# Isolation and Control of *Pythium aphanidermatum* Causing Cucumber Root Rot in Babylon Province Using Biological and Nutritional Methods.

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**Abstract**:

The results of isolation and diagnosis from the roots of infected cucumber plants showing symptoms of wilting revealed the presence of five fungal genera: *Pythium* sp., *Verticillium* sp., *Fusarium* sp., *Macrophomin*a sp., and *Alternaria* sp. The fungal isolates varied in their frequency of occurrence. *Pythium* sp. showed the highest frequency among the isolated fungi, with a rate of 67.136%, followed by *Fusarium* sp. with a frequency of 12.10%. The isolates of *Verticillium* sp. and *Alternaria* sp. recorded frequencies of 4.16% and 4.12%, respectively, while *Macrophomina* sp. showed the lowest frequency at 1.22%. These fungi possess the ability to produce large numbers of reproductive units, which enhances their ability to survive under unfavorable environmental conditions.

Pathogenicity tests showed that all isolates caused a significant reduction in the germination percentage of radish seeds grown on Water Agar medium, with clear variation in the impact of each isolate. Germination rates ranged from 0% to 24%. The isolate *Pythium* sp. (Py2) resulted in the lowest germination rate, which was 0.00%, compared to the control treatment which achieved 100% germination.

Morphological and molecular diagnosis using Polymerase Chain Reaction (PCR) technology and nucleotide sequence analysis confirmed the presence of the pathogenic oomycete *Pythium aphanidermatum*, isolated from the roots of infected cucumber plants, and identified as the most frequent isolate in the samples. The morphological and molecular diagnosis also confirmed the isolation of the biocontrol fungus *Trichoderma virens* from soil using the serial dilution method for biological control applications.

Antagonistic capability tests showed that the bacterium *Bacillus paramycoides* exhibited the highest antagonistic activity against the pathogenic oomycete *P. aphanidermatum* under laboratory conditions, achieving 100% inhibition. This was followed by the biocontrol fungi *T. virens and T. harzianum*, which recorded inhibition rates of 97.22% and 93.05%, respectively.

The results also showed that the use of certain nutrients (Boron, iron, and agricultural gypsum) contributed to the inhibition of the pathogenic fungus. The treatment with boron gave the best results, achieving 100% inhibition at a concentration of 2 ml/L, compared to the control treatment with the pathogen, which showed 0.0% inhibition.

**Introduction**:

Cucumber (*Cucumis sativus* L.) , a member of the Cucurbitaceae family and, a nutritious vegetable. 100 g of fresh cucumber contains approximately 96 grams of water, 3 grams of carbohydrates, 1 gram of protein, 12 calories,

1 milligram of phosphorus, 0.03 milligrams of iron, 1 gram of vitamin B, 0.04 milligrams of vitamin B2, 0.20 milligrams of niacin, and 8 milligrams of ascorbic acid (15). It has many medicinal uses, including maintenance of skin radiance, reduction of nervous disorders, and relief of headaches. It also contributes to detoxification in the body (25). China ranked first in cucumber production, accounting for 70% of the total, followed by Turkey, Iran, and Russia. In Iraq, the production of cucumbers of all kinds reached (196) thousand tons, an increase of (5.6%) in 2022 compared to the previous year's production, which was estimated at (185.5) thousand tons (33). Many problems have been found with the use of chemical pesticides to combat plant diseases because these compounds can pollute the air, water, soil, and food. Their presence can also change the qualitative characteristics of parts of the biosphere and have harmful effects on humans and animals. Therefore, many international organizations, including the World Health Organization (WHO), the Food and Agriculture Organization (FAO), and the US Environmental Protection Agency, have all responded to the health risks that pesticides may pose, and warnings have been repeated about them (5). Bio control agents are alternative to pesticides. These organisms are used as biopesticides and fertilizers to sustain, enhance, and protect crop production. Biological agents are often isolated from soil (21). Species belonging to the genus Bacillus spp. have received significant attention from researchers in many countries around the world, where they have been introduced as a key component in bio control programs to control many pathogens on various agricultural crops (42). Furthermore, the role of beneficial microorganisms in increasing soil fertility and enhancing plant characteristics, which contribute to increased organic agricultural production, is well known. This is particularly true for the fungus *Trichoderma* spp., which is considered a resistance factor to many pathogens (8) and has the ability to stimulate plant control against pathogens (22). In addition to its benefits in improving plant growth traits, such as dry and fresh weights of root and shoot tissues, branching, and yield (7), this study aimed to determine the inhibitory capacity of some bio control agents, nutrients, and the herbicide metalaxyl in inhibiting the pathogenic fungus *P. aphanidermatum* on Potato Dextrose Ager-(PDA) culture medium in the laboratory.

