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APPLIED AND NATURAL SCIENCE FOUNDATION

Journal of Applied and Natural Science
17(3), 1325 - 1332 (2025)
ISSN : 0974-9411 (Print), 2231-5209 (Online)
journals.ansfoundation.org

Research Article

Characterization of newly recorded *Pythium mamillatum* AJA, causing cucumber root rot in Iraq: Implications for disease management in sustainable agriculture

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Article Info
https://doi.org/10.31018/jans.v17i3.6796
Received: May 03, 2025
Revised: August 25, 2025
Accepted: September 05, 2025

How to Cite
Al-Janabi, J. K. A. *et al.* (2025). Characterization of newly recorded *Pythium mamillatum* AJA, causing cucumber root rot in Iraq: Implications for disease management in sustainable agriculture. *Journal of Applied and Natural Science*, 17(3), 1325 - 1332. https://doi.org/10.31018/jans.v17i3.6796

Abstract
Pythium mamillatum is an emerging pathogen responsible for root rot and wilt diseases in cucumber (*Cucumis sativus*), resulting in significant crop losses in Iraq. The present study examined the occurrence, distribution, pathogenicity, and genetic characterization of *P. mamillatum* strain AJA in Iraq, with broader implications for sustainable agricultural practices. Field surveys were conducted to collect symptomatic cucumber root samples from Al-Tahiria, Al-Azizia, and Al-Badah districts revealed disease incidences of 37%, 35%, and 21%, respectively. Infected plants exhibited symptoms of root rot and wilting. According to molecular research, the Iraqi strain, *Pythium mamillatum* AJA, accession No. MN460315.1 was 100% genetically related to strains from China, the USA, and Canada, indicating a shared evolutionary origin. Through inhibiting seed germination from 10% on the third day to 90% on the seventh day of inoculation, *P. mamillatum* (strain AJA) demonstrated significant losses to local Iraqi cucumber crops. The rapid spread of agriculture, its valuable genetic similarity with strains established in other regions worldwide, and its pathogenic viewpoint highlight its increasing threat. The study emphasizes the importance of employing effective disease control techniques within the framework of sustainable agriculture. Reducing the effect of this infection mostly depends on early identification, seed treatments, and constant pathogen monitoring. Maintaining crop health and guaranteeing long-term agricultural resilience also depend on sustainable farming methods that reduce the use of chemical pesticides and advance integrated pest management (IPM).

Keywords: Cucumber, Molecular characterization, Pathogenicity, *Pythium mamillatum*, Root rot

INTRODUCTION

The genus *Pythium* comprises more than 150 species, known as water molds, with broad host ranges that affect both monocotyledonous and dicotyledonous hosts (Abd-Elsalam, 2020). While *Pythium* species have long been recognized to pose severe threats to dicots, such as cucumbers (*Cucumis sativus*), new publications reveal an increasing impact on monocots, including wheat and maize, thereby expanding their ecological and economic importance (Spies *et al*., 2023). It is well-known that *Pythium* species can adapt to a range of hosts, which raises questions about existing methods of disease control.

The adaptive nature of *Pythium* spp. across various agroecosystems is highlighted by current epidemiological research. With over 86% of isolates in a national investigation done in Oman in the year 2024, *P. aphanidermatum* was found as the main pathogen causing damping-off in cucumbers (Al-Kharousi et al., 2024). Consequently, *P. spinosum* was found to be a singular causal agent of crown and root rot infections in Italian greenhouse cucumbers, thereby causing death to all seedlings in pathogenicity tests (Santilli *et al*., 2023). Different places suffer from root rot disease that results from a number of *Pythium* species; these pathogens have been found in soil samples and diseased plants (Punja *et al*., 2022). Cucumbers have this illness. Most typically occurring species include *P. aphanidermatum*, *P. deliense*, and *P. spinosum* (Toporek and Keinath, 2020). According to Punja *et al*. (2022), such diseases reduce seed germination and cause damping-off both before and after emergence, therefore resulting in major crop losses. Under greenhouse conditions, poor drainage, great soil moisture, and high humidity all aggravate the degree of disease (Sanoubar and Barbanti, 2017). Researchers discovered these species by means of pathogenicity, molecular, and morphological studies (Punja *et al*., 2022). The purpose of the presented study is to investigate the distribution, frequency and pathogenic features of the causal agent of root rot disease in cucumber plants. Through molecular, pathogenicity, and morphological studies, the present study endeavoured to isolate and precisely identify the fungal strain from cucumber farms in the Babylon countryside (Iraq) to evaluate its virulence, and probe environmental elements controlling its spread.

