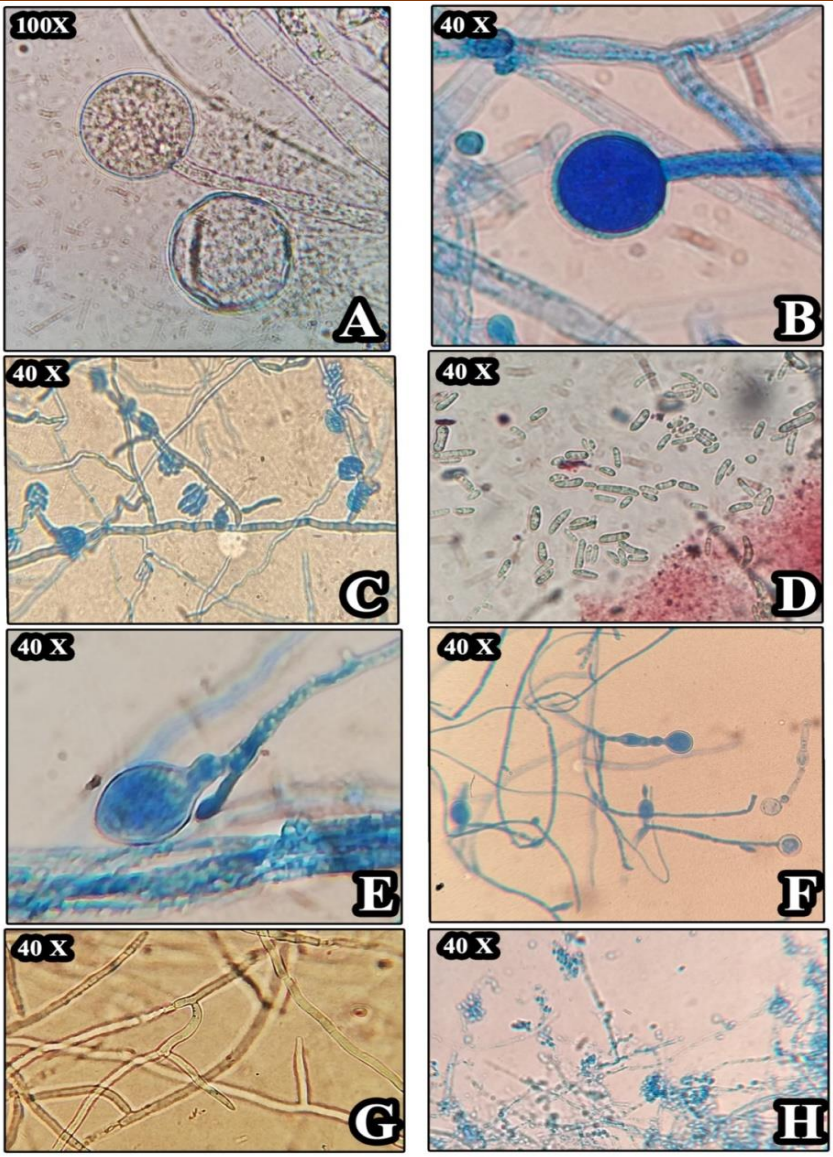

Figure 3 Microscopy slide of fungal isolates; (A) *Globisporangium ultimum*, (B) *Pythium aphanidermatum*, (C) *Fusarium solani* (D) *Fusarium equseti*, (E) *Phytophthora parasitica*, (F) *Phytophthora melonis*, (G) *Rhizoctonia solani*, and (H) *Trichoderma citroviride*.

Molecular Identification: PCR amplification of partial COI gene: 5.8S gene specific primers were designed for using the sequences of ribosomal RNA, available in target fungus species. Synthesized by Micro-gene Company (South Korea), the primers could yield a band of the expected size of 600bp. The PCR product was electrophoresed and visualized by 1.5% Agarose gel. The primers were found to produce ~up to 600 bp band as shown in the figure 4.

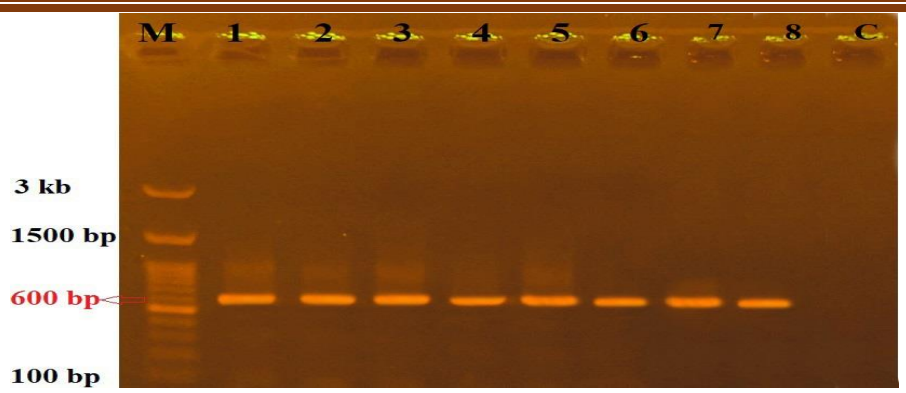

Figure 4 PCR amplification of partial 5.8S rRNA gene from fungi samples first lane is Ladder (3k bp-100 bp) lane numbers 1-8 is band of amplified gene.