Materials and Methods:

1- Field Survey

A field survey was conducted on cucumber fields in Babylon province from March 10, 2024, to May 10, 2024, to determine the incidence of cucumber root rot. Five fields were selected within the five regions, with areas ranging from two to eight dunams. Healthy and infected plants located within the intersection of the diameters in each field were tested. Infected plants were counted based on

visible symptoms, includin wilting, yellowing of leaves, and generally poor growth. Brown, sloughing watery rot was observed in the main and secondary roots. This was performed by randomly uprooting plants located within the intersection of the diameters of each site. Healthy and infected plants were tested and placed in polyethylene bags. They were labeled and stored in a refrigerator at 4°C until the fungi associated with the roots were isolated from each sample (Table (1)). The percentage of infection was calculated using the following equation:

$$\text{Percentage of infection\%} = \frac{(number\ of\ infected\ plants)}{(total\ number\ of\ tested\ plants)} \times 100$$

**Table (1) Temporal, spatial and type distribution of cucumber field sites covered by the field survey in Babylon Governorate**

| Cultivated area in dunums | Sample collection date | cultivars | collection area (Babylon) | numbers |
|---|---|---|---|---|
| 3 dunums | 2024-3-10 | Super Knight | Babylon - Al-Hashimiyah District - Al-Hamzah | 1 |
| 2 dunums | 2024-3-29 | Jana | Babylon - Kutha District - Zabidi | 2 |
| 10 dunums | 2024-6-1 | Shara | Babylon - Kutha District - Dulaimi | 3 |
| 8 dunums | 2024-5-25 | Omiga | Babylon - Kutha District - Al-Imam Branch | 4 |
| 3 dunums | 2024-6-10 | Yekta | Babylon - Kutha District - Al-Himyari | 5 |

The percentage severity of root infection was calculated according to the 5-point pathology index as follows:

0 = healthy plant

1 = more than 0-25% of roots infected

2 = more than 25-50% of roots infected

3 = more than 50-75% of roots infected

4 = more than 75-100% of roots infected

The percentage severity of infection was calculated according to the McKinney (32) equation as follows:

$$\text{Severity of injury\%} = \frac{(Number\ of\ plants\ degree\ 0\times 0+\ldots+Number\ of\ plants\ degree\ 4\times 4)}{Total\ number\ of\ infected\ plants\times 4} \times 100$$

2- Isolation and identification of fungi associated with cucumber roots.

Isolation was carried out from wilted cucumber seedlings taken from greenhouses in some areas of the Babylon Province. Plants showing clear wilt symptoms were uprooted, along with their root systems. The samples were placed in polyethylene bags and brought to the laboratory for isolation and identification. Symptoms included watery and,

soft consistency in the infected root tissue, which led to easy detachment of the outer root cortex with minimal mechanical pressure. This is a characteristic symptom of the infection, as shown in Figure (1). The seedlings were washed under running tap water for several hours, washed several times with sterile distilled water, and then placed on sterile filter paper to remove excess water. to 1-2 cm long pieces of infected seedlings were taken from the crown area and a part of the petiole using a sterile knife and forceps. These pieces were planted at a rate of five pieces per plate, and the cultured plates were incubated in an incubator at 25 ± 2°C. After 2-3 days, the plates were tested and the growing fungal colonies were purified from the growing colonies. They were then examined under a light microscope at 40x magnification. The fungal species were identified based on morphological traits, colony morphology, mycelium type, sporophyte shape and structure, spore shape, and other structures (19). The identified and purified isolates were stored in a refrigerator in P.D.A. slant medium in test tubes for subsequent studies. The replication percentage was calculated using Eequation (17).

$$\text{Frequency percentage}(\%) = \frac{Number\ of\ fungal\ colonies\ that\ appeared\ in\ each\ piece}{Total\ number\ of\ planted\ pieces} \times 100$$

Figure (1) Symptoms of cucumber root rot diseases

3- Isolation of biological resistance elements from soil using the dilution method.

Random samples were collected from the rhizosphere soil of cucumber crops in the Babylon Province. These plants exhibit good and outstanding vegetative growth. The soil was left in the laboratory to air-dry for 24 h , after which it was sieved through a 1 mm sieve. A series of dilutions of soil samples ($10^{-1}$…$10^{-5}$) were prepared. One milliliter of the fifth dilution was transferred to sterile 9 cm diameter Petri dishes containing sterile PDA medium with the antibiotic tetracycline added at a concentration of 250 mg/L, in four replicates. The plates were rotated to ensure even sample distribution and the plates were incubated in an incubator, at 25 ± 1°C for 4 days. The fungal isolates were then purified by transferring portions of the edges of the fungal colony using a sterile inoculation needle to Petri dishes containing sterile P.D.A. The dishes were incubated in an incubator at 25 ± 1°C for 5 days (31). Trichoderma spp. isolates were identified using approved taxonomic keys (39), and antagonistic ability was assessed according to the scale of (12).

degree = traits

1 = The antagonistic fungus covers the entire surface of the plate, preventing the pathogenic fungus from growing.

2 = The antagonistic fungus covers two-thirds of the surface of the plate, and the pathogenic fungus covers the remaining third of the plate.

3 = The antagonistic fungus covers half of the surface of the plate, and the pathogenic fungus covers the other half of the plate. 4 = The antagonistic fungus covers one-third of the plate, whereas the pathogenic fungus covers the remaining two-thirds of the plate.

5 = The pathogenic fungus covers the entire dishes.