MATERIALS AND METHODS

Study area

Between February and April 2023, the presented work was carried out in greenhouse farms situated in the Al-Badah, Al-Azizia, and Al-Tahiria areas of Babylon Governorate, Iraq's Al-Musayyib district. These areas are well-known for their heavy vegetable cultivation in protected environments, so they are prone to several soil-borne diseases. The survey concentrated on plants showing symptoms such as wilting, root rot, and yellowing, which are usually linked to diseases brought on by *Fusarium spp*., *Pythium* spp., and *Rhizoctonia solani* (Hussein *et al*., 2024). Further research was carried out to ascertain the frequency and spread of the disease among different farms, therefore offering basic data needed to create effective management plans.

Sample collection

To collect the samples of root rot from infected cucumber plants, the root systems were gently removed and cleansed under tap water to remove soil particles and limit harm to impacted tissues (Paul *et al*., 2021). Root samples were collected and forwarded to the Advanced Mycology Laboratory, Department of Biology, College of Science, University of Babylon, Iraq, for isolation and identification to ascertain the causal agent of the disease.

Isolation of *Pythium* species

From an infected cucumber, five-millimetre pieces of root rot tissue were carefully washed in distilled antiseptic water and then dried under filter paper. Following a minute of soaking in 1% sodium hypochlorite, the segments were surface-sterilized three times in sterile distilled water to eliminate impurities (Hussein *et al*., 2022). The segments were placed on a PDA enriched with antibiotics to minimize bacterial contamination after decontamination (Senda *et al*., 2009). Van West *et al*. (2003) claim that *Pythium* species generate aseptate, coenocytic mycelium in Petri plates incubated in the dark at 22–25°C over a period of three to five days.

Identification of the causal agent

The microscopic, morphological, and molecular features of the causative agent of wilting and root rot disease were used to identify it. Usually fluffy and white, colonies have spherical or subspherical sporangia. In afflicted plants, the pathogen was associated with root rot, reduced growth, and leaf yellowing.

Microscopically, *P. mamillatum* produces non-septate, filamentous hyphae and globose sporangia. It thrives in cool, wet environments and poorly drained soils, conditions that promote the spread of infection and disease. Morphological identification was performed by examining colony structure, hyphal characteristics, sporangial formation, and zoospore development under a light microscope at 10× and 40× magnification (Dick, 2001).

Molecular identification

Genomic DNA Extraction

Using the FavorPrep Genomic DNA Mini Kit, genomic DNA has been extracted from fungus samples according to the manufacturer's instructions. After being transferred to a 1.5 mL micro-centrifuge tube, fungal or yeast cultures (1–5 × 10^6 cells) have been resuspended in 1 mL of FA Buffer and centrifuged for 2 minutes at 7,500rpm (5,000 × g). After removing the supernatant, the resultant pellet was dissolved in 550.0 µL of FB Buffer with 50 µL of lyticase solution, swirled, and incubated for 30 minutes at 37 °C.

