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ISOLATION AND IDENTIFICATION OF LOCAL PGPR ISOLATES AND EVALUATION OF THEIR EFFICIENCY IN INHIBITING THE PATHOGENIC FUNGUS RHIZOCTONIA SOLANI ON BROAD BEAN IN LABORATORY CONDITIONS

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Abstract

This study was conducted to evaluate the efficiency of some PGPR isolates as biological agents in controlling root rot disease (Rhizoctonia in broad beans) caused by the fungus *Rhizoctonia solani*. Twenty-six isolates were obtained through the Plant Growth Promoting Rhizobacteria sampling from different areas. Two isolates demonstrated a high antagonistic activity against the pathogenic fungus isolate. The identification of two isolates, *Alcaligenes faecalis* and *Acinetobacter radioresistens*, was confirmed using VITEK technology and nucleotide sequencing. The two strain sequences were deposited in the GenBank under accession numbers AR1 (OR101916) and AR2 (OR101917). The antagonistic activity was 100% for both isolates, *A. faecalis* and *A. radioresistens* scored 58.92 and 57.14% inhibition at a dilution 10^{-7} and 10^{-6} respectively. Secondary metabolites test of *A. faecalis* and *A. radioresistens* scored 93.33 and 85.55% inhibition rate at a concentration of 10%

Keywords: Broad bean, *R. solani*, PGPR, *A. faecalis*, *A. radioresistens*.

عزل وتشخيص عزلات محلية من بكتريا PGPR وتقييم كفاءتها في تثبيط الفطر الممرض *Rhizoctonia solani* على الباقلاء تحت الظروف المختبرية

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الخلاصة

اجريت هذه الدراسة لتقيم كفاءة بعض العوامل الاحيائية في الحد من مرض تعفن الجذور الرايزوكتوني في الباقلاء المتسبب عن الفطر *Rhizoctonia solani* تم الحصول على 26 عزلة بكتيرية من الـ Plant Growth Promoting Rhizobacteria (PGPR) من مناطق جمع العينات، اظهرت النتائج ان عزلتين اثبتت مقدرة تضادية عالية ضد عزلة الفطر الممرض وتم تشخيصها باستخدام تقانة فايتك والذي اثبت ان العزلتين هما *A. faecalis* و *A. radioresistens*، فضلاً عن ذلك تم تأكيد تشخيص تلك البكتريتين باستعمال تقنية الـ PCR وقد تم إيداع العزلتين في بنك الجينات وفقاً للأرقام التسلسلية AR1 OR101916 وAR2 OR101917، وأوضحت اختبارات المقدرة التضادية للبكتريا *A. faecalis* و*A. radioresistens* ضد عزلة الفطر الممرض *R. solani* على الوسط الزرعي PDA كفاءة تضادية عالية وبفارق معنوي عن بقية العزلات قياساً بمعاملة السيطرة ، اذ بلغت الفعالية التضادية 100% لكلتا العزلتين، فقد حققت عزلتي البكتريا *A. faecalis* و *A. radioresistens*تثبيطاً بنسبة 58.92 و57.14% عند التخفيف 10^{-7} و10^{-6} على التتابع.

كلمات مفتاحية: الباقلاء، *R. solani*، PGPR، *A. faecalis*، *A. radioresistens*.

Introduction

The broad bean *Vicia faba* L, belonging to the Fabaceae family, is one of the most economically important leguminous plants in many countries. It has originated from Algeria (14 and 39). Its seeds contain a high percentage of protein and carbohydrates, ranging from 25–40% and 56%, respectively, in most varieties. Besides its nutritional values, the crop improves soil by fixing atmospheric nitrogen through establishing symbiosis with PGPR bacteria, resulting in root nodules formation (6 and 32). The broad bean plant is grown in Iraq, nationwide, as the cultivated area in 2022 was about 20,811 dunums, and the production was 40,586 tons, at a rate of 1,950.2 kg/dunum (10). The crop is infected by many fungal diseases, including root and stem rot disease. This disease impacts leguminous crops in many regions of the world, causing significant losses (5, 6 and 19). In recent years, production decreased due to many biotic and abiotic factors, including those caused by soil pathogens mainly *Rhizoctonia solani*, *Fusarium* spp, especially *Fusarium solani*, *Macrophomina phaseolina*, and

Pythium spp. The fungus *R. solani* is one of the most important pathogenic soil fungi that infects the broad bean crop, causing seedling death, rotting of roots and stems of plants, wilting disease and seed rot (28, 33 and 40).

