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ORIGINAL ARTICLE

ISOLATION AND IDENTIFICATION OF THE PATHOGENIC FUNGUS *VERTICILLIUM NUBILUM* THAT CAUSES VERTICILLIUM WILT DISEASE ON EGGPLANT AND THE EFFECTIVENESS OF SOME BIO CONTROL ELEMENTS IN ITS INHIBITION IN LABORATORY

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Abstract: The results of isolation and identification of fungal growths on the culture medium PDA showed obtaining several different isolates from different regions of the pathogenic fungus *Verticillium nubilum*. The results of the PCR transfer on an agarose gel were shown by giving a band size of 500-650 bp to the pathogenic fungus *Verticillium nubilum*. The isolates of the pathogenic fungus *V. nubilum* caused a significant decrease in the percentage of germination of eggplant seeds grown on water agar medium, compared to the control treatment, which amounted to 100%, where the germination rate ranged between (1-73%). The results also showed that there was a high antagonistic ability and high significant differences between the pathogenic fungus *V. nubilum* and the bio-control elements, compared to the control treatment, which amounted to 0.0%. The treatment of *Bacillus subtilis* gave the highest inhibition rate, reaching 96.75%. Followed by the treatment of the fungus *Trichoderma harzianum*, which reached 91.07% and the treatment of Beltanol gave an inhibition rate of 100%.

***Key words*:** Eggplant, *Verticillium nubilum, Trichoderma harzianum, Bacillus subtilis*.

Cite this article

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1. Introduction

The eggplant crop (*Solaum melongena* L.) is considered one of the important vegetable crops for human health because it contains a high percentage of minerals and vitamins. It was classified in terms of nutritional value as the third largest crop after tomatoes and potatoes of the Solanaceae family [Chapman (2020)]. Eggplant has been known since ancient times, as it was growing in India and China, which are considered its original home, and it is cultivated in temperate and hot regions of the world. As mentioned by Priscilla (2013) that eggplant spread across Iran from India to the Arab Islamic lands. Eggplant in open fields and greenhouses is affected by many viral, fungal and bacterial pathogens that reduce the yield qualitatively and quantitatively and cause severe damage to it. Wilt diseases caused by some fungi are among the most important and widespread diseases, and the extent of loss resulting from infections is largely related to the density of the pathogenic fungus pollen present in the soil, the sensitive host, and the planting date, in addition to environmental factors [Sadeghi *et al*. (2008)]. Symptoms of verticillium wilt are systemic, represented by drooping and bending leaves, and in some cases a typical V-shaped yellowness appears. In some cases, yellow spots develop on the leaves between the veins, which later turn into brown necrosis [Klosterman (2009)]. The many uses of chemicals have led to many health and environmental problems for humans and animals. In addition to what was mentioned, its frequent use works to develop new strains that cause plant diseases that are resistant to the effect of these

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pesticides. Therefore, the research aim was to reveal the antagonistic ability of some biocontrol elements against the pathogenic fungus *V. nubilum* in the laboratory.

2. Materials and Methods

2.1 Isolation of the pathogenic fungus, *Verticillium* spp

Samples of eggplant plants showing signs of infection were collected to 4 locations of fields located within the Babylon province (Table 1), where infected and healthy plants were tested randomly, located within the intersection of the diameters of each location. Some of the infected plants were placed in polyethylene bags and transported to the laboratory. The fungi were isolated from the infected roots. The roots were washed with running water for 10 minutes to remove the dust stuck in them, and small pieces were taken and sterilized using sodium hypochlorite solution (2% free chlorine) for 2-3 minutes. Then, it was transferred to sterile distilled water for two minutes and washed well, then placed on sterile filter paper inside the hood to dry it of excess water. Then the infected root parts were transferred using sterile forceps and planted in 4 plant pieces in petri dishes with a diameter of 9 cm containing 15-20 ml of culture medium (PDA) Potato Dextrose Agar to which the antibiotic Tetracycline was added at a concentration of 200 mg /L. After sterilizing it with an autoclave at a temperature of 121°C and a pressure of 1.5 kg/cm^2 for 15-20 minutes, then the dishes were incubated in the incubator at a temperature of 25±1°C for 4 days, after which the examination was carried out for the growing fungal colonies by taking small parts from each colony. The fungal colonies were purified into new Petri dishes containing culture medium (PDA) and incubated at a temperature of 25±1°C for 7 days to be used in subsequent experiments. The infection rate was calculated and the percentage of infection was calculated for each field in the light of the symptoms appearing on the plants using the following equation:

$$\text{Infection percentage \%} = \frac{\text{The number of infected plants}}{\text{The total number of tested plants}} \times 100$$

The severity of the injury was calculated according to the following gradation.