According to the methods described in the FavorPrep Fungi/Yeast Genomic DNA Extraction Mini Kit, a 10-minute centrifugation at 7,500 rpm, the pellet was resuspended in 350µl of TG-1 Buffer, and the supernatant has been disposed of. After transferring the mix to a bead tube and pulse-vortexing it for five minutes, 20.0 µL of Proteinase K (10mg/mL) was added, and the mixture was then incubated for 15 minutes at 60 °C with intermittent vortexing every 5 minutes. A total of 200 µL of the supernatant was transferred to a new 1.5mL microcentrifuge tube, combined with 200 µL of TG-2 Buffer and 200 µL of 96–100% ethanol, and then pulse-vortexed for 10 seconds. Following a 1-minute centrifugation at 7,500 rpm, the sample was ready for use. After that, the sample was placed in a collecting tube on a TG Mini Column and centrifuged for one minute. After centrifugation and

Table 1. Internal Transcribed Spacer (ITS) primer pair

Primer	Sequence 5′-3′	Reference
ITS3 F	GCATCGATGAAGAACGCAGC	Wahyunigsih et al., 2000
ITS4 R	TCCTCCGCTTATTGATATGC	

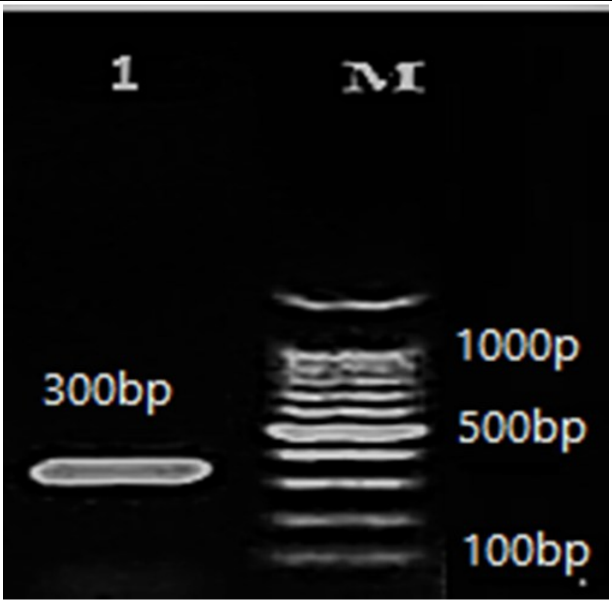

Fig. 1. *0.7% agarose gel electrophoresis at 72 volts for 60 minutes of the PCR-amplified ITS amplicon (300 bp). M: DNA marker (100 bp); (1-8): fungal samples*

washing with 450 µL of W1 Buffer, the flow-through was discarded. After washing again with 750 µL of Wash Buffer, the flow-through was removed, and the sample was centrifuged for 1 minute. It was then centrifuged again for 3 minutes to ensure complete drying.

The TG Mini Column was then placed in an elution tube, and the middle of the membrane was filled with 50–100µL of ddH□O or elution buffer. Centrifugation was used for 2 minutes to elute the genomic DNA, following a 3-minute incubation. A NanoDrop 2000 spectrophotometer was used to evaluate the quality and amount of extracted DNA, which was then stored for later use at -20°C.

Primer pairs

Sterile ddH$_2$O was used to dissolve the primer pair (Macrogen, Korea) (Table 1) utilised in the present study. A working stock of 10pmol/µL has been created by combining 10µL of the stock primer with 90µL of ddH2O, while a stock solution (100pmol/µL) has been prepared by the addition of ddH2O to the vial that contains the lyophilized primer.

Agarose Gel Electrophoresis

The most reliable method used for isolating DNA segments was agarose gel electrophoresis.The sizes of DNA fragments that need to be separated determine how much agarose is present in a gel. ranging from 0.50% to 2%. A 0.70% gel was utilized in order to achieve good separation of genomic DNA (5-10kb) after extraction, while a 1.50%-2% gel was utilized to obtain good resolution for small PCR product fragments (0.20-1kb). Yet, a certain weight of agarose was combined with 100ml of 1× TBE buffer, and the mixture was then cooked in a microwave until it became transparent. Once the agarose had cooled to between 50 and 55°C, 5 µL of safe dye (10mg/mL) was added to 100 µL of melted agarose gel to reach a final concentration of 0.5 µg/mL (Green and Sambrook, 2019).