Biological agents are considered antagonistically effective when they exhibit an antagonism degree of ≤ 2 against the pathogenic fungal isolate under study.

4- Phenotypic Identification of the Fungi Under Study.

A- Phenotypic Identification of Pathogenic Fungi Isolated from Infected Plants:

The isolated fungi were identified from the root group to the genus level based on the morphological characteristics of the colonies, including color, shape, colony diameter, and height, in addition to the pigments produced in the culture medium (potato dextrose agar). Microscopic characteristics were also examined, including the nature of the fungal hyphae, shape and structure of the spores, size of the conidia, color, and other structures. The classification process was carried out according to approved taxonomic principles, and relied on taxonomic keys for each.

B- Pathogen Identification.

The fungus was identified after identifying the physical structures of the asexual and sexual reproductive organs and the characteristics of the mycelium, based on the taxonomic key provided by (19). This identification was supervised by Assistant Professor Kazem Zaghir Khudair of the Department of Biocontrol Techniques.

C- Phenotypic Identification of the *Trichoderma* spp. fungal isolate.

The first *Trichoderma* sp. fungus isolate was regrown in 9 cm diameter Petri dishes containing potato dextrose agar (PDA) medium, which was obtained from the Pathology Laboratory of the Department of Biocontrol Techniques/Technical College/Al-Musayyab and was previously identified. The second isolate (*T.virens*) was obtained by dilution from the soil medium of cucumber plants, which had large and distinct vegetative growth. The plates were incubated at 25 ± 2°C. After 5 days, the fungus was identified based on the characteristics of the fungal culture, nature of the fungal mycelium, and spores formed, using approved taxonomic keys.

5- Molecular identification of the fungi isolated from the soil using Polymerase Chain Reaction (PCR) technology.

A- DNA extraction of the isolated fungi:

This extraction experiment was carried out in the laboratory of Wahj Al-Dana Molecular Research Company, where the genetic material (DNA) of the fungi *T.virens* and *P. aphanidermatum* was isolated for identification. After the fungi were grown in Petri dishes containing PDA culture medium, the ZR-Fungal/Bacterial DNA MiniPrepTM kit, produced by the American company Zymo Research, was used, following the established basic steps.

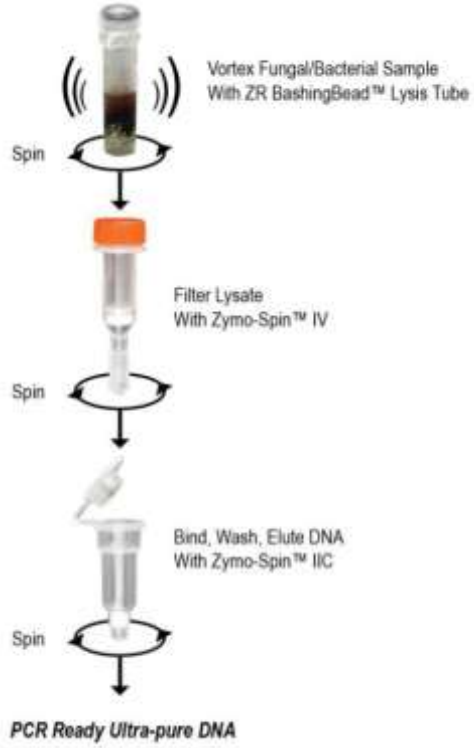



Figure (2) Stages of DNA extraction from isolated fungi.

B- Polymerase chain reaction (PCR) technique.

To conduct the PCR, I used ITS1 and ITS4 primers for fungi manufactured by Integrated DNA Technology, as shown in Tables (2) and (3).

**Table (2) Sequence of the specialized primer for detecting the ITS gene in the fungi *T.virnes* and *P.aphanidermatum.***

| Product size | GC (%) | $T_m$ (°C) | Sequence | Primer |
|---|---|---|---|---|
| 650 - 800base pair | 50 % | 60.3 | 5′- TCCGTAGGTGAACCTGCGG -3′ | Forward |
| | 41 % | 57.8 | 5′ TCCTCCGCTTATTGATATGC-3′ | Reverse |

The dried primer culture was dissolved in standard water (free from DNA-degrading enzymes) according to the manufacturer's instructions to obtaina final concentration of 100 pmol/ µL and served as a stock solution. The working solution was prepared by diluting the stock solution to 10 pmol/ µL.

Third: Preparation of the Master Mix.

The reaction mixture was prepared in a final volume of 25 microliters by mixing the components according to the instructions of the Korean manufacturer, Intron Biotechnology, with a final volume of 25 microliters. The contents of the reaction mixture were mixed for several seconds, and the tube was then placed in a thermocycler. DNA amplification was performed according to the program shown in Table (3) for fungi.

