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Efficiency of some Types of Bacteria Associated with Cucumber Roots Against the Rhizoctonia Solani

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ABSTRACT

This study was carried out in the laboratories of the Plant Protection Department, Ministry of Agriculture, to isolate bacteria associated with cucumber roots from greenhouses in Baghdad, Salah al-Din, Sulaymaniyah, and Basra, and to evaluate their ability to inhibit Rhizoctonia solani, the causal agent of cucumber root rot, on Potato dextrose agar (PDA) medium. A total of 12 bacterial isolates were obtained from healthy cucumber root-associated soil. These isolates varied in type depending on collection site and environmental conditions. All isolates demonstrated high inhibitory activity against R. solani, with fungal colony diameters ranging from 1.17 to 6.63 cm and inhibition rates between 26.3% and 87.0% compared to the control. Molecular identification using Polymerase Chain Reaction (PCR) was performed on three isolates: Bacillus cereus, Bacillus thuringiensis, and Bacillus rugosus. Their nucleotide sequences were submitted to the GenBank database under accession numbers PP935479.1, PP935461.1, and PP934669.1, respectively. These findings highlight the potential of native bacterial isolates as biocontrol agents against cucumber root rot.

Key words: Bacillus sp, biological control, Plant Growth-Promoting Rhizobacteria, root rot disease.

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.INTRODUCTION

Cucumber *(Cucumis sativus)* one of the most important plants of the cucurbitaceous family and of great economic importance, is grown in most countries of the world, including Iraq, and is grown with two seasons spring and autumn loops under conditions of open and protected agriculture. It is a plant rich in vitamins B, A and C and contains a large percentage of water up to 96%, carbohydrates by 3% and 1%, protein and some minerals such as iron, manganese, copper, calcium and potassium, which is low in calories (Sujatha & Kalarani, 2012). The cucumber plant is exposed to many pathogens endemic in the soil, which cause great economic losses. The most important of these diseases are seed rot and the death of seedlings before and after the eruption, root rot and wilt diseases. The most important of these pathogens is *Fusarium* spp. *R.solani*, *Pythium spp*, *Mamacrophominaphaseolina*, *Phytophthora spp.* and *Sclerotium rolfsii* (Elagamey, etal,. 2020; Gabrekiristos & Demiyo 2020; Ibrahim & Al-Juboory, 2024). Soil-borne pathogens are one of the most dangerous and harmful fungi on crops, as they are located far from human sight. Their pathological symptoms usually appear on the vegetative complex after they have completely decimated their root group (Nicolopoulou-Stamati et al., 2016), which increases their danger. Many of them have a wide family range. They also have the ability to resist inappropriate environmental conditions and can stay in the soil and residues of infected plants for a long time (Zalma& El-Sharoud, 2021). The use of chemical pesticides in resistance to plant pathogens is the most efficient method, but the exposure of pathogens to electoral pressure as a result of repeated use of pesticides led to the emergence of resistance in them (Afifi et al., 2017;

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Szalay, 2017). In order to reach the goal sought by all, which is to reduce the incidence of pathogens and find new ways and means in the control of plant pathogens because these pesticides are expensive and not environmentally friendly, the importance of using biological control to combat plant pathogens has emerged as an easy strategy and does not cause environmental imbalance (Omara etal., 2020), as well as it is based on the joint action of different resistance methods to reduce the damage of these pathogens. Therefore, biological control has gained wide acceptance in various parts of the world and enjoys continuous encouragement and wide and advanced application in the field. It works to reduce the risk of crop damage caused by pathogens (Fasusi et al.,. 2021; Vignatti et al., 2020). It has become one of the most important pillars in resisting these pathogens, including the use of anti-pathogenic biota, which leads to reduce the density and activity of pathogenic communities. It has used a number of bacteria species that encourage plant growth Promoting Rhizobacteria (PGPR) in several mechanisms and has been found to accompany the roots of different plants in the Rhizospher region such as *Bacillus spp*. , *Actinomyces sp*. Pseudomonas spp. and *Azotobacter sp*. These biological agents are highly antagonistic towards pathogens, and on the other hand, they are catalysts for plant growth; beneficial bacteria live free in the soil (Prasad et al., 2019; Singh etal., 2018) . Based on this concept, this study aimed to obtain environmentally friendly biological controlagents that carry the specifications of biocides against cucumber root rot disease.