The excessive use of chemical pesticides and their impact on human health and the environment has given priority to develop efficient eco-friendly pesticide alternatives to combat diseases (1, 22 and 37). Bio-agents have been applied to reduce the inoculum of pathogens, promote plant growth, increase production in quantity and quality, and play a role in inducing systemic resistance in plants. At the forefront of these factors are plant growth-promoting rhizobacteria (PGPR) (26 and 29). (42) found that using the bacterium *Alcaligenes faecalis* could efficiently inhibit the fungus *R. solani*, causing sheath blight in rice by 60.26%. (30) indicated that the use of *Acinetobacter radioresistens* enhanced systemic resistance against leaf rot disease in cacti caused by *Fusarium oxysporum* as it inhibited fungal growth by 66% and increased plant growth and root colonization. Therefore, the current study aimed to evaluate the inhibitory activity of local PGPR isolates against the pathogenic fungus *R. solani* under laboratory conditions.

Materials and Methods

Soil sampling: Fifteen soil samples (15 g/sample) were collected from different broad bean fields in Baghdad, Babylon, and Salah Al-Din Provinces. The samples were taken from the rhizosphere of broad bean plants at a depth of 0–30 cm and mixed well to form one homogenized sample representing each field. It was packed in clean and sterile polyethylene bags, transported to the laboratory, and kept in the refrigerator before being used in the isolation process.

PGPR bacteria isolation: PGPR bacteria were isolated by preparing dilutions of soil samples. About 10 g of selected soil samples was added to 90 ml of sterile distilled water in 250 ml glass flasks and mixed well. One ml of the Previously prepared dilutions was taken to inoculate each test tube containing 9 ml of sterilized Nutrient broth. The tubes were incubated at a temperature of 28 ± 2 °C for two days, and the tubes were examined for bacterial growth. Inoculum was taken by a loop from bacterial growth and spread on the surface of a Petri dish containing nutrient agar (NA) medium. The dishes were incubated at a temperature of 28 ± 2 °C for two days. The isolation was repeated on a new nutrient medium several times to obtain pure colonies. The isolates were kept in slant tubes containing (NA) medium and incubated for 24 h, then incubated at a temperature of 4 °C (27).

pathogenicity test of *R. solani*: The pathogenic fungus was isolated from the roots of broad bean plants that showed symptoms of wilting and yellowing by removing the plants with their entire root system and placing them in polyethylene bags. The fungus isolated was diagnosed based on the taxonomic characteristics reported by (9 and 34). Then isolates were molecularly confirmed and deposited in GenBank under the accession code number R1 (OR100344). The isolate was maintained by transferring part of the mycelium to Petri dishes containing PDA culture medium, and incubated at 25 ± 2 °C. The pathogenicity of the isolate was tested according to the method (3).

In Vitro testing the antagonism activity of PGPR isolates against *R. solani*: The antagonism activity of 26 PGPR isolates obtained was tested against *R. solani*. The pathogenic fungus isolates grown on PDA were tested. One ml suspension from each bacterial isolate grown on NB medium was spread on the surface of a PDA plate. About 0.5 cm diameter was placed in the center of the plate. Three plates were used for each treatment, and three plates were left without adding bacteria as a comparison. The plates were incubated for 5 days at 25 ± 2 °C (4). The inhibitory percentage was calculated as follows:

$$\text{Inhibition \%} = \frac{\text{Average comparison diameter} - \text{Average treatment diameter}}{\text{Average comparison diameter}} \times 100$$

Accordingly, the bacterial inoculum was added to both isolates separately, grown on solid NA activation medium for 2 days, by placing lines on the PDA medium using a loop inoculating needle in a linear, straight line at a distance of 2 cm from one edge of the plate, and cultured. A 0.5 cm diameter disk of the pathogenic fungus was cut from the fungal culture grown on PDA medium at 5 days of age, at a distance of 3.5 cm from the bacterial line and 3.5 cm from the other edge of the plate, and four replicates were used for each treatment and 4 plates left without bacteria as a control, and the plates were incubated at 25 ± 2 °C and after 5 days, the percentage of inhibition was calculated using the following equation: (36).