**Table (3) Polymerase program for DNA fragment amplification of T.virens and P.aphanidermatum fungi.**

| No. of cycle | Time | Tm (°C) | Phase | No. |
|---|---|---|---|---|
| 1 cycle | 5 min. | 94°C | Initial Denaturation | 1 |
| 35 cycle | 45sec | 94°C | Denaturation -2 | 2 |
| | 45sec | 52°C | Annealing | 3 |
| | 45sec | 72°C | Extension-1 | 4 |
| 1 cycle | 7 min. | 72°C | Extension -2 | 5 |

h-agarose gel electrophoresis of DNA. 1- DNA was electrophoresed on an achromatographic gel to confirm its quality after extraction or after polymerase chain reaction (PCR) using different gel concentrations depending on the purpose of the migration. A 0.8% (w/v) achromatographic gel was used to carry the DNA, whereas a 1.5% gel concentration was used to detect PCR products.

2- The achromatographic gel was prepared according to a previously described method. (41) by dissolving it in 1XTBE solution and

heating it in a microwave for 3 min to complete the lysis. It was then left in the laboratory until it reached 50°C. Two microliters of Red Safe stain was then added with gentle stirring to mix the dye with the gel. The gel was then gently poured into an electrophoresis plate to avoid the formation of bubbles within the gel, which would impede the movement of DNA, after pre-fixing the comb.

3- The electrophoresis plate was immersed after the gel solidified, and the combs were removed using XTBE1 solution. Five microliters of DNA was mixed with 3 microliters of 6x loading dye inside the wells.

4. The DNA was carried on an achromatic gel for half an hour, while the PCR products were carried on an achromatic gel for an hour (70 volts, 65 volts), as the DNA began to move from the negative electrode to the positive electrode.

5. The PCR products were electrophoresed on an achromatic gel with a known molecular weight indicator called a DNA ladder (Kapa, USA). This ladder comes in 18 different bands, starting with the following molecular weights: 100, 150, 200, 300, 400, 500, 600, 800, 1000, 1200, 1600, 2000, 4000, 5000, 6000, 8000, 10,000) bp.

6- Upon completion of the migration, the bands were observed and imaged using a UV transiluminator at a wavelength of 360 nm.

*1XTBE was prepared by diluting 10 ml of 10XTBE solution (the base solution) in 90 ml of sterile distilled water to obtain a concentration of 1XTBE.

G- DNA sequencing.

The nitrogenous base sequences of the polymerase chain reaction (PCR) products of the ITS1 and ITS4 genes of the fungus were determined by sending the reaction products to the Korean company Macrogen to determine the fungal DNA sequence and identify the fungal species.

The sequences obtained from the Korean company Macrogen were analyzed using the global website of the National Center for Biotechnology Information (NCBI) under the BLAST subwindow, followed by the nucleotide BLAST subwindow.

6- Evaluation of the antagonistic potential of the fungi *T.virens* and *T. harzianum* against the pathogenic fungus *P. aphanidermatum* on PDA culture medium.

The antagonistic ability of *T. virens* and *T. harzianumm* against the pathogenic fungus P. aphanidermatum was tested using the double-culture method. Sterilized PDA medium was prepared in an autoclave under sterile conditions at 121°C and 1 atm and poured into sterile 9 cm Petri dishes. The dishes were divided into two equal parts. The first part of the dish was inoculated with the pathogenic fungus. A 0.5 cm diameter disc was taken from a 7-day-old fungal culture, while the other part of the dish was inoculated with a 0.5 cm diameter disc from a 7-day-old *T. virens* culture. The experiment was conducted with

four replicates. The dishes were placed in an incubator at 25 ± 1°C for one week. The percentage inhibition of fungal growth was calculated usingthe following equation (35). The following: -

inhibition%

=

$$\frac{\text{Average diameter of control colony} - \text{Average diameter of treatment colony}}{\text{Average diameter of control colony}}$$

×100

The same experiment was repeated with the *T. harz*ianum funga isolate, and the inhibition rate was measured according to the equation above.

7- Evaluation of the effectiveness of the nutrients boron (Bo), iron (Fe), agricultural gypsum (calcium sulfate) (CaSo4.2H2O) in inhibiting the growth of the pseudo-fungus *P. aphandermatum* on PDA culture medium.

The poisoned food method was used to estimate the effectiveness of nutrients (boron, boron, iron, iron, agricultural gypsum, and calcium sulfate CaSo4.2H2O). Concentrations (0.5, 1, 1.5, and 2 mL) of each nutrient were prepared by adding 0.5, 1, 1.5, and 2 ml of each element to 1 liter of PDA medium in a glass flask after sterilizing the medium in an autoclave. The flasks were shaken well to ensure the homogeneity of the mixture. The mixture was poured into sterile Petri dishes (9 cm in diameter) and allowed to solidify. Each dish was inoculated with a 5 mm diameter disc taken from the edge of a 9-day-old *P. aphandermatum* colony using a cork piercer, previously grown on PDA medium. The disc was placed in the center of each poisoned plate, upside down, with the part containing the fungal hyphae perpendicular to the center of the poisoned plate. The plates were incubated at 25 ± 2°C until the control treatment reached the edge of the plate alone. (4) Plates were used for each concentration, whereasthe control treatment included the pathogenic fungus without nutrients. The percentage of inhibition was calculated using Eequation (35) mentioned in paragraph (6). The effective concentration was chosen for subsequent experiments.