MATERIALS AND METHODS

Isolating Bacteria from Cucumber roots

Soil samples were collected from the Rhizosphere area (the area around the roots) of well-grown cucumber plants that did not show symptoms of disease planted in green houses from different locations in the governorates of Baghdad ,Salah al-Din ,Basra and Sulaymaniyah to isolate the plant growth-stimulating root bacteria Promoting Rhizobacteria (PGPR) . The samples were randomly taken from each green house and placed in polyethylene bags separately. The required informations were included recordeing the sample number, place and date of collection (Table 1). The samples were transferred to the Disease Laboratory/Plant Protection Department to conduct the isolation process in a dilution manner. 10 g of each soil sample were added separately in a 250 ml glass carafe containing 90 ml of sterile distilled water and were well shaken. A series of dilutions was carried out by transferring 1ml of soil stuck and adding it to a test tube containing 9 ml of sterile distilled water to dilution $1x10^{-9}$ and taking 1 ml of the last three dilutions above and added in Petri dishes with a diameter of 9 cm containing the Nutrient Ager (NA) sterilized medium with the buffer (at a temperature of 121 ˚ and a pressure of 5.1kg/cm for 20 minutes) before hardening it, the dishes were requested to homogenize the medium. She hugged at a temperature of 28 C for one day and bacterial growth was observed and purified. She took swabs of bacterial growth with a sterile loop needle and planned it on a new culture medium to obtain pure isolation from each bacterium.

Table 1. Governorates and areas from which the collection of samples took place and the date of collection**.**

Province	Region	Collection date
Sulaymaniyah	Sulaymaniyah	2023/6/8
	Dujail	2023/10/12
	Al-Ishaqi	2023/10/12
	Balad	2023/10/18
Salah al-Din	aldulueia	2023/10/18
	Samarra	2024/2/5
	Yusfia	2023/10/3
Baghdad	Abu-ghraib	2024/1/29
Basra	Basra	2023/11/14

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Efficiency of Bacterial Isolates in the Growth and Inhibition of *Rhizoctonia solani*

In vitro: The antagonistic ability of twelve unknown bacterial isolates was tested and they were given codes from B1 to B12. obtained from the isolation process from greenhouses planted with cucumber plants for the agricultural season (2023-2024) from nine sites in the governorates of Sulaymaniyah, Salah al-Din, Baghdad and Basra was tested against the isolation of the fungus *R.solani*, which was isolated from the roots of cucumbers and the tests of pathological ability proved its high efficiency in reducing the percentage of cucumber seeds. As the bacterial isolates grew on the NA culture medium and incubated for 24 hours and then a growth line was made in a straight line by taking a swab of bacteria using a needle with a Loop knot (sterile) 2 cm from the edge of a petri dish containing the culture medium PDA and placing a 0.5 cm diameter tablet taken from a newly growing colony of the pathogenic fungus 3.5 cm from the second edge of the dish and by 5 repetitions per transaction, the dishes were incubated at a temperature of 25 ± 2C until the dish growth was completed in the comparison transaction, the results were taken by calculating the average diameter of the developing colony and the inhibition ratio was extracted according to the following equation (Helgason etal.,2000 ; Montealegre etal.,2003):

% to inhibit fungal growth= [1- (Fungal growth of Bacteria treatment / (fungal growth of control treatment)] 1×00

Molecular Identification of Bacterial Isolates from Soil by Polymerase Chain Reaction (PCR): The samples of bacteria obtained, which showed a high rate of inhibition, were sent to the Central Public Health Laboratory in Baghdad for the purpose of diagnosing them by chemical methods By Vitek device and ensuring that they are not pathogenic to humans. Pure isolates of bacteria were were grown on NA, Blood Ager and MacConkey agar, incubation at a temperature of 28C for 24 hours. Molecular identification was made for three isolates of bacteria (B7, B11 and B12) that showed antagonistic efficiency against the pathogenic fungus *R.solani*, which were not diagnosed through biochemical tests in central health laboratories because It is not pathogenic to humans. The three isolates were individually purified by grown on the NA culture medium, and the individual isolates were selected for each isolation and replanted in a planning manner by the knotted inoculation needle (Loop) to ensure the purity of the isolates. The DNA extraction process was carried out to isolate the three bacteria It is not pathogenic to humans. The growths were collected from the surface of the medium containing bacteria at the age of 1-2 days and were placed in special tubes prepared for this purpose sterilized individually. They were diagnosed based on the 16SrRNA gene in using PCR reaction. The DNA was extracted by following the steps on the extraction kit produced by the Taiwanese company Geneaid, and the DNA was kept at a temperature of 4 C until use. The prefixes used in the PCR are 27f ('5-AGAGTTTGATCCTGGCTCAG-3') and R149 '5TACGTTACCTTGTTACGACTT-3) Non-specific Universal Primer used for 16s rRNA gene amplification. These primers were prepared by the Korean company Macroge. . For the purpose of detecting the amplified extracted DNA bands and comparing it with the standard Ladder, Ethidium bromide was used to photograph the stained bands in a 1% agarose gel and the images were taken using 350 nm UV light. (Prashanthi etal.,2021). The amplified 16S rRNA gene products were sent to the Korean company Macrogen for the purpose of analyzing the nitrogenous base sequence of the double-stranded nucleic acids from the bacterial isolates using the Genious software program. (Šišić etal., 2018). DNA sequencing for each bacterial isolation was entered separately in the database at the US National Center for Biotechnology Information (NCBI). The Basic Local Alignment Search Tool (blast) was used for the purpose of comparing the results of the standard sequences found in NCBI, and BioEdit was used. The phylogenetic tree of the nitrogenous base sequences of the double-stranded nucleic acids of the bacterial isolates was also drawn using the MEGAX program.