0 = No infection.

1 = Weak infection, symptoms of wilting covering 1-25% of plant leaves.

2 = Medium infection with symptoms of wilting, including 26-50% of the leaves of the plant.

3 = Severe infection, Symptoms of wilting include 51-75% of the leaves of the plant.

4 = very severe infection, death of the plant, symptoms of wilting, including 76-100% of the leaves of the plant, or the death of the entire plant. The severity of infection was calculated as follows

$$\text{Infection severity \%} = \frac{\text{(The number of grade plants 0×0 +... +The number of grade plants 4×4}}{\text{The total number of plants tested} \times 4} \times 100$$

Table 1: The temporal and spatial distribution, isolation symbol and cultivar of the sites of eggplant fields and cultivation that were covered by the field survey in Babylon province.

Sample number	Location(s)	Cultivars	Isolation symbol	The date of taking the sample is 2022	The area cultivated in dunums
1	Babylon / AL MUSSAIB Project	Barcelona	V1	4-5-2022	1 dunum
2	Babylon / Sridip	Barcelona	V2	28-5-2022	8 dunum
3	Babylon \aljadwal algharbiu	Barcelona	V3	28-5-2022	10 dunum
4	Babylon \ al-Azzawiya	Barcelona	V4	12-6-2022	2 dunum

2.2 Diagnosis of the pathogenic fungus: the person of the fungus based on the following traits

2.2.1 Visual and microscopic traits: A disc with a diameter of 0.5 cm was transferred from the edge of the new colony of pure pathogenic fungal isolates to the middle of the Petri dish containing PDA culture medium, and the dishes were incubated at a temperature of 25 ± 1°C for a period of 10-15 days. The fungus was diagnosed after the end of the incubation period at the species level after the emergence of fungal growths, based on the nature of the mycelium, the traits of the fungal colony and the

Table 2: Standard kit for DNA extraction.

Contents	Size/number
ZR BashingBead™ Lysis Tubes (0.1 & 0.5 mm)	50
Lysis Solution	40 ml
Fungal/Bacterial DNA Binding Buffer	100 ml
DNA Pre-Wash Buffer	15 ml
Fungal/Bacterial DNA Wash Buffer	50 ml
DNA Elution Buffer	10 ml
Zymo-Spin™ IV Spin Filters (Orange Tops)	50
Zymo-Spin™ IIC Columns	50
Collection Tubes	150
Instruction Manual	1

manufacturer's instructions to give a final concentration of 100 picomoles/microliter and prepare a stock solution. As for the daily working solution, it was prepared by diluting the stock solution to 10 picomoles/ microliter.

Prepare the master mix: The reaction mixture was prepared in a final volume of 25 microliters by mixing the components shown in Table 4.

According to the instructions of the Korean company, Intron Biotechnology, then the contents of the reaction mixture were mixed for several seconds, then the tube was placed in a thermocycler. The DNA amplification reaction of the samples was conducted according to the program shown in Table 5.

2.2 Electrophoresis on an agarose gel for nucleic acid

Electrophoresis was performed to detect the results of the PCR reaction while in the presence of a standard DNA bundle size characterization. The acarose gel was prepared by adding 1.5 gm of acarose in 100 ml buffer solution (Tris - Borate EDTA TE) (its concentration is 1.5%). Heat to boiling point, then leave to cool until reaching 45-50°C. Pour the gel into the gel hardening trough carefully to avoid the formation of bubbles, then immerse the comb in the trough to make holes in the gel and leave the gel to solidify. Gently lift the comb and immerse the gel in a TBE buffer after it has fully solidified in the electrolytic relay basin. PCR products were injected into the wells of the immersed gel plate at 5 µl per well in addition to the standard Ladder solution. It was electrocuted at 700 volts/hour (for 21 hours). The stained DNA pieces were tested with a

Table 3 : Primers sequencing specific for the detection of the ITS gene in the pathogenic fungus *Verticillium*.

