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A new species of *Pythium hydnosporum* as a pathogen associated with vine decline of melon (*Cucumis melo* L.) in Iraq

Rebwar A. Mustafa

Bakrajo Technical Institute, Sulaimani Polytechnic University, Sulaimani, Iraq

*Email: Rebwar.mustafa@spu.edu.iq

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Abstract

Several pathogenic fungi have become an important vine decline of cantaloupe melone (*Cucumis melo* L.). In 2021, root rot on cantaloupe was observed during the harvest phase in the fields in Penjwen and Shahrazoor in Sulaimani governorate of Northern Kurdistan region Iraq. This present study was conducted to isolate the causal agent of fungi in lesions of root rot of cantaloupe. A total of 16 fungal isolates were obtained of which isolates *Monosporascus cannonballus, M. eutypoides, Acremonium vitellinium, Fusarium oxysporium, F. equiseti, Macrophomina phaseolina, Saccharomyces kudriavzevii, F. robinianum, Rhizopus arrhizus, Botrytis cinerea, Cytospora eucalypticola, F. falciforme, Pythium hydnosporum, Alternaria tenuissima, Rhizoctonia solani* and *Phytophthora colocasiae* were obtained from root melon. Identification of all fungal isolates was based on using both morphological characteristics and molecular analysis, on internal transcribed spacer (ITS1, ITS4, LSU) are primers that are used for identification. DNA sequences of the fungal pathogen were identified as *Pythium hydnosporum* a new pathogenic fungi causal agent of cantaloupe vine decline. A pathogenicity test was conducted to verify Koch's postulates and *P. hydnosporum* was observed to cause root rot of melon, symptoms of the disease were similar to those seen in the field.

Keywords

Cantaloupe; deterioration; fungi; melon root rot; *Pythium hydnosporum*

Introduction

The melon fruit known as the cantaloupe (*Cucumis melo* L.) is a member of the Cucurbitaceae family. Grey-green skin with white streaks netted orange flesh. Melon has recently become quite popular in Iraq, especially in the Kurdistan area. According to the Department of Agricultural Planning, Ministry of Agriculture and Water Resources, cantaloupe cultivation has expanded in Iraq by around 2019 (1). One place where there is a significant temperature variation between day and night is Penjwen, where the high humidity levels result in the spoilage of cantaloupe fruit. However, at all phases of cantaloupe development, similar circumstances may also favor the germination of fungal infections. Fruits are a well-known essential dietary item that is both nutritionally and economically significant. Fruits are fundamental to human nutrition because they provide the key vitamins and minerals that people need each day to develop and maintain normal health. All cantaloupe development phases are in danger of infection because fruits are

extensively spread in nature. For example, the *Didymella bryoniae*-caused sticky stem scourge is a critical foliar sickness of muskmelon at the seedling stage (2). Because of restricted stockpiling and transportation foundations, postharvest misfortunes are many times more extreme in immature countries. Fruits might get contaminated with fungi out of the blue, including during the developing season, collection, taking care of, travel, post-reap capacity and showcasing conditions or after a client has bought them. Fruits are particularly interesting to growth-made deterioration due to their high sugar wholesome substance and low pH values (3). Fungi that spoil are much of the time considered poisonous or unsafe. Fruits not doing so great have been displayed to contain poisonous parasites (4). A few molds might make mycotoxins when refrigerated. Then again, pathogenic parasites could bring about sensitivities or diseases (5). Because of the way that *Aspergillus* spp. are known to deliver various harmful metabolites, including malformins and naphthopyrones as well as the capacity to create ochratoxin (OTA), a mycotoxin that is a vital poison universally because of the gamble it postures to human and creature wellbeing (6). However, there haven't been many explorations on melon postharvest fruit rot in Iraq. Soil survival of *Pythium hydnosporum* sporangia has been reported to persist for fewer than 21 days in naturally infested soil. Oospores as the survival structure of homothallic *Pythium* species in soil are well established. However, oospores of heterothallic species in naturally infested soil are rare.