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RESULTS AND DISCUSSION

Isolating and Identification Bacteria from Cucumber roots: The results of isolation and bacterial identification showed that there are 12 bacterial isolates accompanying the roots of healthy cucumber plants from the soil of green houses with cucumber plants collected from several governorates, namely Baghdad, Sulaymaniyah, Salah al-Din and Basra (Table 2), as the bacteria varied in their genera and types. This difference may be due to the difference in the places of collection and environmental conditions between the areas from which the samples were collected, as well as the nature and origin of the bacteria with the revival of other soils and the difference in the type of soil and animal fertilizers added , as well as agricultural practices that have a role in changing the physical and chemical properties of the soil, which in turn leads to the difference and diversity of the bacterial community in the soil (Jangid etal.,2008). This has been confirmed by several studies when isolating several bacterial genera from the soil of greenhouses of several crops (Hussein et al., 2024).

Table 2. Bacterial isolates according to biochemical test probability

No.	Isolation Code	Bacteria Type	Probability (%)
1	B1	*Providenci rettgeri*	99%
2	B2	*Providenci stuartii*	99%
3	B3	*Citrobacter braakii*	99%
4	B4	*Enterobacter cloacae*	95%
5	B5	*Citrobacter braakii*	95%
6	B6	*Staphylococcus vitulinus*	97%
7	B7	*Bacillus sp*	___
8	B8	*Pseudomonas fluorescens*	99%
9	B9	*Pseudomonas oleovorans*	95%
10	B10	*Pseudomonas putide*	99%
11	B11	*Bacillus sp*	___
12	B12	*Bacillus sp.*	___

Efficiency of Isolated Bacteria in the Growth and Inhibition of the *R.solani* on the PDA Culture: The results showed that all the isolates of the 12 bacteria that were isolated from the soil of greenhouses planted with cucumber plants had a high inhibitory efficiency in the direction of growth of the pathogenic fungus *R.solani*. Growth rates and percentages of inhibition varied according to the different isolates of bacteria compared to the control treatment. The growth rates of the fungus colony ranged between (1.17-6.63) cm. The inhibition rates of the pathogenic fungus ranged between26.3 - 87.0% as shown in Table (3). Three bacterial isolates achieved the highest significant reduction in the growth rate of fungus colonies and the percentage of fungus inhibition. Bi12, Bi8, Bi1 and Bi3 isolates, which gave the lowest growth rate of fungus colonies and the highest inhibition rates on all tested fungus isolates, reached (1.17,1.2, 1.23, 1.5) cm and (87.0, 86.6,86.3 and 83.3%) respectively. Bi12,Bi8, Bi1 and Bi3 did not differ with each other, but they differed with all remaining isolates after analyzing them at a probability level of 5%. The reason for the difference in the efficiency of bacterial isolates in reducing the growth of isolates of the pathogenic fungus *R.solani* may be due to its ability to produce secondary metabolites and the production of enzymes that degrade the walls of fungal cells, including Chitinaese, Proteases, B-1,3-glucanse, antibiotics, organic compounds and hydrogen cyanide, as well as the competition of bacteria with pathogenic fungus for food and place (8), as well as the production of bacteria a number of antifungal compounds such as Pyrolnitrin, Phenazines, Siderophores, 2.4-diacetylphoroglucinol (Bhattacharya etal.,2020). These results were

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similar to (Al-Fadhal etal.,2019) and (Vignatti etal.,2020) found when using *Pseudomonas sp*.and *Bacillus* sp. It was effective in inhibiting the growth of the *R.solani*, and these results agreed with (7)mentioned that the bacteria *Bacillus subtilis* and *Pseudomonas fluorescens* have the ability to inhibit the growth of the *R.solani* and the *F solani*. in the lab and experiment with green containers.