Fungal growth inhibition = A / A + B x 100

A = the distance between the bacterial line and the end of the fungal growth.

B = the fungal expansion towards the bacterial line.

Identification of two isolates of PGPR that showed the highest antagonistic activity against *R. solani* using the Vitec 2 technology: The Vitek2 Compact System device was used, which consists of a Cassette holder and Reagent Cards containing 64 holes, each hole represents the basic material or medium for conducting the test. Based on the cultural and microscopic examinations, the diagnostic card or kit was chosen and prepared by the French company Biomeriux. The diagnosis process was carried out in the laboratories of ASCo Learning Center / Baghdad according to the manufacturer's instructions.

Molecular diagnosis of *Alcaligenes faecalis and Acinetobacter radioresistens* isolates. Using polymerase chain reaction (PCR) Technique and determining the sequences: The DNA of the two bacterial isolates was extracted at Jisr Al-Musayyab Company/ Baghdad. A special diagnostic kit prepared by Geneaid Biotech was used, and the extraction was carried out according to the instructions of the company.

The concentration and purity of DNA extract: After performing the DNA isolation process, the DNA concentration was estimated using a Nano drop spectrophotometer at two wavelengths (260/280) nm, according to the following equation: (41)

Nucleic acid concentration (µg/ml) = absorption at 260 x 50 x dilution factor

Then the purity of the nucleic acid was estimated using the following equation:

Purity = Absorption at 260/Absorption at 280.

Amplify the 16S rRNA gene using the polymerase chain reaction (PCR) technique: Polymerase chain reaction (PCR) technology was used to amplify the 16S rRNA gene using a master mix prepared by Bioneer Company. Primers 1942R were added at a concentration of 1µL for each primer and a DNA template at a concentration of 5µL to the reaction mixture, and then transferred to the PCR Thermo cycler according to the table below.

Table 1: Polymerization reaction conditions (cycles, temperatures, time).

N	Steps	N. of cycles	Temperature	Time
1	Initial Denaturation	1	95c^{o}	min 5
2	Denaturation	35	95c^{o}	30 sec
3	Annealing		55c^{o}	30 sec
4	Extension		72c^{o}	1 min
5	Final extension	1	72 c^{o}	5 min

The reaction products were electrophoresed on a 1% agarose gel. Electrophoresis stopped when the blue loading solution reached approximately 2 cm before the end of the gel.

The gel was examined by placing it on a UV transluminator at a wavelength of 260 nm to view the DNA bands and estimate its molecular size compared with the ladder, pictures taken. The results were then sent to the Korean company Macrogen to analyze the results and to carry out the gene sequencing.

Pedigree tree: The sequences were edited in BioEdit software and saved in FASTA format (17). *A. faecalis* and *A. radioresistens* diagnosed during this study were compared with other isolates previously registered in the National Center for Technology Information (NCBI), Clustal W used in the MEGA6 program to draw the genetic tree and align the sequences of the nitrogenous bases for all the isolates (24). The Neighbor joining method included in the MEGA6 program was used to perform genetic evolutionary analysis of the bacterial isolates (16 and 38).

Preparation of the PGPB bacterial inoculum: The two bacteria, *A. faecalis* and *A. radioresistants*, were grown in autoclaved liquid NB medium to multiply. After placing them in flasks of capacities 100 and 50, respectively, and sterilizing them in an autoclave, each flask was inoculated with bacteria individually. Then, it was incubated for 48 hours at a temperature of 28 ± 2 °C, using a shaking (100 Rev/ min) in the incubator.

Production and extraction of secondary metabolites: To obtain the secondary metabolites, the bacteria were grown in 150 ml of culture NB / in flasks of 250 ml capacity and incubated with shaking (100 Rev/ min) f) for 72 h at 28 ± 2 °C (7). Secondary metabolites were extracted. The method has been adopted. First the liquid culture was centrifuged using 10000 rpm/min for 15 min at 4°C (7). The supernatant was collected 100 ml of ethyl acetate extraction solution was added and the mixture was left for 24 hours at room temperature and occasionally stirred. The extraction solution was removed using a rotary evaporator at 25°C. The resultant secondary metabolite solution was lyophilized using a freeze dryer in order to obtain a pure compound