Primer	Sequence	Tm (^{0}C)	GC (%)	Product size
Forward	5′-TCCGTAGGTGAACCTGCGG-3′	60.3	50 %	500-650 base pair
Reverse	5′-TCCTCCGCTTATTGATATGC-3′	57.8	41 %	

structures it forms, and using the approved taxonomic keys.

2.2.2 Molecular diagnosis of the fungus *Verticillium* spp. using the Polymerase Chain Reaction (PCR) technique: 1- DNA extraction of fungi for the pathogenic fungus:

The extraction experiment was conducted in the laboratory of the DNA Glow Company for Molecular Research, where the genetic material (DNA) of the fungus to be diagnosed (*Verticillium* spp.) was isolated. After growing the fungus in a 9 cm diameter petri dish containing Potato Dextrose Agar (PDA) The ZR Fungal/Yeast/Bacterial DNA MiniPrep™ kit produced by the American company Zymo research was used using the materials shown in Table 2 and followed the recommendations of the private company that supplied the standard kit in extracting the DNA of the fungus isolates.

1. Polymerase Chain Reaction (PCR) technique

Primer: To perform the amplification chain reaction (PCR), it used the ITS1 and ITS4 primers manufactured by Integrated DNA technology as shown in Table 3.

The dried primers was dissolved in standard water (free of DNA-degrading enzyme) according to the

Table 4: Diagnostic specific reaction (PCR) mixture ((P[g..)

Components	Concentration
Taq PCR PreMix	5 µl
Forward primer	10 picomols/µl (1 µl)
Reverse primer	10 picomols/µl (1 µl)
DNA	1.5 µl
Distill water	16.5 µl
Final volume	25 µl

Table 5: The polymerase program to replicate the DNA segments of the pathogenic fungus *Verticillium* spp.

No.	Phase	Tm (^{0}C)	Time	No. of cycle
1	Initial Denaturation	94^{0}C	3 min.	1 cycle
2	Denaturation -2	94^{0}C	45sec	35 cycle
3	Annealing	52^{0}C	1 min	
4	Extension-1	72^{0}C	1 min	
5	Extension -2	72^{0}C	7 min.	1 cycle

gel document device under UV rays.

2.3 Determine the sequence of nitrogenous bases (DNA Sequencing)

To determine the sequence of the nitrogenous bases of the DNA replication segments in the products of the DNA amplification reactions of the isolates, the amplification products were sent to Macrogen company for the purpose of determining the nucleotide sequence of the recombinant DNA of the fungus. Testing the pathogenicity of isolates of the pathogenic fungus Verticillium spp using eggplant seeds and on the culture medium (water agar).

The pathogenicity of four isolates of *Verticillium* spp. was determined by inoculating Petri dishes with a diameter of 9 cm containing 15-20 cm^3 of culture medium and water agar to which the antibiotic Tetracycline was added at a rate of 250 mg/L after sterilizing it with an autoclave at a temperature of 121°C and a pressure of 1.5 kg/cm^2 for 20 minutes with a 5 mm disc. It was taken by a cork puncture near the edges of fungi colony at the age of 10 days. The disc was placed in the center of dish. 4 dishes were used for each isolate. As for the control treatment, a container was left on the medium W.A. Only then the dishes were placed in the incubator at a temperature of 25±1°C for 4 days. After that, local eggplants were planted (their germination percentage was previously tested) and sterilized superficially with sodium hypochlorite solution (2% free chlorine) for two minutes, by 25 seeds/dish, in a circular manner near the edge of the dish, approximately at equal distances. Four dishes were used for each isolate in addition to the control treatment without pathogenic fungus. The dishes were incubated after planting the eggplant seeds in the incubator at a temperature of 25±1°C for 15 days, after which the percentage of seed germination was calculated according to the following equation.