8- Preparation of the *B. paramycoides* bacterial suspension.

The *B. paramycoides* isolate was obtained from the Pathology Laboratory of the Biocontrol Techniques Department/Technical College/Al-Musayyab, and was previously diagnosed. It was propagated on Nutrient Broth medium in sterile 500 ml glass flasks in an autoclave at 121°C and 1.5 kg/cm² for 15 minutes. The medium was then inoculated with the desired bacteria by taking a sterile swab from the previously prepared 48-hour-old bacterial growth on Nutrient Agar. The flask components were mixed well and incubated at 32 ± 3°C for 3-4 days.

A- Determining the effective concentration of the *B. paramycoides* inoculum that inhibits the growth of *P. aphidermatum*.

A series of dilutions ($10^{-1}$....$10^{-8}$) of *B. paramycoides* bacterial inoculum was prepared

by adding1 ml of the bacterial inoculum grown on the liquid nutrient broth medium at the age of 48 husing a sterile pipette (micropipette) and adding it to a test tube containing 9 ml of sterile distilled water. This process was repeated until a 10-8 dilution was reached. Potato dextrose agar (PDA) was prepared without adding antibacterial tetracycline. Then, the plates prepared for the experiment were inoculated with1 ml of each dilution of *B. paramycoides* bacterial inoculum. They were left for 24 h in an incubator at a temperature of 32 ± 3 °C. Subsequently, a disc with a diameter of 0.5 cm was placed in the center of the plate from the edges of a semi-isolated colony of the fungus *P. aphandermatum* grown on potato dextrose agar (PDA) medium at the age of 9 days, at a rate of four plates for each dilution. Four plates of fungi left without inoculation with bacteria were added to 1 ml of sterile distilled water for comparison. The plates were incubated at a temperature of 25±2°C until the fungi in the comparison treatment reached the edges of the plate. the percentage inhibition of fungal growth was calculated according to the following equation:- (35), as mentioned in paragraph (6):-

B- Calculating the Population Density of *B. paramycoides*

After obtaining an inhibitory dilution of the *B. paramycoides* inoculum for the pathogenic *P. aphandermatum*, a set of sterile Petri (,-9 cm in diameter), was prepared containing Nutrient Agar. These dishes were inoculated with 1 ml of the inoculum suspension for each dilutions 10-6, 10-7, and 10-8, with four replicates for each dilution. The dishes were incubated at 25 ± 2°C for 48 h. The number of colonies in the 10-7 dilution was counted to ensure that the colony count was clearly determined. The number of bacterial cells was calculated as follows:

Number of bacteria/ml of the original sample = number of colonies in the dish × reciprocal of the sample dilution (Al-Kar'awi, 2022).

Results and Discussion:

1- Field survey of cucumber root rot disease.

The results in Table (4) show the presence of root rot disease in all areas covered by the survey in cucumber crop fields and in protected agriculture in Babylon province , with varying infection rates ranging between 29% and 56% and infection severity. 19% - 39.25%, as samples from the areas of Zubaidi area, affiliated with Kuthi district, gave the highest percentage and severity of infection, which amounted to 56% and 39.25%, respectively, followed by samples from al-Dulaimi area, also affiliated with Kuthi district, which amounted to 47% and 39.25%, respectively. Samples from the Hamza area, affiliated with the Al-Hashimiya district, had the lowest 29% and severity (19%) of, infection. The widespread spread of cucumber root rot disease may be due to repeated cultivation inside greenhouses and in the same fields, that is., they are specialized for growing cucumber plants without following agricultural

rotations, which leads to the accumulation of fungal inoculum of pathogens, or perhaps as a result of the presence of strong pathogenic fungal strains or because of the availability of suitable environmental conditions, especially temperatures suitable for the growth of pathogenic fungi, which play a major role in increasing the spread of fungal inoculum. In addition, weeding and weeding operations may cause wounds in the roots, which facilitate the invasion of fungi. In addition, the severity of infection varies according to crop and soil maintenance, irrigation regularity, control methods, and unbalanced fertilization, in terms of quantity or quality. Oosomes, which are the main structures responsible for the initial infection of the fungus, are found at a depth of 5-10 cm in the soil and can remain dormant in dry soil for 12 years under laboratory conditions., They play a role in the survival of the pathogen and the spread of infection (24).

**Table (4) Field survey of cucumber root rot disease in some areas of Babylon province 2024**

| No. | Region | Infection rate % | Infection severity % |
|---|---|---|---|
| 1 | Babylon - Kutha District - Zabidi | %56 | %39.25 |
| 2 | Babylon - Kutha District - Dulaimi | % 47 | %31.9 |
| 3 | Babylon - Kutha District - Al-Himyari | %43 | 34% |
| 4 | Babylon - Kutha District - Al-Imam Branch | %35 | %26.37 |
| 5 | Babylon - Al-Hashimiyah District - Al-Hamza | %29 | %19 |

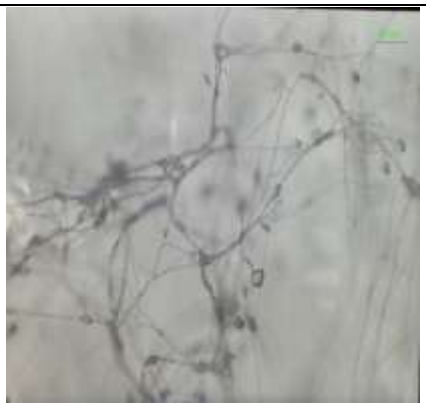
2- Isolation and identification of fungi associated with the roots of infected cucumber plants.