The comb's tips were sealed, the agarose was put into the gel tray, and it was then allowed to dry. The samples have been placed in a second gel well with a marker in the first. Genomic DNA requires 45 mins of agarose gel electrophoresis, while PCR products require 1 hour and 30 minutes. The run was applied according to gel size and percentage, and the electrodes had been correctly attached (Fig. 1).

Pathogenicity

Commercial cucumber seeds were purchased from local markets in Hilla City, Babylon Province, Iraq, thoroughly cleaned, and then soaked for 2 minutes in a 1.50% (v/v) sterile sodium hypochlorite solution to disinfect their surface. After that, the seeds were air-dried for ten minutes at room temperature (25 ± 2 °C) after being rinsed 3 times with sterile distilled water (El Hartiti *et al*., 2016). 15 mL of water agar medium containing chloramphenicol was used to produce sterilized Petri dishes, each measuring 9 cm in diameter. Five-day-old *P. mamillatum* colonies cultured on water agar were infected with 5-mm discs cut from the margins of each plate. In addition to control plates devoid of the fungus, twenty seeds per plate were placed in a circle close to the edge, with four replicates per treatment. At 25 ± 2° C, the Petri plates were incubated after a fully randomized

The Petri plates were cultured using a completely randomized design (CRD) at 25 ± 2°C. After inoculation, germination percentages were measured three, five, and seven days later (Hussein *et al*., 2024).

RESULTS

Pythium mamillatum in Iraqi cucumbers

P. mamillatum strain AJA (Accession No. MN460315.1) was first identified during the 2023 growing season in cucumber greenhouses located in the Al-Tahiria, Al-

Azizia, and Al-Badah areas of the Al-Musayyib region, Babylon Province, Iraq. This recently documented event highlights the pathogen's recent appearance in regional agricultural systems, underscoring the importance of determining its prevalence and impact on the region's cucumber crop.

Field disease prevalence

The percentage distribution of wilt and root rot disease caused by *P. mamillatum* in cucumber plants in Al-Tahiria, Al-Azizia, and Al-Badah in Al-Musayyib districts during 2023 reveals a progressive increase over time (Table.2), demonstrating the continuous spread of the pathogenic fungus. Al-Tahiria showed a consistent rise in disease incidence, reaching the highest proportion (37%) by 15th March; Al-Badah, starting with 0% on 1st February, showed a fast rise to 35% by the same date. Conversely, Al-Azizia displayed the lowest incidence (21% by March 15th) and the slowest rate of development, suggesting either less suitable conditions for pathogen growth or superior disease control.

Al-Badah's dramatic rise in illness incidence between February 1st and 15th (from 0% to 15%) suggests a late, yet aggressive , start of infection, which may be resulting from the recent introduction of a pathogen or abrupt environmental changes that favour disease development. The data indicate that P. mamillatum is still spreading in most areas; Al-Badah and Al-Tahiria are particularly affected. These results highlight the need of quick disease surveillance and efficient management plans to reduce disease impact and lower crop losses.

Disease symptoms and morphological Characteristics

Plant diseases caused by *P. mamillatum* included root rot, with symptoms that vary depending on host and environmental factors. The stem near the soil line becomes shriveled and drenched with water. Although they received enough water, the affected roots wilt and grew more slowly. They were also brown, soft, and wet. Because they were unable to absorb nutrients, leaves might turn yellow.

