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First record of *Paramyrothecium roridum* as a causative agent of melon stem blight in Iraq

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Abstract

The study was conducted in the Plant Pathology Laboratory of the Department of Plant Protection, College of Agriculture, University of Basrah, during the period 2023-2024. The study aimed to isolate and identify the pathogen *Paramyrothecium roridum* associated with melon stem blight disease morphologically and molecularly, evaluate its pathogenicity, and test the effect of chitosan on fungal growth in vitro conditions and the effect of the Bioagent *Trichoderma viride* on the pathogenic fungus. The results of the morphological diagnosis showed that the fungus consists of fungal colonies in the form of concentric circles and an identification color. As the colony ages, the color becomes darker and the nature of the fungus growth is slow. It takes 10 days for the fungus to fill the entire plate. The results of the pathogenicity showed that the tested isolate caused the death of melon seedlings grown in pots. The germination rate and seedling death reached 56.66 and 36.33%, respectively, compared to the control treatment, which had a germination rate of 80.00 and seedling death of 4.00. The results showed The inhibition of chitosan at concentrations of 2%, 3% and 5% g/L the concentration of 5% excelled by achieving a high inhibition rate, as the inhibition rate of fungal growth reached 100.00%, while the results of the antagonism with the bio fungus *T. viride* in the dual cultivation experiment were that the bio fungus *T. viride* has a high antagonistic ability in the PDA culture medium against the pathogenic fungus, as the antagonism degree reached 1.

Keywords: Trichoderma fungus, melon, stem blight, Paramyrothecium fungus

I. Introduction

Melon (*Cucumis melo L*) is a warm-season plant (Farcuh et al., 2020). melon production in Iraq amounted to (181) thousand tons, a decrease of (12%) from last year's production, which was estimated at (205) thousand tons (Central Statistical Organization, 2023). It is a major economic fruit crop in many countries of the world, such as China (Yang et al., 2019). It is often Available in the markets from late summer to early fall, it is considered one of the most popular fruit crops in the world and an important food source with health benefits because it is rich in ascorbic acid, carotene and folic acid and provides about 12% of the daily requirement of potassium and contains 100% of the recommended value of vitamin C and vitamin A in addition to many other bioactive compounds (Thakur et al., 2019; Yavuz et al., 2021; Tabassum et al., 2021). High consumption of fruits leads to the production of a large amount of waste, such as peels and seeds, which are still rich in polyphenol molecules, carotenoids and other bioactive components that have a positive effect on human health. Sustainable development can be achieved in the agricultural food and agro-industrial sectors by reusing and valuing these wastes, which in turn can reduce their environmental impact. Melon residues are a good source of natural phytochemicals useful for many purposes, such as components of the food, cosmetic or pharmaceutical industries, and the production of fertilizers and animal feed. (Vella et al., 2019) Orange watermelon contains carotene, which benefits heart health and the immune system, while green melon contains vitamin B6 to maintain strong bones and teeth (Vanoli, 2015; Agus, 2018; Sánchez et al., 2021). In a study, it was indicated that the fungus *Paramyrothecium* eichhorniae was recorded in Thailand and is considered a pathogen of leaf blight in water lily plants (Pinruan et al., 2022). In a study, it was indicated that *Paramyrothecium* contains many important secondary

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metabolites and toxins, including macrolides of the trichothecenes type, such as roridin and verrucarin, which play a fundamental role in some medical and biotechnological applications (Liu et al., 2016; Elkhateeb and Daba, 2019). Melon plants are infected with several fungal, bacterial, nematode and viral diseases. These pathogens negatively affect and cause significant losses in crop production (Singh et al., 2016; Al-Daghari et al., 2021). Melon is one of the plants grown in large areas in Basra. Studies have been conducted on diseases that affect Melon, including fusarium wilt and root knots in melon plants. (Al-Samar, 2003 and Al-Musaidi, 2014) and the death and fall of seedlings (Al-Lami, 2012).