The results of isolation and identification from the roots of infected cucumber plants that showed wilt symptoms revealedthe presence of five fungal genera: *Pythium* sp., *Verticillium* sp., *Fusarium Macrophomina* sp., and *Alternaria* sp. (Table (5)). The fungal replication rates of the isolates varied. *Pythium sp*. showed the highest replication rate among the isolated fungal isolates (,67.136%), followed by *Fusarium* sp. (,12.10%). *Verticillium* sp. and *Alternaria* sp. had replication rates of 4.16 and 4.12%, respectively. *Macrophomina* sp. produced the lowest replication rate (,1.22%). These fungi can produce large numbers of reproductive units, which enhances their ability to withstand adverse environmental conditions. The high frequency of *Pythium* sp. may be due to the fact that when environmental conditions are unsuitable, the fungus produces thick-walled Oospores in order to survive for a longer period, as these spores remain dormant in the soil until they germinate when suitable conditions are available (24). or because of root exudates (27). Alternatively, the spores remain for a short period, and through the

asexual phase, a sporangium is formed, which can either germinate directly or indirectly by the formation of thin-walled, water-borne, swimming spores. This is the primary method of dispersal of these spores, as a result of the secretion of substances by these hairs that act as chemotaxis inducers, attracting the fungus towards them. These spores encyst, germinate, and cause infection by attacking sensitive young seedlings and newly formed root tissues through direct penetration (26; 4).

**Table (5): Isolation and identification of fungi associated with cucumber roots.**

| No. | fungus | Frequency% |
|---|---|---|
| 1 | *Pythium* sp. | 67.13 |
| 2 | *Fusarium* sp. | 12.10 |
| 3 | *Verticillium* sp. | 4.16 |
| 4 | *Alternaria* sp. | 4.12 |
| 5 | *Macrophomina* sp. | 1.22 |



Microscopic form of the fungus *Pythium* sp.

Colony shape of the fungus *Pythium* sp.

Figure 2: Morphological and microscopic appearance of *Pythium* sp.

3- Testing the pathogenicity of pathogenic fungal isolates using radish seeds on a water agar medium.

The results of Table (6) for the pathogenicity test showed that all isolates caused a significant reduction in the germination percentage of radish seeds grown on water agar, with a clear difference in the effect of these isolates compared to the control treatment, which reached 100%. The germination percentage ranged between 0-24%, with *Pythium* sp. (Py2) fungal isolates producing the lowest germination percentage, reaching 0.00%. This is because of their high pathogenicity. As a result of infection, the

fungus spreads between and within host cells, where it secretes pectolytic enzymes that decompose pectin in the host cells (37). This was followed by the isolate (Py3), which reached 1.00%.,The Fusarium sp. isolate produced (Fu3) had the highest germination rate for radish seeds, reaching 24.00% (Figure (3)). The reason for the difference in pathogenicity among these isolates is genetic differences in pathogenicity, or the difference in the effect of fungi on germination rates is due to several environmental and biological factors, in addition to the variation in the ability of these fungi to produce enzymes and toxins, which are the primary factors causing infection. These results are consistent with thosereported previously (29) and (1). Another important factor is the toxins produced by fungi, as these toxins can hinder basic cellular processes, such as respiration and photosynthesis, leading to disrupted seed growth and preventing proper germination (36).

**Table (6) Detection of pathogenic fungal isolates using radish seeds.**

| Germination percentage from radish seeds (%) | Number of germinated seeds from radish seeds (%) | Isolation symbol | fungus | No. |
|---|---|---|---|---|
| 5.00 | 1.25 | Py1 | *Pythium* sp. | 1 |
| 0.00 | 0.00 | Py2 | *Pythium* sp. | 2 |
| 1.00 | 0.25 | Py3 | *Pythium* sp. | 3 |
| 6.00 | 1.50 | Py4 | *Pythium* sp. | 4 |
| 15.00 | 3.75 | Fu1 | *Fusarium oxysporum* | 5 |
| 21.00 | 5.25 | Ve | *Verticillium* sp. | 6 |
| 19.00 | 4.75 | Al | *Alternaria* sp. | 7 |
| 23.00 | 5.75 | Ma | *Macrophomina* sp. | 8 |
| 20.00 | 5.00 | Fu2 | *Fusarium* sp. | 9 |
| 24.00 | 6.00 | Fu3 | *Fusarium* sp. | 10 |
| 100.00 | 25.00 | Control | | 11 |
| 9.3703 | 2.3426 | L.S.D $_{0.05}$ | | |


Figure (3) Isolation of the fungus Pythium sp. (Py2) with the control treatment on radish seeds.