$$\text{Germination percentage} = \frac{\text{The number of germinated seeds}}{\text{The total number of seeds used}} \times 100$$

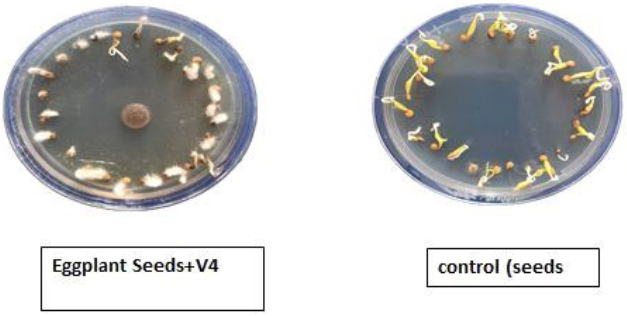
2.4 Statistical Analysis

A Completely randomized design was used in this research, and the GenStat statistical program was used in analyzing the data to study the effect of different treatments on the studied traits, and the significant differences between the means were compared with the Least Significant Difference (LSD) test.

3. Results and Discussion

3.1 Isolation and identification of the pathogenic fungus, *Verticillium nubilum*

The results of the survey (Table 6) showed the presence of wilt disease of the eggplant plant in all areas of Babylon provainc that were covered by the survey, with a percentage of infection ranging between 13-52% and a severity of infection ranging between 26-72%. The field of the western creek area, amounting to 44%. The reason for the high average of infection in these areas is due to the fact that they are areas dedicated to the cultivation of eggplant, in which the crop is grown annually and repeatedly or because of the cultivation of crops belonging to the Solanaceae family in these fields, which led to the accumulation of pollen of pathogenic fungi, and with regard to the Sclerotia, which live in the soil for a long time and to suit their environmental conditions, especially temperatures, their survival period in the soil may reach five years [EL-Mougy *et al.* (2011)]. The reason for this is the difference in the percentage of infection due to the variation in the use of agricultural operations, the widespread and repeated use of chemical pesticides for plant pathogens, and the type and method of adding organic and chemical fertilizers. It is also known that environmental factors such as humidity and temperature have a great effect on increasing the fungus pollen, as



Picture 1: shows the pathogenicity of the fourth isolate in its effect on eggplant seeds

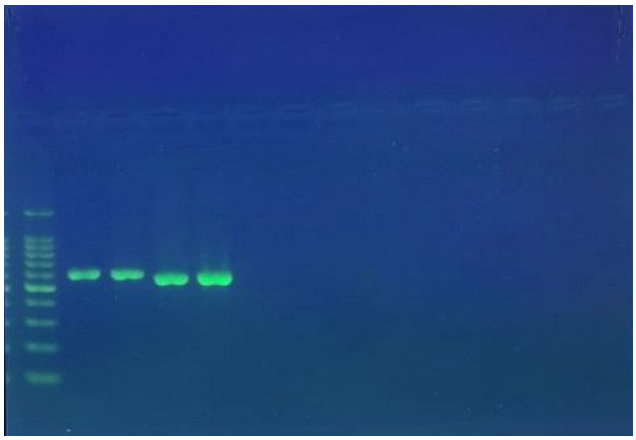


Fig. 1: Electrophoresis of the genetic material of *Verticillium* spp. isolates. Nucleotide sequencing study

in Jableh district samples. The reason for this is due to the difference in the locations of the fields that were surveyed, the difference in environmental factors, and the fact that these areas were planted with the eggplant crop for the first time, in addition to interest in soil and crop service operations.

3.2 Pathogenicity test

3.2.1 Detection of the pathogenic isolate of Verticillium spp. using eggplant seeds: The results showed (Table 7) the isolates of *Verticillium* spp. The experiment caused a significant decrease in the percentage of germination of eggplant seeds. It was noted that there was a large variation in the pathogenicity of the fungus isolates, where the Babylon/Azzawiya (V4) isolate was excelled in its pathogenicity to the isolates, which had a clear effect in reducing the percentage of germination, reaching 4% (Picture 1), compared to the isolates of the large project / Dulaimi, Sreideb and aljadwal algharbiu belonging to the province of Babylon (V2, V1, V3), which gave a germination rate of 73% -72% - 44%, respectively. The reason for the variation of the isolates in their effect on the percentage of daughters of eggplant seeds may be due

Table 6: Field survey of verticillium wilt disease of eggplant plants for some fields in Babylon province for the agricultural season 2022-2023.