The maintenance of seed health constitutes a crucial element in disease control, as seeds afflicted with infections exhibit diminished viability, reduced germination rates, diminished vigor and lower overall yield (7, 8). The vitality of seeds devoid of pathogens is paramount for achieving the desired plant population and ensuring a bountiful harvest. The health of seeds can be compromised either through direct infection by pathogens or via contamination by pathogenic entities. Such contamination may occur within, on, or in association with the seeds, presenting itself as concomitant contamination (9). The infection of seeds by pathogenic organisms and the presence of pathogenic propagules within a seed lot assume critical significance, as the germination failure of infected seeds or seed lots can lead to the transmission of infections to seedlings and established plants. Consequently, the preservation of seed health emerges as an indispensable factor for the triumph of successful crop production.

Materials and Methods

Sample collection

Disease surveys were conducted during May, June and August of the years 2020 to 2022 from 198 different melon farms' fields in various areas of Iraqi Kurdistan regions (IKR) to report the incidence and distribution of vine decline melon disease at various melon production area and different weather conditions. The survey was conducted in 198 Cantaloupe fields distributed within 44 collection sites belonging to 11 districts belonging to Sulaymaniyah, Garmian, Halabja and Erbil provinces which vary in altitude and climate. The presence of disease incidence as shown in Fig.1 was represented via percentage by randomly choosing 5 cantaloupe plants from each field using the crossing diameter method.

Fig. 1. Symptoms of cantaloupe root.

Molecular identification of Pythium hydnosporum isolates

DNA extraction of *Pythium hydnosporum* was extracted from fungal mycelium according to Favorgen Biotech Company of Taiwan. The concentration of DNA was measured by nano-drop spectrophotometer and adjusted to 18-53 ng/uL PCR Assays, PCR amplification was performed using a Taq DNA Polymerase kit (Favorgen Biotech Company/Taiwan) and 3 types of primers and the ITS region was amplified primer general for all fungi (10). ITS1/ITS4 Forward (50-TCC GTA GGT GAA CCT GCG30) and Reverse (50-TCC TCC GCT TAT TGA TAT GC-30). And Lociprimer pair forward/ reverse, LSU 5' -ACC CGC TGA ACT TAA GC-3' LR5 5'- CGC CAG TTC TGC TTA CC-3& 39; Vilgalys and Hester (11). Fornon-sporulation fungi, Glass and Donaldson (12).

PCR reaction, mixture consisted of 50 ng/µL DNA, 2 µL forward primer 2 µL reverse primer, 18 µL di ionic distilled water and 25 µL master mix in small tubes. The mixture containing the general primer was. The PCR mixture containing the general primers ITS1/ITS4 was denatured at 95 °C for 40 seconds (40 cycles), annealed at 55 °C for 50 seconds, extended at 72 °C for one min and final extension at 72 °C for 8 min (1 cycle). The PCR products were analyzed by electrophoresis on 1.5 % agarose gel at 80 V for 45 min and then visualized by staining the gel in ethidium bromide solution and photographed under a UV transilluminator. The DNA fragment was extracted and submitted to sequencing by ABI Prism Terminator Sequencing Kit (Applied Biosystem) at Macrogen Molecular Company of Korea.

Pathogenicity testing

Greenhouse incubation was done at 32 ± 2 °C for 62 days followed by inoculation of muskmelon seedlings (Taj genotype) with *Pythium hydnosporum* isolates. Specific primers were employed for re-isolation and pathogen identification (13).

Fungal identification

Morphological study

As indicated by strategies created by Wang et al. (14), Crous et al. (15) and Wang et al. (16), the morphological highlights of parasitic segregates were recognized. Following a multi-week of hatching in obscurity at 25 ± 2 °C, settlement elements, for example, state structure, pigmentation and smell were seen on PDA, Oatmeal agar (OA; Difco, Le Pont de Claix, France) (17).

DNA extraction and PCR amplification and sequencing

The Favorgen Biotech Corp. of Taiwan's Fungi/Yeast Genomic DNA Extraction Small Unit was utilized to disengage genomic DNA from tests of fungi.