4- Phenotypic identification of the fungi under study.

A- Phenotypic identification of the pseudofungus *Pythium aphanidermatum.*

Pseudofungus *p.aphanidermatum* is characterized by its coenoctic, translucent, branched, and rapidly growing fungal hyphae on potato dextrose agar. It grows rapidly, has a spherical or oval appearance, is unspecialized, and contains thick-walled, spherical sexual organs (oospores) (2). These results are consistent with those of (20), who demonstrated that *Pythium* sp. It forms a white, undivided, cottony mycelium, called a compact cell. Hyphae weare highly branched and grew rapidly. The mycelium forms sporangia, whereas the sporophytes are of varying lengths, and their branches are in the form of swellings. These sporophytes are > 20 µm wide and bear irregular, finger-shaped sporophytes.

B. Morphological diagnosis of *Trichoderma virens.*

It is characterized by fungal hyphae that are divided by transverse septa and are initially transparent. It grows rapidly on the dictrose potato agar medium. The colonies weare initially white and, then turned green to dark green because of spore production. The colonies had acottony or powdery textures. Unicellular conidia may appear spherical to oval in shape, green in color when mature, and form short branches. Conidia weare typically branched, forming a tree-like pattern. This is consistent with the findings of (39). 5- Molecular diagnosis of the fungi under study using Polymerase Chain Reaction (PCR) .

Table (7) shows the results of the molecular diagnosis of the fungal isolates under study, matching the phenotypically identified species, according to the classification criteria adopted for phenotypic description, with the molecular diagnosis results based on the ITS1 and ITS4 regions. The isolate of the pathogenic fungus *P. aphanidermatum* was registered under

accession number PV579842, and this isolate matched the Oman isolate registered under accession number MT510400.1 with a 91.57% match rate. The isolate of the biotrophic fungus *T. virens* was registered under accession number PV579841, and this isolate matched the Chinesea isolate registered under accession number MK870572.1, with a 99.72% match rate.

**Table (7) shows the codes of the isolates registered in GenBank along with their matching isolates.**

| Country | Global isolation symbol | Identity percentage | Isolation symbol | fungus name | No. |
|---|---|---|---|---|---|
| MT510400.1 | Oman | 91.57 | PV579842 | *Pythium aphanidermatu* | 1 |
| MK870572.1 | China | 99.72 | PV579841 | *Trichoderma virnes* | 2 |

6- Results of isolating bio control elements from soil using the dilution method.

A group of fungi was obtained, including an isolate of the biotrophic fungus *T. virens*, which was selected because it was highly effective against pathogenic fungi. Its growth occupied the entire surface of the plate, and it recorded a degree of (1) antagonism according to the (12) scale against the pathogenic fungus *P. aphanidermatum.*

7- Evaluation of the effectiveness of the nutrients boron = Bo, iron = Fe, and agricultural gypsum = (hydrated calcium sulfate) CaSo4.2H2O in inhibiting the growth of P. aphanidermatum on the PDA culture medium.

The results in Table (8) show that significant differences were obtained when using some nutritional elements to inhibit the diameter growth and percentage of inhibition of the pathogenic fungus *P. aphandermatum*, and significant differences were obtained when using some nutritional elements. The boron element outperformed the other nutritional elements, as the diameter growth and percentage of inhibition reached 0.0 cm and 100%, respectively, when using a concentration of 2 ml/liter, compared to the comparison treatment, which reached the diameter growth and percentage of inhibition of 9.0 cm and 0.0%, respectively. Boron treatment also resulted in a high percentage of inhibition against the pathogenic fungus, which reached 100%, that is, the diameter of the pathogenic fungal colony was 0.0 cm. This is attributed to the fact that boron leads to a defect in the fungal cell membrane through a decrease in the expression of antioxidant enzymes and an increase in reactive oxygen species (ROS) in the spores of *Penicillium expansum* and Botrytis cinerea (38). This is consistent with what was found by (45) that boron reduces the activity of antioxidant enzymes and leads to lipid peroxidation in the membrane of P. nicotianae, thus destroying the antioxidant system in *P. nicotianae* is one of the reasons for boron inhibition of sporangia

formation. It was also followed by the treatment of agricultural gypsum (hydrated calcium sulfate) ($CaSo_4 \cdot 2H_2O$), in which the diameter growth and inhibition percentage of the pathogenic fungus reached 7.50 and 52.77 when using the concentration of 2 g/L. This may be attributed to the fact that agricultural gypsum is composed of calcium and sulfur, as calcium reduces the penetration of cells by pathogens (30). It also improves soil aeration and water drainage by loosening the soil and improving its physical structure, which increases aeration around the roots and reduces the spread of these diseases (14). This was followed by iron treatment of the pathogenic fungus, in which the diameter growth and inhibition rate of the pathogenic fungus reached 9.00 and 0.50 at a concentration of 2 g/L. This is attributed to the fact that iron plays a pivotal role in the formation of oxidative enzymes such as peroxidase and catalase, which are involved in antioxidant defenses and help neutralize free radicals generated by the plant's response to pathogens. Iron is also involved in the activation of defense genes duringinfection (30).