II. Materials and methods

Isolation of the fungus associated with stem blight disease in melon

Melon plant samples (stem) showing symptoms of stem blight disease were brought, and the infected parts were washed with running water, then cut with a sterile scalpel into small pieces of 0.5 cm in length and sterilized with a 10% sodium hypochlorite solution (NaOCl) for two minutes, then washed with sterile distilled water to remove traces of sodium hypochlorite solution, dried on sterile filter paper, then planted in sterile Petri dishes with a diameter of 9 cm containing sterile PDA medium prepared in advance with 250 mg/liter of the antibiotic Chloramphenicol added to it, at a rate of five pieces of plant stem/dish, then the dishes were incubated in the incubator at a temperature of 25±2°C for four days, after which the fungal growths around the isolated pieces were examined and purified on new dishes containing the same PDA medium. Morphological diagnosis of fungi

The fungus isolated from the affected stem area was diagnosed to the species level based on the external appearance of the colony such as color, shape, colony diameter and height using PDA culture medium. Microscopic characteristics such as the shape, structure and size of the spores and conidia were also relied upon according to the taxonomic principles mentioned in (Solimann, 2020)

Molecular diagnosis of fungi associated with stem blight disease in melon

A 7-day-old fungal culture was used for each pathological isolate and incubated on PDA medium at 25 °C ± 2. The surface layer of the fungi was scraped off, then rapidly frozen using liquid nitrogen and ground with a mortar and pestle to a fine powder. Up to 25 mg of this powder was transferred to an Eppendorf 1.5 tube for DNA extraction using the gSYNCTM DNA Extraction Kit according to the instructions

The manufacturer's company. (Fayyadh et al., 2024). Then, electrophoresis was performed to detect DNA. After the migration was completed, the gel was examined with a Gel Documentation device to observe DNA bands (Sambrook et al., 1989). The PCR reaction mixture was prepared in a 200-µl Eppendorf tube and the final volume of the components was 25 µl. Then, the tubes were placed in a small centrifuge (snip) to mix the components. Table (1) shows the quantities used in the reaction, Table (2) shows the primers used in this technique, and Table (3) shows the special program for this technique (Abd-Elsalam et al., 2003). After confirming the PCR product amplification process by electrophoresis, a quantity of 20 microliters of the amplification product for each isolate was sent to the Korean company Macrogen for the purpose of determining the sequences of nitrogenous bases in the genes used, then matching them with the National Center for Biotechnology Information (NCBI) and registering them (Manea et al., 2023).

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Table (1) Quantities of materials used in the PCR technique in microliters.

Final size	Nuclease-free water	DNA template	primer	Master Mix	Chemical
25	6.5	5	)1forward + reveres(	12.5	Volume

Table (2) Sequences of primers used in PCR technique.

primer temperature	primer name	primer sequence	No.
64.5	ITS1	F:TCCGTAGGTGAACCTGCGG	1
70.8	ITS4	R:TCCTCCGCTTATTGATATGC	2

Table (3) DNA amplification program.

Number of cycles	Time (minutes(	temperature	Stages
1	5:00	94	Initial Denting
35	0:30	94	Denting
	0:45	58	Adhesion
	2:00	72	Elongation
1	7:00	72	Final Elongation

Then, the molecular identification of the obtained confirmed sequences was processed through Chromas 2.6.5 3 program, and then multiple alignment of each sequence sample was performed separately with the NCBI database using BLAST program (Al-Saad et al., 2018). The processed sequences of the identified isolates were submitted to NCBI for registration.

Testing the pathogenicity of the fungus associated with melon stem blight disease in pots.

A mixture of soil mixed with peat moss at a ratio of 3 soil:1 peat moss was sterilized in an autoclave at a temperature of 121°C and a pressure of 1.5 kg/cm for one hour and two consecutive days. Then, the fungal inoculum of soil-pathogenic fungi was added at a rate of 1% (weight/weight) in 1 kg plastic pots, mixed well and moistened with water for two days before planting. Three pots were planted with the pathogenic fungus at a rate of ten seeds of local melon, leaving three pots without inoculum as a comparison treatment. The pots were watered carefully (Al-Musaidi, 2014). Symptoms were observed after 7, 10, and 14 days of planting, and the germination percentage of the seeds was calculated according to the following equation:

$$\text{germination\%}=\frac{Number\ of\ germinated\ seeds}{Total\ number\ of\ seeds}\times 100$$

After four weeks, the number of dead seedlings was calculated according to the following equation:

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seedling death%=$\frac{Number\ of\ dead\ seeds}{Number\ of\ germinated\ seeds}$×100