Determination of the effective concentration of bacterial inoculum in inhibiting *R. solani* growth. A series of dilutions 10^{-1} to 10^{-9} of each bacterium were prepared in test

tubes. About 0.6 mL suspension of each dilution was used to inoculate PDA plates. The center of each plate was inoculated with a 0.5 cm disk from the *R. solani* fungus colony on the 4th day of growth. Control treatment was inoculated with sterilized distilled water only for comparison. 4 replicates were used for each treatment and the control. All plates were incubated for 4 days at 25 ± 2 °C. Then, the inhibition percent was calculated as follows:

$$\text{Inhibition \%} = \frac{\text{Average comparison diameter} - \text{Average treatment diameter}}{\text{Average comparison diameter}} \times 100$$

Determination of the two bacteria concentration: One ml of each dilution was spread on NA solid medium in a 9 cm Petri dish and the plates were incubated for 48 h at 28 ± 2 °C. The number of colonies was calculated as follows:

The average number of bacterial colonies for concentrations 10^{-7} and 10^{-6} was multiplied by the reciprocal of the dilution (11), Accordingly, the concentration used was 7×10^8 and 8×10^7 (CFU/ml).

Evaluate the efficiency of the bacterial metabolites against *R. solani* growth: Metabolites extracted from each bacterium were mixed with PDA medium at a rate of 10% (2) and sterilized by passing through a 0.45 µ Millipore filter using a vacuum device. The PDA medium containing the filtrates was distributed in Petri dishes, left to Solidify, and then each dish was inoculated in the middle, with 0.5 cm discs of pathogenic fungus *R. solani* growths. PDA free metabolites were used as a control and treatments were incubated at a temperature of 25 ± 2 °C. The inhibitory activity was calculated as follows: the average measurement of two orthogonal diameters for each treatment. The experiment ended after the completion of the growth of the comparison dishes (pathogenic fungus). The experiment was carried out in four replications the inhibition percentage of fungal growth was estimated as follows:

$$\text{Inhibition \%} = \frac{\text{Average comparison diameter} - \text{Average treatment diameter}}{\text{Average comparison diameter}} \times 100$$

Statistical analysis: The statistical analysis was conducted using the statistical program GenStat Version 10.3.0.0, and the completely randomized design (C.R.D) was used in implementing the laboratory experiments and the plastic house experiment, and the averages were compared according to the least significant difference LSD at the probability level (0.05).

Results and Discussion

Pathogenicity test of *R. solani*: The pathogenic fungus *R. solani* significantly reduced the percentage of germination of broad bean seeds under greenhouse conditions, scoring 3.3% (Table 2 and Fig.1) compared to 100% in the control treatment (without pathogenic fungus. The pathogenic fungus could reduce seed germination through its ability to produce enzymes that degrade pectin and cellulose compounds at early stages of plant life. These enzymes, including pectinase, pectin methyl esterase, pectinlyase, and cellulase, enable the penetration of the host and cause the disease. Moreover, the fungus releases some substances that have a toxic effect on plant cells, including phenyl acetic acid derivatives, causing cell lysis and fermentation (8, 28 and 31).

Table 2: Pathogenicity test for isolation of R. solani fungi in pots.

Treatments	% germination
Control	**100**
R. solani	3.3
L.S.D $_{0.05}$	9.25

• Each number represents the average of 3 replicates.

Figure 1: Testing the pathogenicity of R. solani in pots Seedlings infected with the fungus R. solani (left), healthy control (right).

Sampling and isolation showed a variation in the types and numbers of PGPR bacteria based on collection areas (Table 3 and Figure 2). Testing the antagonistic ability of 26 bacterial isolates showed that these isolates varied in their inhibition activity against the pathogenic fungus *R. solani*.

Table 3: Efficiency of bacterial isolates in inhibiting the growth of the pathogenic fungus *R. solani* on PDA.

Governorate	Isolation symb	%Inhibition
Baghdad	B1	68.88
	B2	29.63
	B3	52.60
	B4	16.66
	B5	17.40
	B6	80.46
	B7	45.48
Babylon	Ba8	58.14
	Ba9	100
	Ba10	61.88
	Ba11	50.74
	Ba12	11.48
	Ba13	75.55
	Ba14	31.85
	Ba15	70.51
	Ba16	24.81
	Ba17	24.07
	Ba18	45.55
Salah al-Din	Sa19	62.22
	Sa20	37.77
	Sa21	100
	Sa22	30.74
	Sa23	15.92
	Sa24	8.51
	Sa25	21.11
	Sa26	78.98
	Control	0.0
L.S.D $_{0.05}$		4.26

- Each number represents 3 replicates.