Molecular Identification of damping off Fungi: The eight 5.8 partial rRNA gene sequence samples with size 600 bp are alimented by BLAST program from Gen bank (*http://blast.ncbi.nlm.nih.gov/*) was used to compare our amplified sequences with other stored species of fungus sequences. The results got from the BLAST indicated that the highest identity number query sequence was 100% identity is eight different fungi. This aliment indicates to submission our query sequences inside of NCBI Gen bank and given accession numbers include as shown in table 5:

Table 5 The accession numbers of eight different fungi.

samples	Fungal Identified	Accession Numbers	Query Cover %	Identic Number %	Accession Number of BLAST Identification
1	***Globisporangium (Pythium) ultimum***	OP235917	100 100	100 100	MT177229 MN922782
2	***Pythium aphanidermatum***	OP234320	100 100	100 100	MT598009 KJ162355
3	***Fusarium solani***	ON665766	100	100	MF370942 MK881752
4	***Fusarium equiseti***	ON665765	100	100	MZ960458 LS479416
5	***Phytophthora parasitica***	ON665761	100	100	MN121487 MH537599
6	***Phytophthora melonis***	ON665763	100	100	MG865536 KX965714
7	***Rhizoctonia solani***	ON665762	100	100	MT530327 KJ545578
8	***Trichoderma citrinoviride***	ON665764	100	100	MN547406 MG972800

Phylogenetic inferences: Phylogenetic analysis based on 16S rRNA nucleotide sequence revealed grouping of 15 investigated species of fungi on expected lines. From sequence divergence similarity data and phylogeny constructed, it was revealed that species belonging to respective genera were close to each shown in figure 5:

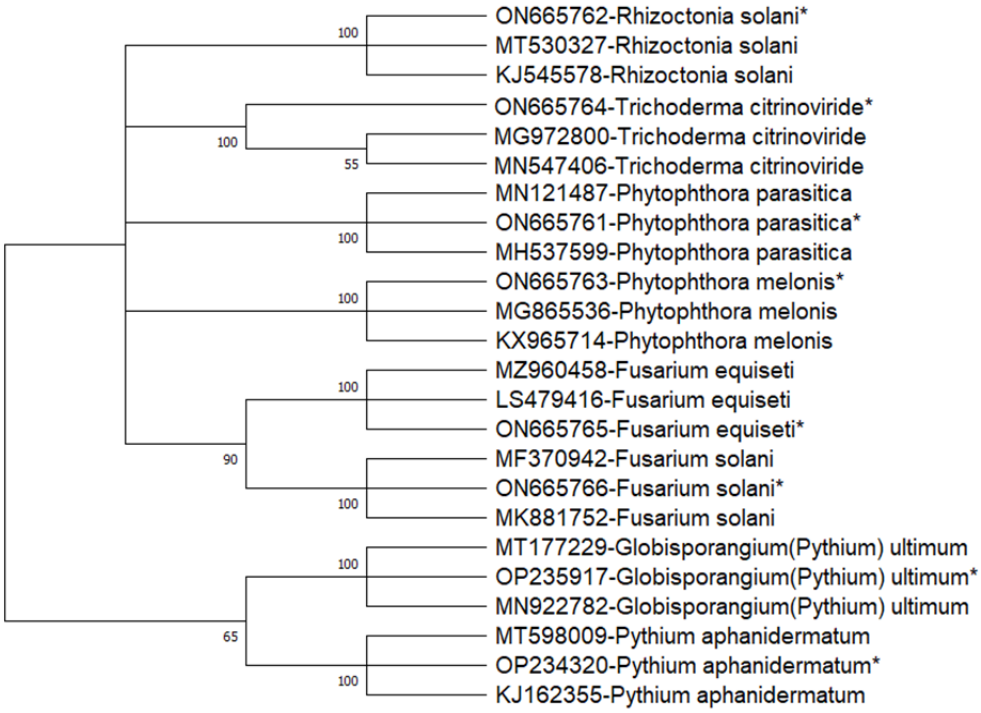

Figure 5 Phylogenic tree of Fungi sp. samples from Iraq: Kurdistan region. The phylogenic tree was constructed using the Maximum Likelihood method based on the Tamura-Nei model in MEGA11 software and bootstrap analysis with 100 re-samplings. Partial DNA sequences of concatenated partial 5.8 ribosomal rRNA genes were used as input data.

Conclusion: In Kurdistan region -Iraq cucumber was planted in a significant amount and it's the highest cultivation crops more than 70% of greenhouses were planted with cucumbers were cultivated in a two seasons (spring and summer), cucumber like any crops is susceptible to cause numerous diseases and the most important disease is damping off disease which cause a huge losses every year.

In this study the results showed that in all isolates *R. solani* is the most virulent fungus with 110 isolates, and the lowest isolates was *Phytophthora parasitica* 46 isolates. Five fungal isolates were the first record and had not recorded yet on cucumber in Iraq, and these isolates were *Globisporangium ultimum, Fusarium equseti, Phytophthora parasitica, Phytophthora meloni, and Trichoderma citrinoviride*.

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