Early symptoms appeared as soft rot with a yellow discoloration near the soil contact point (Fig. 2a). In severe cases, the crown area underwent complete breakdown, followed by wilting in the lower leaves, which eventually affected the entire plant; however, some leaves remained green. The pathogen affected various plant parts, including roots and fruits, making disease management essential in cucumber cultivation. Symptoms on cucumber fruits included rot and internal mycelial growth, resulting in the fruit turning brown (Fig. 2b).

Colonies of *P. mamillatum* typically appeared white or off-white in early growth stages, later developing a pale yellowish due to sporulation. They expanded rapidly across the agar surface, often reaching the edges of a Petri dish within 2 to 5 days. The colony surface was usually smooth or slightly wrinkled, and colony margins were frequently irregular, showing radial growth patterns (Fig. 1c). Under optimal conditions, *P. mamillatum* formed sporangia, which were visible on the colony surface, giving it a slightly rough or granular appearance. The colony also produced significant aerial mycelium, contributing to its dense, cotton-like texture (Fig. 2c & d).

P. mamillatum's mycelium was coenocytic, meaning it lacked septa and -contained multiple nuclei, allowing for continuous cytoplasmic flow. Large, oval, or spherical sporangia were seen alone or in groups. Vesicles were bulbous structures found at the terminals or branches of hyphal organs, aiding in the spread of pathogens (Fig. 2D & E).

***Pythium mamillatum* strains compared to the Iraqi strain AJA (Accession ID: MN460315.1).**

The present Iraqi strain of *P. mamillatum* (ID: MN460315.1) shared complete genetic compatibility (100%) with strains from the USA, Canada, and China, indicating a high degree of genetic similarity among these isolates. This suggests that these strains may have a common origin or have undergone similar evolutionary pressures, resulting in conserved genetic sequences (Table 3).

Strains with 99% compatibility, originating from countries such as Canada, the United Kingdom, South Africa, Switzerland, and Japan, also exhibit close genetic relationships with the Iraqi strain. The slight genetic variations observed may be attributed to geographical separation, environmental factors, or host-specific adaptations.

Moderate compatibility (97-98%) with strains from France, Australia, South Africa, and Switzerland indicates more pronounced genetic divergence. These differences could result from prolonged geographical iso-

Table 2. Percentage distribution of wilt disease caused by *the P. mamillatum* fungus on cucumber plants in Al-Tahiria, Al-Azizia, and Al-Badah districts of Al-Musayyib during the year 2023

Regions	% Distribution			
	1st Feb	15th Feb	28th Feb	15th March
Al-Tahiria	5	12	22	37
Al-Azizia	2	10	18	21
Al-Badah	0	15	27	35

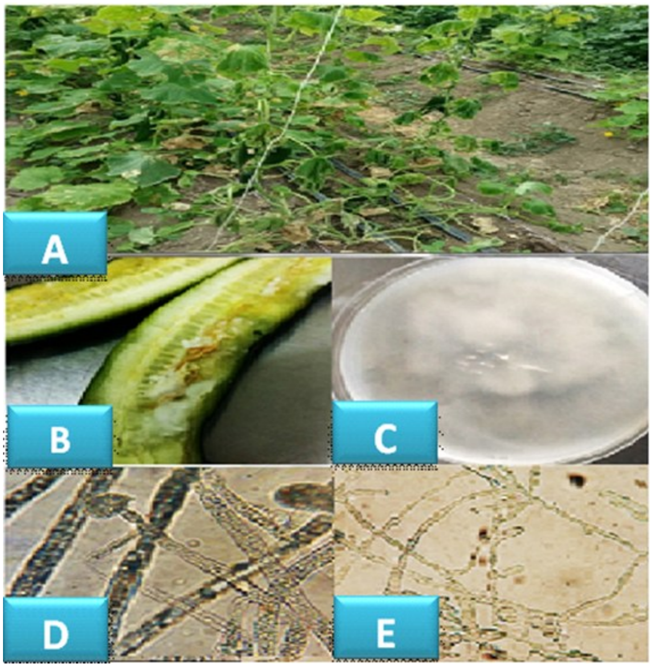

Fig. 2. *Symptoms of cucumber wilt disease caused by P. mamillatum in a greenhouse; (A) Wilt symptoms on cucumber foliage, showing complete wilting of the plant; (B) Longitudinal section of an infected cucumber fruit; (C) White fungal colony of P. mamillatum; D & E: Fungal structures*

lation, distinct ecological niches, or evolutionary adaptations to local environmental conditions.