**Table (8) Evaluation of the efficiency of nutrients in inhibiting the growth of the fungus *P.aphanidermatum* on the PDA culture medium.**

| Inhibition rate % | Colony diameter (cm) | concentration | treatments |
|---|---|---|---|
| | | | |
| 0.13 | 9.00 | 0.5 | Fe+Py |
| 0.25 | 9.00 | 1 | |
| 0.38 | 9.00 | 1.5 | |
| 0.50 | 9.00 | 2 | |
| 38.89 | 7.00 | 0.5 | $CaSo_4H_2o$+PY |
| 44.44 | 6.50 | 1 | |
| 47.22 | 7.50 | 1.5 | |
| 52.77 | 7.50 | 2 | |
| 0.00 | 9.00 | 0.5 | Bo+Py |
| 83.33 | 0.75 | 1 | |
| 100.00 | 0.00 | 1.5 | |
| 100.00 | 0.00 | 2 | |
| 0.00 | 9.00 | 0 | Py alone |
| 16.001 | 0.8492 | LSD 0.05 | |

*Each number in the table represents the average of up to four replicates.

8- Testing the antagonistic capacity of some bio control agents, including the fungi *T.virens* and *T.harzianum*, the bacteria *B.paramycoides*, and the fungicide Metalaxyl, to inhibit the pathogenic fungus *P. aphanidermatum* on PDA culture medium in the laboratory.

The results in Table (9) show a high antagonistic capacity between the bio control agents, which included the fungi *T.virens* and *T.harzianum*, and the bacteria *B.paramycoides*, against the pathogenic fungus *P. aphanidermatum*, compared to its control coefficient. Treatment with *B. paramycoides* resulted the highest inhibition rate for the pathogenic fungus *P. aphanidermatum*, which reached 100%, compared to the control treatment, which reached 0.0%. This may be attributed to the fact that these bacteria possess high enzymatic capabilities that have the ability to analyze the cell walls of pathogenic fungi or antibiotics that can inhibit pathogenic fungi. This is consistent with our findings. (16) found that these bacteria have the ability to produce many biological compounds, such as *Bacillus* and fengycin, which inhibit pathogenic fungi. They also produce the biological compounds macrolacin, bacillaen, dididi, bacilysin, plantazolicin, and amylocyclicin, which suppress and directly kill pathogenic fungi, in addition to the volatile compound Acetoin/2,3-butanediol, which causes systemic resistance in treated plants. The treatment of the two fungi *T.virens* and *T.harzianum* used in the biocontrol achieved a high antagonistic capacity against the pathogenic fungus compared to the control treatment, reachinge 97.215 and 93.525%, respectively, compared to the control treatment, which reached 0.0%. This effect on the variation in the antagonistic capacity of the isolates was attributed to the difference in the types of *Trichoderma* sp. This inhibition may be attributed to the ability of *Trichoderma* sp. It produces many antibiotics such as Gliotoxin, Alamethicins, Viridol, Harzianic acid, and trichoviridine. These antibiotics have a synergistic effect when linked to other enzymes that degrade the cell wall of the pathogenic fungus, thus causing an inhibitory effect on many pathogenic fungi. In addition to having the ability to decompose the hyphae of the pathogenic fungus by producing the decomposing enzymes chitinase, protease,and β-1 glucanase, it is considered a very important means of combating plant diseases. It also has the ability to compete for food sources, because competition for nutrients is one of the most important means of biological resistance (3, 34, 28, and 40). The results inTable (9) also showed that 100% inhibition of the pathogenic fungus was obtained by using the fungicide Metalaxyl, compared to the comparison treatment, which amounted to 0.0%. This result was confirmed by many studies that have shown that the use of Metalaxyl on PDA culture medium completely inhibits the growth of pathogens (23 and 6). The effective effect of metalaxyl is attributed to its highly efficient systemic fungicidale activity against many pathogenic fungi.

Metalaxyl belongs to the phenylamide group and works primarily by inhibiting RNA synthesis within oomycete cells, especially RNA polymerase I, the enzyme responsible for ribosomal RNA (rRNA) production. This inhibition disrupts the production of vital fungal proteins, therebyhalting growth and reproduction (18).

**Table (9) Testing the antagonistic ability of some biological resistance elements represented by the fungi *T.virens* and *T.harzianum*, the bacteria *B.paramycoides*, the element boron, and the pesticide Metalaxyl in inhibiting the isolation of the pathogenic fungus *P. aph*anidermatum on the PDA culture medium.**

| Inhibition rate % | diameter Colony | treatments* | numbers |
|---|---|---|---|
| 97.22 | 0.50 | *T.virens* + Py3 | 1 |
| 93.05 | 0.63 | *T. harzianum* +Py3 | 2 |
| 100.00 | 0.00 | *B.paramycoides* + Py3 | 3 |
| 100.00 | 0.00 | Metalaxyl + Py3 fungicide | 4 |
| 0.00 | 9.00 | Py3 fungicide alone (comparison) | 5 |
| 0.794 | 0.7945 | L.S.D. (5%) | |

*Each number in the table represents the average of four replicates.

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