Koch's postulates were then applied by matching the symptoms caused by *Paramyrothecium roridum* with the symptoms of stem blight present in the field and then re-isolating to confirm the pathogen. Evaluation of the efficiency of chitosan on fungal growth in the laboratory Chitosan was obtained from HIMEDIA Company and prepared by dissolving 20 g of regular chitosan in 50 ml of acetic acid concentration 92% and mixing the solution with a magnetic mixer for one hour to dissolve the chitosan and obtain a clear suspension. The volume was completed to 1000 ml of sterile distilled water and mixed well to obtain the basic solution (Stock solution). Three concentrations of 2, 3 and 5% were prepared. This was done by adding 2, 3 and 5 ml of the basic solution to 98, 97 and 95 ml of sterile PDA medium, respectively. Then the flask was shaken well to homogenize the medium and then poured into sterile dishes with a diameter of 9 cm. When they solidified, they were inoculated with pathogenic fungal discs with a diameter of 0.5 cm. Three replicates of the treatment were used, leaving only a control treatment of culture medium. The dishes were incubated in the incubator at a temperature of 25 ± 2 °C for seven days. The fungal growth and the percentage of inhibition were measured according to For the equation given in Tomlin (1998).

Inhibition percentage = $\frac{(\text{treatment colony diameter} - \text{control colony diameter})}{\text{control colony diameter})}$×100

Evaluation of the efficiency of the fungus *Trichoderma viride* in inhibiting the fungus *Paramyrothecium roridum* in the laboratory

The Daul-culture Technique was used to test the antagonism between the bio fungus *Trichoderma* and the pathogenic fungus. The Petri dish with the sterile PDA medium added to it was divided into two equal parts and the center of the first half was inoculated with a 0.5 cm diameter disk from a seven-day-old colony of the bio fungus *Trichoderma viride* and the second half was inoculated with a 0.5 cm diameter disk from a pathogenic fungus colony with three replicates for each test, leaving three replicates for comparison that were inoculated with the pathogenic fungus only. Then the dishes were incubated at a temperature of 25 +_ 2 C. The degree of antagonism was calculated after the growth in the comparison treatment reached the edge of the dish according to the Bell and others (1982) scale consisting of five degrees as follows: Shown in Table (5) and my agencies:

Table (5) Bell scale for measuring the degree of antimicrobial resistance of *Trichoderma* fungus

Degree	Description
1	biotrophic fungus covers the entire plate
2	biotrophic fungus covers 4/3 of the plate and the pathogenic fungus covers 4/1 of the plate
3	biotrophic fungus and the pathogenic fungus both cover 2/1 of the plate
4	pathogenic fungus covers 4/3 of the plate and the biotrophic fungus covers 4/1 of the plate
5	pathogenic fungus covers the entire plate

The antifungal is effective if the degree of antagonism is 1 or 2. Results

Isolation of the fungus associated with stem blight disease in melon and its morphological and molecular diagnosis

The results showed the possibility of isolating the fungus *Paramyrothecium roridum* associated with stem blight disease in the areas of Abu Sakhir, Abu Al-Khaseeb and Al-Luhais. The results showed Figure (1) that the fungus *Paramyrothecium roridum* The fungus consists of fungal colonies in the form of concentric circles and an olive green color. As the colony ages, the color becomes darker and the nature of the fungus' growth is slow. It takes 10 days for the fungus to fill the entire plate. The results are consistent with several researchers who indicated the

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appearance of sticky, dark olive-black droplets with a concentric distribution on the surface of the fungal colony that produces spores and gradually dries and becomes solid as the fungus ages. Microscopic examination showed that the spores take a rod-shaped shape with slightly rounded ends. The spores appear transparent to light green, especially in young farms, and become dark as the farm ages (Solimann, 2020).