Vitec 2 technology confirmed that the two bacterial isolates selected were *Alcaligenes faecalis* and *Acinetobacter radioresistens.* The two isolates referred to as Ba9 and Sa21, respectively, were isolated from Babylon and Salah al-Din provinces. They showed 100% maximum inhibition compared to other isolates that showed lower inhibition rates. Besides, Chitinase, Glucanase, and Protease production, as well as other metabolites including, organic compounds, Indole acetic acid, fatty peptide, Secondary compounds, and hydrogen cyanide (HCN) varied from other isolates within the same region, could be due to different environmental and genetic conditions (12, 13, 18 and 25).

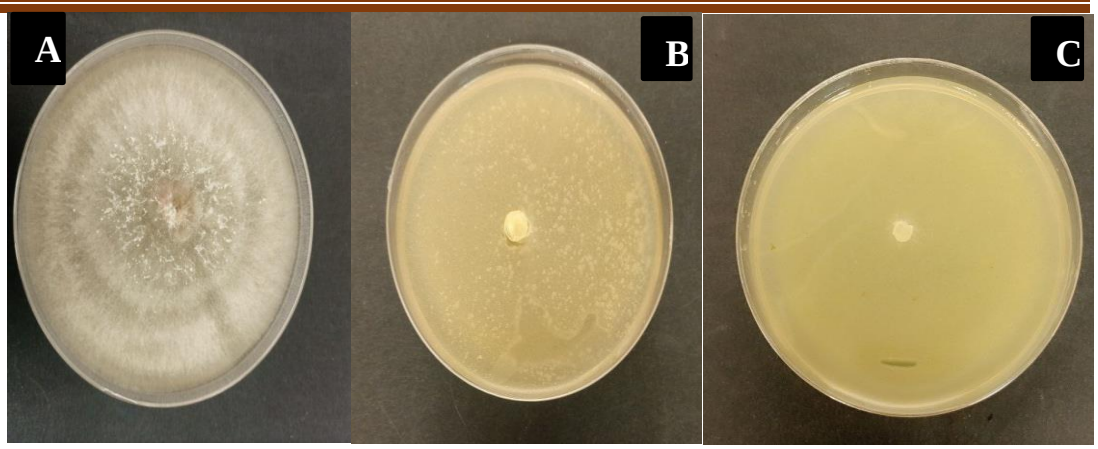

Fig. 2: A- antagonist activity of *Alcaligenes faecalis* and *Acinetobacter radioresistens* against *R. solani* on solid PDA medium.
B- *A. faecalis* against *R. solani.*
C- *A. radioresistants* against *R. solani.*

The results of the polymerase chain reaction (PCR) technique showed the ability of primers 27F and 1942R to diagnose the bacterial isolates *A. faecalis* and *A. radioresistens*, as they were able to Amplify the DNA segments producing bands 1400 bp respectively (fig3) as well as The two isolates were registered in the GenBank, and the sequences of the nitrogenous bases were deposited in the GenBank, and bank number (Accession No.) was given to the two isolates, AR1 OR101916 and AR2 OR101917, respectively, This is the first recording of the two bacterial isolates in Iraq.