The 96% compatibility observed with the Indian strain suggests the highest level of genetic divergence among the compared strains. This could be due to unique evolutionary pathways influenced by India's specific climatic conditions, soil types, or agricultural practices.

Phylogenetic tree of *Pythium mamillatum*

The evolutionary history has been inferred using the unweighted pair group method with arithmetic mean (UPGMA) technique, a hierarchical clustering method primarily employed in taxonomy, phylogenetics, and bioinformatics to generate dendrograms, or tree diagrams, based on distance or similarity data (Sneath and Sokal, 1973). The ideal tree is shown with a total branch length of 12.42289796. The tree is shown to scale, with branch lengths measured in the same units as evolutionary distances that are required to establish phylogenetic connections (Fig. 3). The Maximum Composite Likelihood method was used to compute the distances of evolution, which are expressed in terms of the number of base substitutions per site (Kumar *et al*. 2018).In the present study, eighteen nucleotide sequences were analyzed. The codon locations included first, second, third, and noncoding regions. For every pair of sequences, the pairwise deletion option was used to eliminate any confusing spots. The final dataset comprised a total of 1,477 locations. Using MEGA X, evolutionary studies were performed.

Pathogenicity

The pathogenicity assessment of *P. mamillatum* on cucumber seeds revealed a progressive increase in germination inhibition over time, demonstrating the aggressive nature of the pathogen. As shown in Table 4, germination inhibition was 10% at 3 days, increased sharply to 60% at 5 days, and reached 90% at 7 days post-inoculation. In contrast, the control treatment, which consisted of uninoculated seeds, showed no inhibition at any time point, confirming that the observed

Table 3. GenBank accession numbers, countries of origin, and sequence similarity percentages of *Pythium mamillatum* strains compared to the Iraqi strain AJA (Accession ID: MN460315.1).

ACCESSION	Country	Source	Compat.
ID: MN460315.1	IRAQ	*Pythium mamillatum* strain AJA	100%
ID: AY598703.1	Canada	*Pythium mamillatum*	99%
ID: MN306119.1	USA	*Pythium mamillatum*	100%
ID: MK026904.1	France	*Pythium mamillatum*	98%
ID: MF446828.1	United Kingdom	*Pythium mamillatum*	99%
ID: KF780560.1	Australia	*Pythium mamillatum*	97%
ID: HQ665173.1	Canada	*Pythium mamillatum*	99%
ID: JX944763.1	India	*Pythium mamillatum*	96%
ID: HQ643689.1	Canada	*Pythium mamillatum*	100%
ID: GU071883.1	South Africa	*Pythium mamillatum*	98%
ID: GQ410353.1	South Africa	*Pythium mamillatum*	99%
ID: DQ525086.1	Switzerland	*Pythium mamillatum*	97%
ID: AF290856.2	China	*Pythium mamillatum*	100%
ID: EU240185.1	Switzerland	*Pythium mamillatum*	99%
ID: JX115089.1	Canada	*Pythium mamillatum*	99%
ID: GU071819.1	South Africa	*Pythium mamillatum*	97%
ID: AB512918.1	Japan	*Pythium mamillatum*	99%
ID: DQ528753.1	Switzerland	*Pythium mamillatum*	99%