Polymerase chain reaction (PCR) amplification 5.8S and 28S ribosomal RNA (rRNA)

50 l of response blend, which included 25 l of 2x Taq DNA Polymerase Expert Blend (AMPLIQON A/S Stenhuggervej 22), 2 l of forward groundwork, 2 l of opposite preliminary, 17 l of sans dense water and 4 l of DNA format, were utilized for the PCR intensification of rRNA incomplete qualities by a Bioresearch PTC-200 Inclination thermocycler. Stage one of the temperature profile comprises an underlying denaturation at 95 °C for 5 min, trailed by 35 patterns of a preliminary strengthening at various temperatures as per the introductions for the qualities recorded in Table 1 for 60 seconds, an expansion at 72 °C for 1 min and a last additional expansion at 72 °C for 10 min.

Sequence alignment and phylogenetic analyses

Table 2 records the arrangements from this examination, as well as those recovered before research from the GenBank data set (with 60 % question inclusion and 85-100 % grouping likeness). MUSCLE (18) was utilized to do different grouping arrangements and BioEdit v. 6.0.7 (19) was utilized to make any expected upgrades. Utilizing a blend of the tef-1, cam and rpb2 datasets, a phylogenetic examination was performed. The outgroup included *Fusarium camptoceras* CBS 193.65 and *F. neosemitectum* CBS 115476 from the *F.camptoceras* species complex (FCAMSC). Utilizing the greatest probability (ML) and Bayesian derivation (BI) methods, a phylogenetic tree was made. The GTRCAT model with 25 classifications and 1000 bootstraps (BS) replications (19, 20) was exposed to ML investigation utilizing RAxML v7.0.3 utilizing the web-based stage CIPRES Science Door v. 3.3. (21) BI examination was done utilizing the Windows (22) program MrBayes v3.2.6. The Akaike data measure was utilized utilizing jModel Test 2.1.10 (23) to anticipate the ideal substitution models for BI and ML investigation (AIC). The GTR + I + G model filled in as the establishment for the ML and BI evaluations. Over the BI investigation, six simultaneous Markov chains with irregular starting trees were led for 1000000 ages, with tests taken each 1000 ages. The initial 2000 trees were wiped out involving a consume-in stage and the excess trees were then used to construct the half-democratic agreement phylogram with assessed Bayesian back likelihood (PP). In Fig Tree v1.4, the tree geographies were shown (24).

Table 1. Information about the study's acquired PCR product and primers.

Primers				Annealing Temperature °C	Fragment Size (bp)
Forward		Forward			
ITS1	TCCGTAGGTGAACCTGCGG	ITS4	TCCTCCGCTTATTGATATGC	55	650
LSU-F	ACCCGCTGAACTTAAGC	LSU-R	CGCCAGTTCTGCTTACC	58	1200

Stage one of the temperature profile comprises an underlying denaturation at 95 °C for 5 min, stage 2 is 40 patterns of denaturation at 95 °C for 40 seconds, stage 3 is an expansion at 72 °C for 1 moment and stage 4 is a second augmentation at 72 °C for 8 min.

Sequencing

Perception of DNA Sections: Following 30 min in an electric field of electrophoresis, 1.5 % dissolved agarose gel is added to 1X TBA support. Band areas are then assessed by examining the gel under a UV trans-illuminator.

DNA Sequencing: At the Microgene Center in Korea, tests of PCR items with fragmented qualities were sequenced utilizing the ABI Crystal Eliminator Sequencing Unit (Applied Biosystem). Utilizing the Finch television program, chromatograms of nucleotide qualities were altered and base calls were checked.

Succession Arrangement and Accommodation: The quality groupings were applied to Essential Nearby Arrangement Search Device (Impact), a web search tool that utilizes the succession arrangement technique (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to look at and adjust research facility or question arrangement with other natural successions to find greater likeness with different targets.

Table 2. Reagents for PCR amplification.