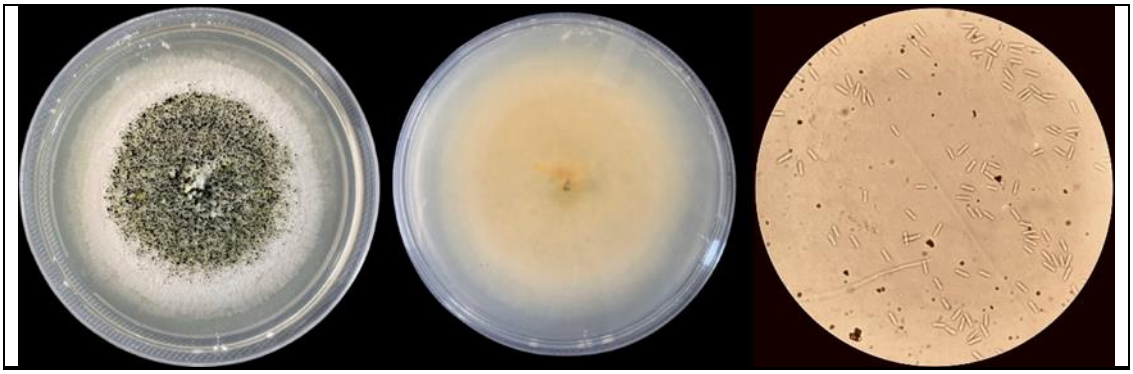

Figure (1) The morphological and microscopic appearance of the fungus *Paramyrothecium roridum*

Molecular diagnosis of the fungus

The results of the detection Figure (2) using the electrophoresis technique of the PCR product of the fungus sample isolated from the stem of the melon plant showed the appearance of bands ranging between 500-700 base pairs. The results confirmed Table (3) The conformity of the fungal isolate diagnosed morphologically according to the taxonomic keys based on the morphological appearance with the results of the molecular diagnosis based on reading the sequences of the nitrogenous bases of the ITS1-ITS4 interphase region. The fungus *Paramyrothecium roridum* was registered in the Gene Bank under the accession number PQ325449.1 and the results were identical to the isolate registered under the accession number MG988391.1 with a matching rate of 99.26%. The sequence of the nitrogenous bases was deposited in the American National Center for Biotechnology Information (NCBI). There was no study or data indicating the registration of the fungus on the melon plant in Basra Governorate, but the fungus *P. roridum* is considered It is a known cause of stem spot and blight diseases that affect many plants including soybeans, strawberries and melon (Solimann, 2020).

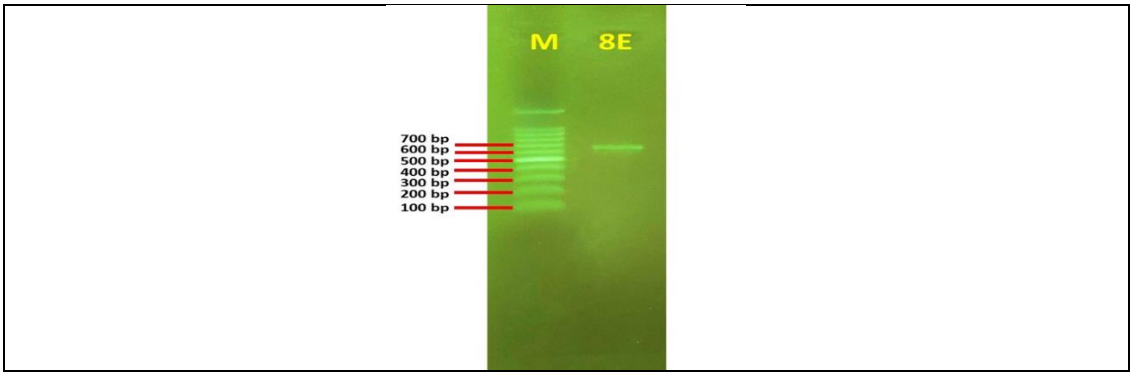

Figure (2) The amplified sample in PCR for the ITS1-ITS4 region on agarose gel

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Testing the pathogenicity of the fungus associated with melon stem blight disease in pots.

The results of the pathogenicity test Figures (3) and (4) showed that the tested isolate caused the death of melon seedlings grown in pots. The germination and seedling death percentages reached 56.66 and 36.33, respectively, compared to the control treatment, which had a germination percentage of 80.00 and seedling death of 4.00. It also showed symptoms similar to those of melon stem blight disease when applying Koch's hypotheses. Aumentado and Balendres (2022) reported their study on the isolation of the fungus *Paramyrothecium* from melon plants and they proved that the fungus has a high pathogenic capacity. Moreover, the fungus *Paramyrothecium* sp is the pathogen of cucumber and melon seedlings, causing stem canker (Huo et al., 2021).