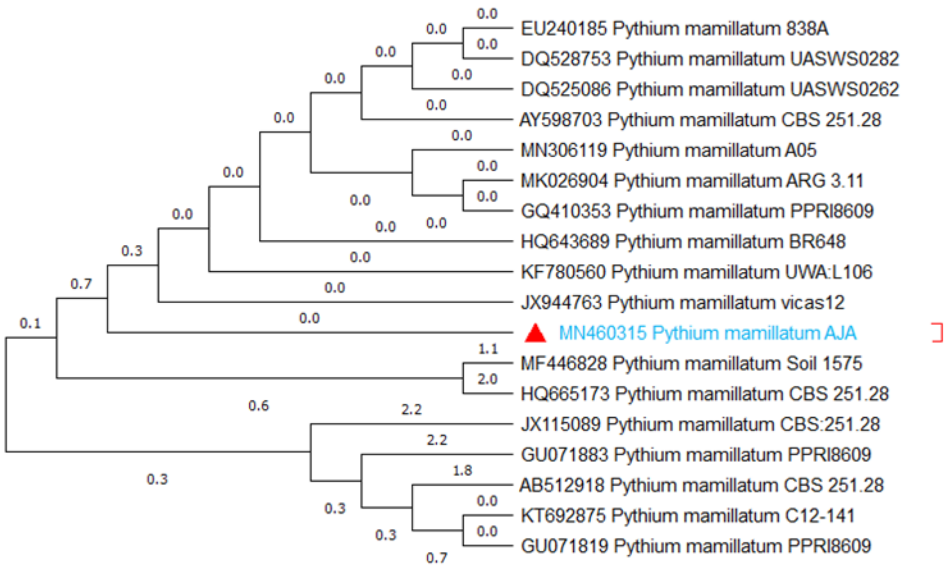

Fig. 3. *Evolutionary analysis by the Maximum Likelihood method*

Table 4. Percentage suppression of cucumber seed germination on water agar at 25 ± 2°C, 3, 5, and 7 days following *Pythium. mamillatum* inoculation

Cucumber seeds	**% inhibition**		
	3 days	**5 days**	**7 days**
Seeds + *Pythium mamillatum*	10	60	90
Control treatment*	0.0	0.0	0.0

* cucumber seeds placed on water agar only

effects were solely due to fungal infection.

The significant rise in inhibition from days three to five revealed that *P. mamillatum* colonized promptly and significantly decreased seed sustainability. The approximately complete inhibition perceived by day seven (90%) highlights the pathogen's high virulence and its severe effect on seed germination under the tested conditions. The rapid development of the disease emphasizes the urgent need for early detection methods to avoid substantial seed and seedling losses in cucumber cultivation.

These findings demonstrate the strong inhibitory effect of *P. mamillatum* and emphasize the importance of effective disease management strategies, such as seed treatments and pathogen monitoring, to diminish its impact. Early detection and control measures are essential for minimizing germination failure and improving crop establishment.

DISCUSSION

Naturally occurring in water and soil, fungal-like organisms—more especially, the *Pythium* species—are sometimes indicated as water molds. Some species cause major illnesses in greenhouse vegetable crops, therefore causing significant crop losses. In the present work, trends in *P. mamillatum*-caused wilt and root rot diseases in cucumber plants in Al-Azizia, Al-Tahiria, and Al-Badah showed similarities to those observed in other cucurbit diseases associated with *Pythium*. Inadequate drainage, high soil moisture, and rising humidity—all of which promote *Pythium* growth—probably produced the claimed disease outbreak (Toporek and Keinath, 2021).

The use of biocontrol agents, including *Streptomyces murinus*, and other successful disease management strategies, is shown to help inhibit *Pythium*-induced damping-off in cucurbits (Ge *et al*., 2023). Appropriate water management practices help decrease disease outbreaks, according to Weiland (2011). *Pythium mamillatum* has been proven to be pathogenic, as indicated by other studies on Pythium species that affect cucurbits, such as *P. ultimum*, *P. irregulare*, and *P. aphanidermatum* (Weiland *et al*., 2015; Punja *et al*., 2022). Several studies have identified the unique physical traits of P. mamillatum, particularly its ornamented oogonia (Paul *et al*., 2006).