No.	PCR Components	Concentration	Volume (µL)
1	Master Mix	2x	25
2	Forward Primer	10 Pmol	2
3	Reverse Primer	10 Pmol	2
4	DNase Free Water	-	18
5	Template DNA	50 ng/µL	3
		Total	50

Statistical analysis

The occurrence of disease in the control and microbe vaccination medicines was looked at utilizing one-way examination of fluctuation (ANOVA) utilizing Collaborator V.7.6 beta (25) and contrasts were resolved utilizing the Understudy's t-test. A p-esteem of .05 was used to decide importance.

$$DSI\ (\%) = \sum (\text{Score Amount of Plants}) \text{ Maximum score} \times \text{Total number of plants} \times 100.$$

Results

The isolated *Pythium hydnosporum* was achieved by studying injured melon roots cultivated in the areas of Iraqi Kurdistan. The physical features of the isolates were used for identification and this was confirmed by amplifying 650 bp and 1200 bp of the ITS and LSU sections respectively, using specified primers (Fig. 2). Soil-borne fungal pathogens such as *Monosporascus* species, *Acremonium vetillinum*, *Fusarium oxysporum*, *Rhizoctonia solani*, *F. falciforme*, *F. equiseti*, *Phytophthora colocasiae* and *Macrophomina phaseolina*, were all isolated as contributors to the vine decline complex. Most *Phytophthora* sp. have caused vine decline of melon, however, this species of *Pythium hydnosporum* is the first species reported. *P. hydnosporum* produces microscopic, asexual spores called sporangia. These sporangia are oval, hyaline, semi-papillate (tip of spore is not pointed), deciduous (spores fall from the colony) and have a short stalk or pedicel attached to the base of the spore. These sporangia release swimming spores called zoospores when water or sufficient moisture is present. The sporangia can also germinate directly by producing germ tubes that penetrate the host. Zoospores can swim for hours and are attracted to organic matter or host tissue. These spores will stop swimming, encyst (the tails or flagella are lost) and produce a germ tube to penetrate the host (26).

Sequencing and phylogenetic analyses

In table 3 was shown that the partial 5.8S rRNA (ITS) gene amplification by polymerase chain reaction (PCR) in fungal samples is shown in Fig. 3. Lanes 1–28 show the PCR result from the fungus; M is the DNA ladder (3K bp–100 bp) and C is the negative control. Isolated fungus samples (one per lane) are labeled with numbers from 1 to 30, representing 30 different fungal species. Bands of the correct sizes, as shown in Fig. 4, were successfully produced using the primers tested.

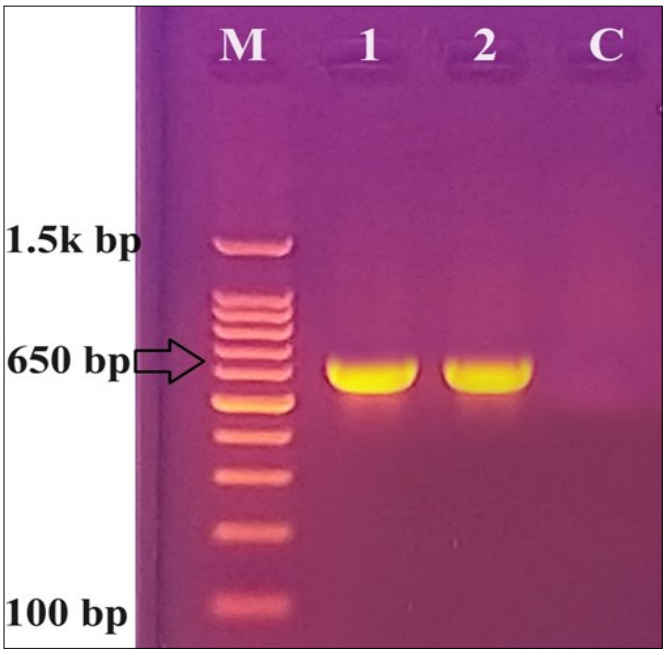

Fig. 2. Amplified 650 bp and 1200 bp of the ITS and LSU Sections Using Specified Primers.