Figure (3) Testing the pathogenicity of the fungus in the germination of melon seeds in pots where:

G = *Paramyrothecium roridum* Con = Control

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Figure (4) Symptoms of stem blight on melon caused by the fungus *Paramyrothecium roridum*

Evaluation of the efficiency of chitosan on fungal growth in the laboratory

The results showed in Figure (5) that the fungus achieved a high inhibition rate of 79.00, 88.33 and 100.00 for the three concentrations of 2%, 3% and 5%, respectively. These results are consistent with what was stated by Lopez-Moya et al. (2019) that chitosan has antibacterial and antifungal activity ranging from growth inhibition to lethal effect. The inhibitory ability of chitosan may be due to the disruption of the activity of some enzymes and proteins essential for fungal growth, in addition to the increased permeability of the cell membrane as a result of the interaction of positively charged chitosan with the negatively charged cell membrane of fungi, which leads to the inhibition of the synthesis of proteins and essential enzymes as a result of a change in the DNA structure and a lack of essential nutrients for fungal growth (Rinaudo and Younes, 2015). This study is consistent with previous studies, as Al-Ramahi (2021) indicated that the addition of regular chitosan to the culture medium at concentrations of 250, 500, 1000, 2000, 3000, 4000, 5000, and 6000 ppm led to the inhibition of the growth of Aspergillus flavus on the PDA culture medium with inhibition rates of 28.23, 31.37, 32.54, 45.09, and 49.02, 61.17, 64.70 and 82.35% respectively compared to the control treatment, and the highest inhibitory activity was shown at the concentration of 6000 ppm.

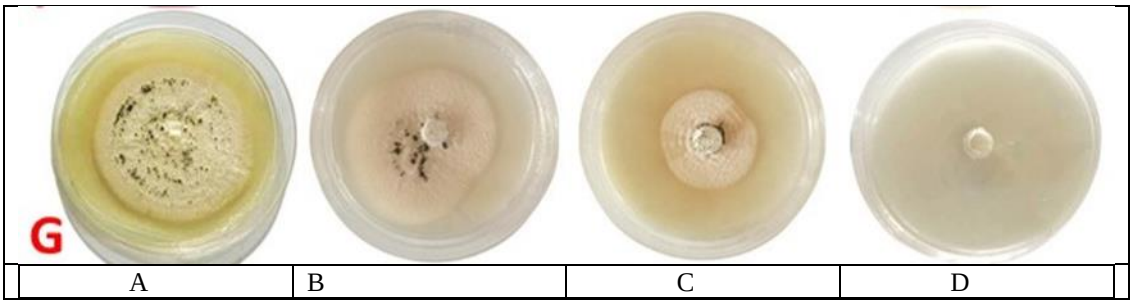

Figure (5) Effect of chitosan concentrations on the pathogenic fungus *Paramyrothecium roridum* isolated from melon stems where:-

A: Control

B: Chitosan concentration 2%

C: Chitosan concentration 3%

D: Chitosan concentration 5%

Evaluation of the efficiency of the fungus *Trichoderma viride* in inhibiting the fungus *Paramyrothecium roridum*

The results of sub culture experiment Figure (6) showed that the bio fungus *T. viride* has a high antagonistic ability in the PDA culture medium according to the Bell and others (1982) scale, as the antagonistic degree reached 1 against the pathogenic fungus *Paramyrothecium roridum*. The results were consistent with Matroud (2015), who showed the ability of the bio fungus *T. viride* to inhibit the pathogenic fungus *Macrophomina phaseolina*. The antagonistic ability of the fungus *Trichoderma* spp. is due to the production of enzymes that decompose the cell walls of the pathogen, such as B-1-3-gluconase, chitinas, protease, and cellulase, in addition to its various mechanisms such as parasitism on the fungal hyphae, penetration, and feeding on the fungal contents (Hermosa et al., 2012)

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Figure (6) The effect of the bio fungus *Trichoderma viride* on the pathogenic fungus isolated from the melon stem

Conclusions

The pathogenic fungus *Paramyrothecium roridum* is characterized by its slow growth in laboratory conditions and its high ability to show symptoms of stem blight in melon after applying Koch's postulates to this pathogen. Also, the effectiveness of chitosan at a concentration of 5% caused 100% inhibition of fungal growth. In addition, the vital fungus *Trichoderma viride* showed high antagonistic ability against the pathogenic fungus in the dual cultivation experiment. melon plant is susceptible to infection by new fungi in addition to the fungi latent in the soil that repeatedly infect the plant.

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