Moreover, the gene content of *Pythium* species has shown a clear variation that influences pathogenicity (Kittichotirat *et al*., 2023). Comparative genomic studies of *P. mamillatum* have found genetic variants in compatibility that can affect adaptability and virulence (Chimote *et al*., 2010).

Genetic grouping of *P. mamillatum* strains shown by UPGMA suggested a common ancestor. Even though UPGMA assumes that the rate of molecular evolution is constant, strain-to-strain variations in evolutionary rates may affect tree topology, suggesting the need for further methods to consider this heterogeneity (Tamura & Nei, 1993).

To investigate *P. mamillatum*'s pathogenicity, cucumber seeds have were inoculated; over time, the results revealed a growing reduction of seed germination. The rise in germination inhibition—from 10% at 3 days to 90% at 7 days after inoculation—confirmed the aggressive character of the pathogen and its propensity to lower seed viability. The lack of inhibition in the control treatment implied that the fungal infection was the only one generating the noted effects. This result aligns with previous research on Pythium species, which have been proven to substantially harm seeds (Rahman *et al*., 2012; El Hartiti *et al*., 2016).

The rapid increase in inhibition between days three and five suggests that *P. mamillatum* is aggressively colonizing the area, most likely by generating infectious zoospores. The high pathogenicity of Pythium species, which cause root rot and seedling disease, is well recognised (Johnston and Ristaino, 1999). Stopping the spread of diseases calls for effective management strategies, such as biological agent treatments applied to seeds or fungicides. Early identification and disease monitoring is essential to both lowering seedling losses and improving crop establishment (Lamour *et al*., 2012). Effective control of P. mamillatum and other soil-borne diseases requires a comprehensive strategy that combines resistant cultivars, chemical treatments, and cultural methods (Syed *et al*., 2020). Knowing such components will enable the development of targeted disease control plans, therefore boosting general crop health and productivity and reducing the detrimental effects of root rot on cucumber output.

Conclusion

In the present study, the distribution, pathogenicity, and genetic variation of *Pythium mamillatum*—the causal agent of root rot disease in cucumber (*Cucumis sativus*)—were investigated in the areas of Al-Azizia, Al-Tahiria, and Al-Badah within the Al-Musayyib district, Babylon Governorate, highlighting the pathogen's aggressiveness and its significant threat to crop yield. Severe root rot and wilt resulted from high soil wetness, inadequate drainage, and humidity, which accelerated the spread of the disease. Pathogenicity tests demonstrated 90% germination suppression by day 7, thereby suggesting the great virulence of the pathogen. Molecular studies revealed degree of genetic similarity between the Iraqi isolate *Pythium mamillatum* strain AJA (Accession No. MN460315.1) and foreign strains, suggesting shared evolutionary pressures. The present results highlight the need for integrated management strategies to mitigate the effects of P. mamillatum and protect crop yields, including early disease diagnosis, pathogen monitoring, and the use of fungicides and biocontrol techniques. From a sustainability perspective, it is crucial to develop disease control plans that minimize environmental damage while effectively controlling pathogens. By combining chemical, biological, and cultural control strategies, an integrated pest management (IPM) approach will help preserve soil health over time and reduce the need for chemical fungicides. In the face of growing plant diseases, this sustainable approach may reduce the ecological impact of farming, the ensuring the resilience of agricultural systems and enhancing food security.

ACKNOWLEDGEMENTS

We sincerely thank the Head of the Biology Department and the Deanship of the College of Science at the University of Babylon, Iraq, for their generous support and for providing the necessary facilities that made this study possible.

Funding

The authors declare that no specific grant or financial support was received from the university or any external funding agency for the conduct of this research.

Conflict of interest

The authors declare that they have no conflict of interest.

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