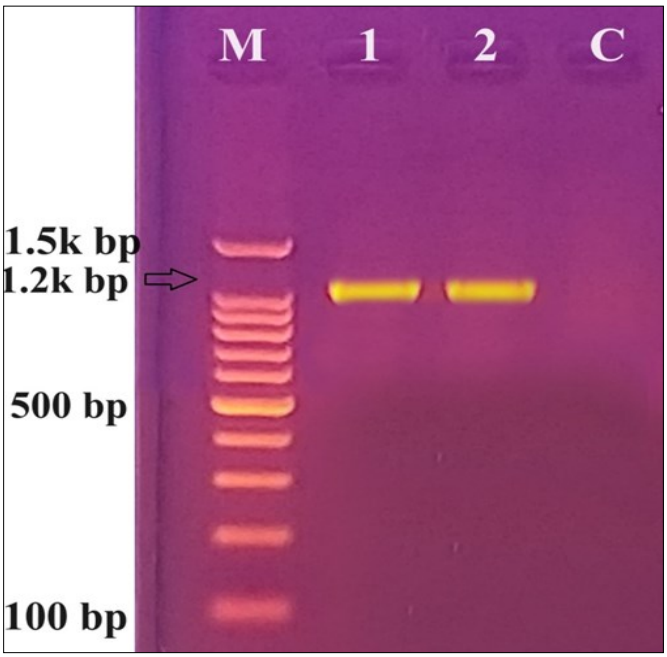

Fig. 3. Partial 5.8S rRNA (ITS) Gene Amplification by Polymerase Chain Reaction (PCR) in Fungal Samples.

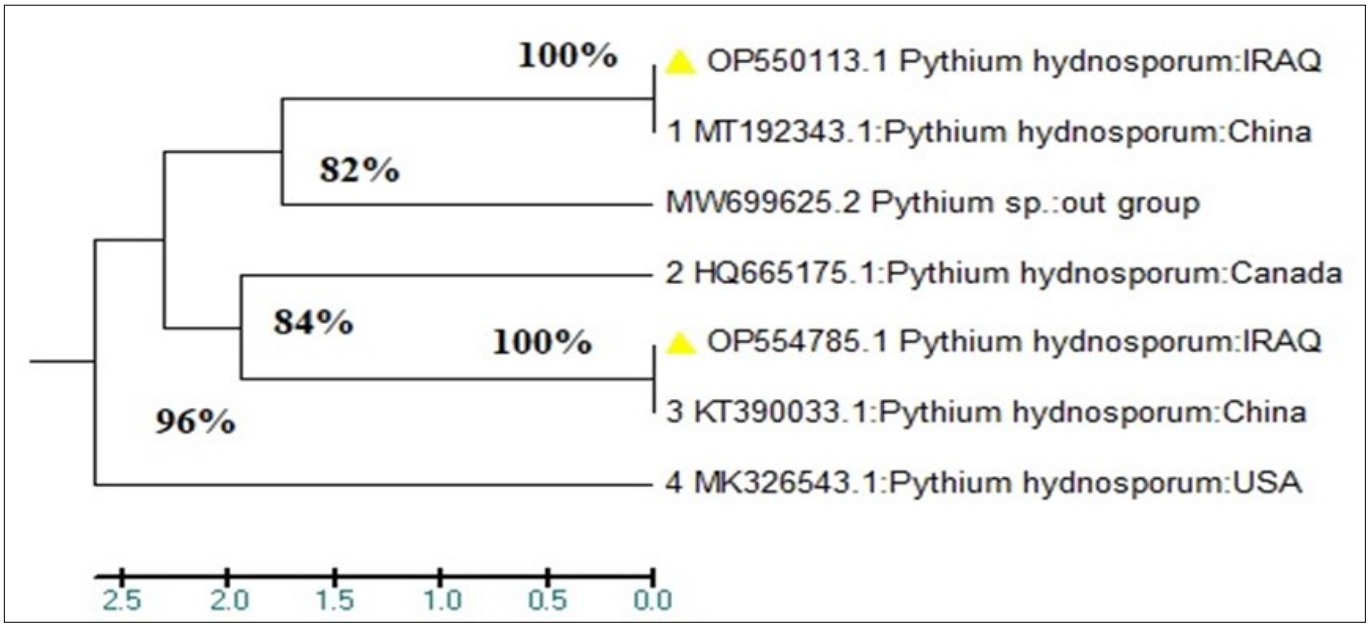

Fig. 4. Evolutionary Relationships of Taxa.