Sample number	Location(s)	Cultivars	Field area/ dunam	Infection rate (%)	Infection severity (%)
1	AL MUSSAIB Project, Babylon	Barcelona	1	13	26
2	Sridip, Babylon	Barcelona	8	20	49
3	Aljadwal Algharbiu, Babylon	Barcelona	10	44	70
4	Al-Azzawiya, Babylon	Barcelona	2	52	72

Table 7: shows the pathogenicity test of the four isolates of the pathogenic fungus, *Verticillium* spp and its effect on eggplant seeds on PDA culture medium.

Treatments	Number of germinated seed	Germination %
V1	18.25	73
V2	18	72
V3	11	44
V4	1	4
Control	25	100
L.S.D.	1.67	6.54

well as increasing the pathogenicity of fungi, as all these factors affect the plant, making it more sensitive. As the results showed, the lowest infection rate was found to the genetic difference between the isolates collected from different regions. The reduction of germination of eggplant seeds was also due to the treatment of *Verticillium* spp. For its ability to produce lytic enzymes that have the ability to break down the structural barriers of plant cells, which have an important role in pathogenic processes, in addition to the toxic effects that this fungus produces on plant cells.

3.3 Isolation and diagnosis of the pathogen

3.31 Molecular Diagnosis using Polymerase Chain Reaction (PCR) Technology: The result of electrophoresis of DNA extracted from mushrooms under study on an agarose gel showed the presence of two bands with molecular weight (500 bp - 650 bp).

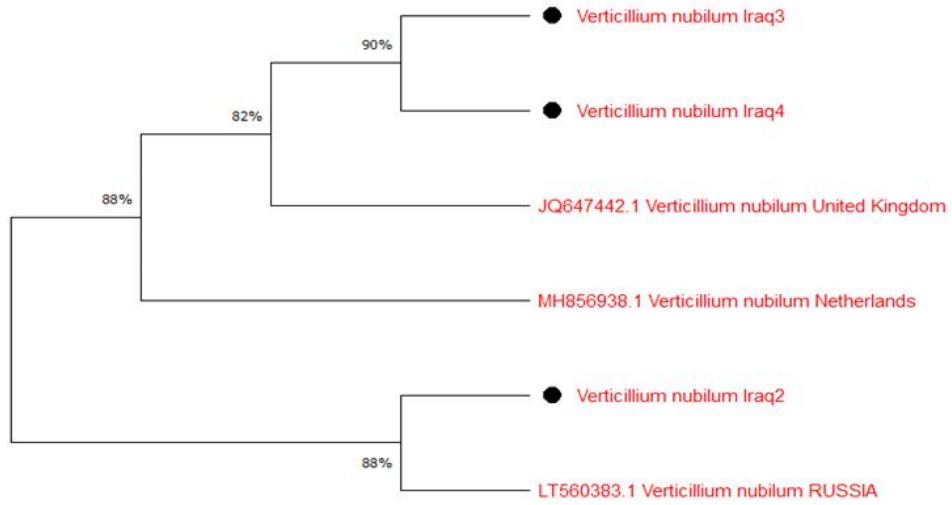


Fig. 2: Genetic tree of *Verticillium nubilum*

	1	2	3	4	5	6
1. MH856938.1 Verticillium nubilum Netherlands						
2. JQ647442.1 Verticillium nubilum United Kingdom	0.34115					
3. Verticillium nubilum Iraq2	0.35728	0.38430				
4. Verticillium nubilum Iraq3	0.25755	0.24204	0.32554			
5. Verticillium nubilum Iraq4	0.26946	0.24632	0.32946	0.00965		
6. LT560383.1 Verticillium nubilum RUSSIA	0.32449	0.33839	0.23107	0.27310	0.28717	

Fig. 3: Similarity percentage between nitrogenous base sequences of PCR-amplified products from *Verticillium nubilum* isolates in this study.