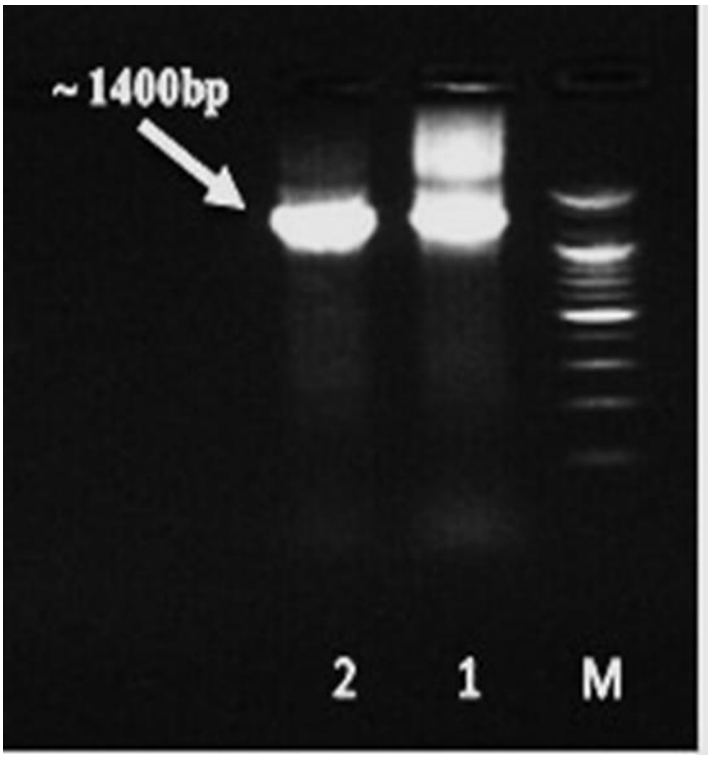

Fig. 3: Agarose gel electrophoresis products of DNA fragments with molecular weight (1400 bp) representing the isolates *Alcaligenes faecalis* and *Acinetobacter radioresistens.*

Analysis of nucleotide sequence products of *Alcaligenes faecalis and Acinetobacter radioresistens:* The results of the nucleotide sequence analysis of the sequences of the two bacterial isolates that were Amplified confirmed that they are isolates belonging to the two species*: A. faecalis and A. radioresistens,* after they showed 99% sequence identity with the global isolates present in GenBank (NCBI) when compared with the nucleotide sequences retrieved from GenBank. This is the first deposit of the isolate A. *faecalis* in Iraq (Fig. 4). As for the isolation of *A. radioresistens*, it was isolated and diagnosed for the first time from the rhizosphere of the roots of bean plants in Iraq. The two isolates had a high ability to inhibit the pathogenic fungus *R. solani*.

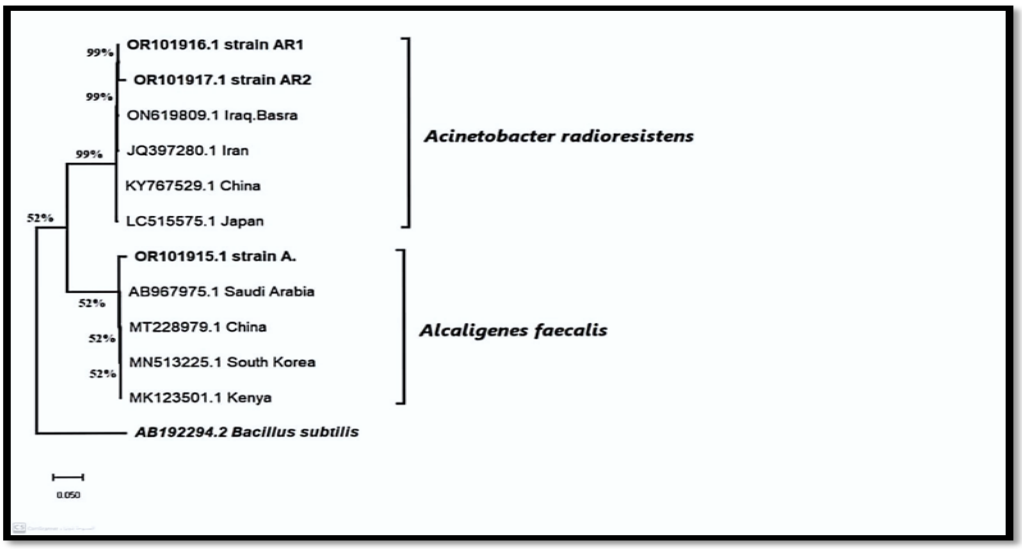

Fig. 4: The genetic relationship of the two bacterial isolates, *Alcaligenes faecalis and Acinetobacter radioresistens*. The pedigree tree of the connective type, next to each other, and then building it from the nucleotide sequences, as it explains the relationship between the Iraqi isolates and their counterparts retrieved from the gene bank.

The results also showed that the use of two bacterial isolates, *A. radioresistens* and *A. faecalis* as biological control agents could inhibit the growth of the pathogenic fungus, *R. solani*, scoring 57.14 and 58.92% inhibition respectively, Table 4 and Figure 5. Similarly, (20) who demonstrated the ability of the bacterium *Alcaligenes faecalis* to inhibit the growth of pathogens, mainly *R. solani*. (18) found that the use of isolates from root bacteria, including *Acinetobacter radioresistants*, was highly effective in inhibiting many pathogens, including *Pythium ultimum, Fusarium oxysporum* and *Alternaria alternate*.