The evolutionary history was inferred through the implementation of the UPGMA method (26). The resultant optimal tree, characterized by a sum of branch length equal to 0.67423313, is presented herein. Alongside the branches, the percentages denoting the frequency with which the associated taxa clustered together in the bootstrap test (conducted with 500 replicates) are provided (20).

Table 3. *Pythium hydnosporum* phylogenetic tree based on the ITS and LSU-rDNA sequences.

No.	Isolate Species	GenBank acc. No.	Strain No.	Host (Source)	Geographic Location	Similarity
1.	*Pythium hydnosporum*	OP550113	Re-28	Melon root	Iraq	100 %
2..	*Pythium hydnosporum*	MT192343	JZB3410002	Strawberry root	China	100 %
3..	*Pythium hydnosporum*	MW699625	LT4	Nematodes	Bosnia and Herzegovina	Out group
4.	*Pythium hydnosporum*	HQ665175	CBS 253.60		Canada	91.71 %
5.	*Pythium hydnosporum*	OP554785	R-28	Melon root	Iraq	96.20 %
6.	*Pythium hydnosporum*	KT390033	ZX14-3-61	South China Sea	China	96.20 %
7.	*Pythium hydnosporum*	MK326543	C-MICO2_5-13	Corn	USA	100 %

Discussion

This is the species' first report from Iraq. Furthermore, the species that was isolated from melon roots for the first time in history displayed vine decline and root rot. *Pythium hydnosporum* is a soil-borne fungal pathogen that can cause a variety of diseases, such as stem and root rot, charcoal rot and seedling blight. This study used genetic analysis to characterize a set of isolates to gain a better understanding of the genetic diversity and geographic distribution of *Pythium hydnosporum*. Seven *P. hydnosporum* isolates that were obtained from different hosts and locations are shown in the table below. The isolates were traced back to their unique hosts, environments and geographic regions using their GenBank accession codes and strain numbers. With results ranging from 91.71 to 100 %, the isolates' genetic similarity was quite high. Genetically similar to *P. hydnosporum* OP554785, another isolate of melon roots, *P. hydnosporum* OP550113 was isolated from Iraqi melon roots. *P. hydnosporum* appears to be a common pathogen in Iraq that affects melon roots. Since it was found in strawberry roots in China, *P. hydnosporum* MT192343, another isolate, has been proposed as a pathogen of strawberry plants.

It was found that 11 distinct species of *Pythium* associated with maize in agricultural settings after comparing their findings with those of other researchers (27). For *Pythium strains*, summarized the general pathogenic characteristics and management strategies (28). The research findings indicate that a high count of hyphae that are germinating may facilitate *Pythium* infection of the roots during the seedling stage of the host plant. *Pythium* mostly comes from infectious sources called microsclerotia. Moreover, a developed qPCR assay may be used to detect and quantify *Pythium* levels in rhizosphere soil and plant tissues in real-time (29). Based on this data, *P. hydnosporum* is a globally widespread, highly adaptable fungus that can colonize a wide variety of hosts and ecosystems, including plants.

Conclusion

Melon infections causing root rot and vine decline were discovered in nearly every investigated location throughout three provinces in the Iraqi Kurdistan region: Erbil, Sulaymaniya and Hallabja. *Monosporascus cannonballus, M. eutypoides, Machrophomina phaseolina, Fusarium oxysporum, F. falciforme, F. robinianum, Acremonium potronii, A. vitellinum, Phytophtora colocasiae, Pythium hydnosporum, Trichoderma effusum, T. viridi, Alternaria tenuissima, Cytospora eucalypticola* and *Botrytis cinerea* were isolated and identified from melon roots showing vine decline and root rot disease, whereas, *Fusarium equiseti* and *Rhizoctonia solani* were detected from rhizosphere soil of melon roots. ITS and LSU primers for the isolated fungal species were used in molecular methods and phylogenetic analysis to verify the morphological identification. *Pythium hydnosporum* was reported in Iraq as a potential complex agent that may contribute to the decline of melon vines and root rot disease.

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Compliance with ethical standards

Conflict of interest: Author do not have any conflict of interests to declare.

Ethical issues: None.

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