Picture 4: The antagonistic ability of *B. subtillus* bacteria, the fungus *Trichoderma harzianum* and Beltanol against the pathogenic fungus *Verticillium nubilum* with the control treatment are placed on PDA culture media

Fig. 1 using clonal spacer primers ITS1 and ITS4 and this result confirmed the ability of these primers to amplify the rDNA of fungi, where they were used in many studies [Mirtalebi *et al*. (2019)].

The results of the nucleotide sequence of the fungus showed that the first isolate, V1, of type cephalosporum V, showed a 92% match with the global isolates in the NCBI global gene bank. While the V2-V3-V4 isolates

Table 8: shows the conformity of isolates of the pathogenic fungus V. nubilum to the genetic tree.

	ACCESSION ID	Gene	Country	Source	Compatibility
1		RNA gene	Iraq3	*Verticillium nubilum*	90%
2		RNA gene	Iraq4	*Verticillium nubilum*	90%
3	JQ647442.1	RNA gene	United Kingdom	*Verticillium nubilum*	82%
4	MH856938.1	RNA gene	Netherlands	*Verticillium nubilum*	88%
5		RNA gene	Iraq2	*Verticillium nubilum*	88%
6	LT560383.1	RNA gene	Russia	*Verticillium nubilum*	88%

Table 9: represents the test of the antagonistic ability of some bio-control elements represented by the bacteria *B. subtilis*, the fungus *T. harzianum* and the fungicide Beltanol against the pathogenic fungus *V. nubilum* in laboratory conditions.

No.	Treatments	Colony diameter (cm)	Inhibition %
1	B.S + V.n	0.3	97.15
2	T.H + V.n	0.8	91.07
3	V.n + Bentanol	0.0	100
4	Fungi alone	9.0	0.0
5	L.S.D	0.23	2.58

matched 95% with the type of *V. nubilum*, the nucleotide sequences of the fungus were registered in the International Genebank Organization and accession number MH864644.1 and LT560383.1 were obtained and became a reference for Iraq, the Middle East and the world (Fig. 3). As shown by the genetic tree of the pathogenic fungus *V. nubilum* (Fig. 2), the third and fourth Iraqi isolates matched each other by 90%, the first isolate matched 82% to the United Kingdom and 88% to the Netherlands and the second Iraqi isolate matched 88% to the Russian isolate (Table 8).

3.4 Pathogenicity test

3.4.1 Testing the antagonistic ability of *B. subtillus* bacteria, *Trichoderma harzianum* and Beltanol against the pathogenic fungus *Verticillium nubilum* on PDA: The results (Table 9) of this test showed that there was a high antagonistic ability of the biological resistance elements represented by *B. subtillus* and *T. harzianum* against pathogenic fungi in the laboratory. The results showed that the bacteria *B. subtillus* (22.8×10^{10} CFU/ml) tested had a significant effect in obtaining the highest rate of inhibition of the growth of the pathogenic fungus, where the inhibition rate was 97.15% for the pathogenic fungus compared to the control treatment (the fungus alone) without adding the bacterial suspension, which amounted to 0.0%. This is due to the mechanism of its effect, which is through the production of antibiotics that are secreted outside the bacterial cells and volatile hydrocarbons, which are responsible for inhibiting the growth of pathogens [Sorokulova (2013)]. The bacterium, *B. subtilis*, produces more than 66 types of antibiotics, most of which are peptides, including subsporin, neocidin, eumycin, bacillomycin, subtilin, mycosubtilin, bacilysin and others. The results of the investigation of the biological fungus *T. harzianum* used in the bio-control also showed a high antagonistic ability against the pathogenic fungus, of which the percentage of inhibition was 91% compared to the control treatment, which amounted to 0.00. As shown in the results in Table 9, obtaining a percentage of 100% inhibition of the pathogenic fungus using the fungicide Beltanol, compared to the comparison treatment, which amounted to 0.0%, as shown in Picture 2, which shows the antagonism of the elements of the bio-control and the fungicide Beltanol against the fungus *Verticillium nubilum*.

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