Table 4: Antifungal test for against the pathogenic fungus according to the method (38) in vitro.

Treatments	%Inhibition
A. radioresistants	**57.14**
A. faecalis	**58.92**
Control	**00.00**
L.S.D $_{0.05}$	**17.65**

- Each number represents 4 replicates.

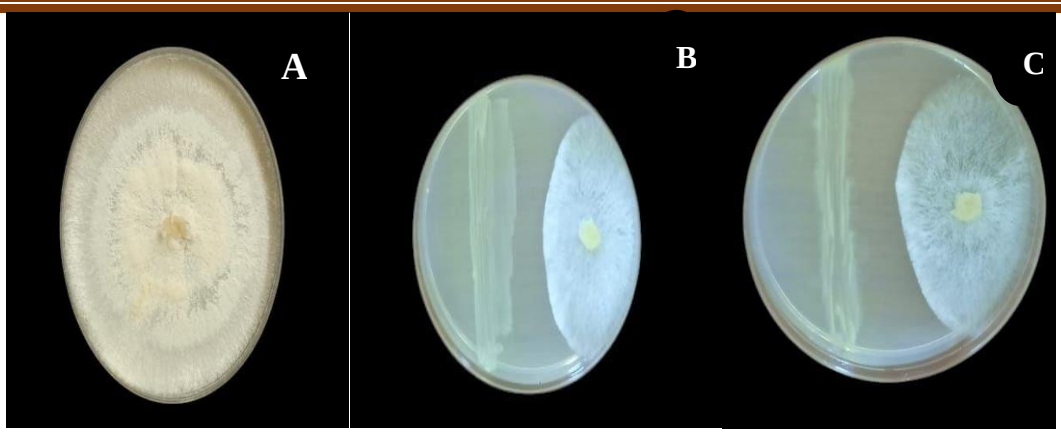

Figure 5: Antifungal test for *Alcaligenes faecalis and Acinetobacter radioresistens* against the pathogenic fungus *R. solani* s according to the method (38) in vitro.

1. *R. solani*. 2. *A. faecalis* against R. *solani*. 3. *A. radioresistants* against *R. solani*.

Secondary compounds of *Alcaligenes faecalis and Acinetobacter radioresistens* inhibiting the growth of *R. solani*: The use of secondary compounds extracted from the bacteria *A. faecalis* and *A. radioresistens* significantly reduced the growth rate of the pathogenic fungus compared to the control treatment on PDA Table 5. Secondary compounds produced by root bacteria stimulate plant growth to produce volatile organic compounds, enabling the growth inhibition of pathogens. These compounds are able to spread in the soil, with the advantage of working remotely (15, 23, 30 and 35). (21) confirmed that the PGPR metabolites, including *Pseudomonas fluorescens*, *Bacillus megaterium, B. subtilis*, and *B. cereus*, play a pivotal role in controlling root rot and wilting diseases in broad bean plants resulting from the fungi *Rhizoctonia solani* and *Fusarium. solani*, *F. oxysporum*, and *Macrophomina phaseolina* infection.

Table 5: The effect of secondary compounds of *Alcaligenes faecalis and Acinetobacter radioresistens* on inhibiting the growth of *R. solani*.

Treatments	Concentration%	Inhibition %
Sc *A. radioresistens*	**10**	**85.55**
Sc A. faecalis		**9333**
Control	**0**	**0.0**
L.S.D $_{0.05}$		**4.51**

Each number represents 4 replicates.

Conclusions

Two isolates of PGPR demonstrated a high antagonistic activity against the pathogenic fungus isolate. The identification of two isolates Alcaligenes faecalis and Acinetobacter radioresistens was confirmed using VITEK technology and nucleotide sequencing. The two strain sequences were deposited in the GenBank under accession numbers AR1 (OR101916) and AR2 (OR101917). The antagonistic activity was 100% for both isolates in the first method. While in the second method, the two isolates of *A. faecalis* and *A. radioresistens* scored 58.92 and 57.14% inhibition at a dilution 10^{-7} and 10^{-6}, respectively.

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Author 1; methodology, writing original draft preparation, Author 2 and Author 2 writing review and editing. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest:

The authors declare no conflict of interest.

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