Table3. Antagonistic Estimation of Bacterial Isolates Isolated from Soils of Cucumber Plants in Growth rate and percentage of inhibition of the *R.solani.*

Isolate code	Fungal growth rate cm	%Inhibition Ratio
Providenci rettgeri	1.23	86.30
Providenci stuartii	2.40	73.30
Citrobacter braakii	1.50	83.30
Enterobacter cloacae	3.30	63.30
Citrobacter braakii	5.70	36.60
Staphylococcus vitulinus	6.63	26.30
Bacillus sp	2.57	71.40
Pseudomonas fluorescens	1.20	86.60
Pseudomonas oleovorans	3.87	57.00
Pseudomonas putide	4.10	54.40
Bacillus sp	6.00	33.30
Bacillus sp.	1.17	87.00
(control) R.solani	9.00	0.00
L.S.D.	0.52	5.8

Molecular Identification of Bacterial Isolates by PCR

The results of sequens of DNA by the polymerase chain reaction of the bacterial isolates B7,B 11 and B12 isolated from the soil of greenhouses planted with cucumber plants showed that the DNA amplification band of B7 belonged to the bacteria *Bacillus cereus*, while B11 and B12 belonged to the bacteria *Bacillus thiensis* and *Bacillus rugosus* respectively. The 16SrRNA gene proved its ability to diagnose bacteria, and the result of electrophoresis on the agarose gel of the DNA chain reaction products showed packages with a molecular weight of approximately 1200 bp (Figure 1).

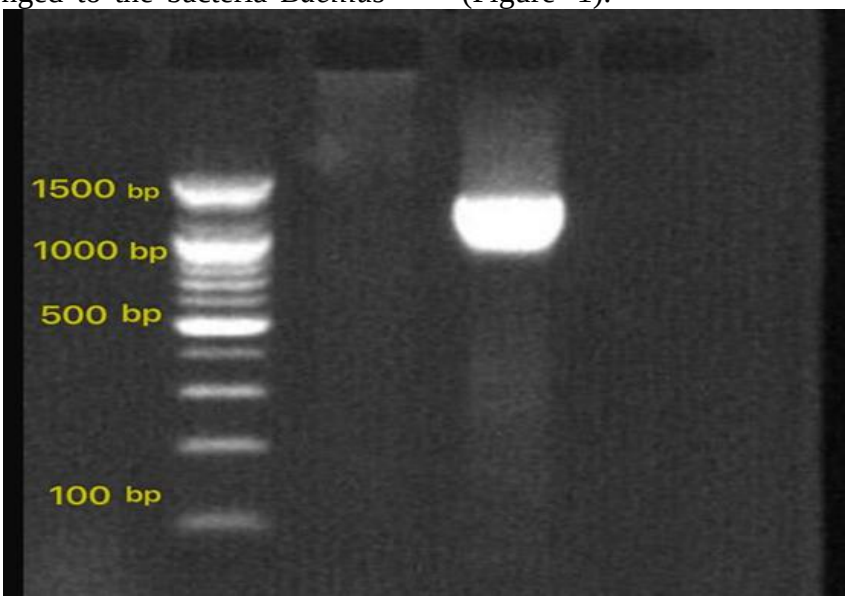

Figure 1. Electrophoresis results of the polymerase chain reaction of DNA strands of *Bacillus rugosus* isolate.

Studies have also shown that the use of the 16SrRNA gene has proven its ability to diagnose Bacillus bacteria (Frank etal.,2008). The results of the nucleotide sequence analysis

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of the bacterial isolates Bi7, Bi11, and Bi12 using the MEGA11 program (Molecular Evolutionary Genetics Analysis Version 11 (Kumar etal.,2018), showed that they were due to the bacteria *Bacillus, cereus Bacillus thuringiensis*, and *Bacillus rugosus*, respectively. The nitrogenous base sequences of *Bacillus cereus* , *Bacillus thuringiensis* and *Bacillus rugosus* were deposited in the gene bank under accession numbers PP935479.1 , PP935461.1 and PP934669.1, respectively. The results showed that *Bacillus cereus*, which is one of the ubiquitous soil bacteria, is an opportunistic pathogen that is a common cause of food poisoning, especially those with weakened immunity, newborns, and postoperative patients. It also causes pollution problems in the dairy industry and paper factories (Carroll etal.,2021). *Bacillus thuringiensisis* special in its work against insects, while *Bacillus rugosus*, a number of researchers have indicated that it can be isolated from the soil (Wang etal.,2015) and its high antagonistic capacity against many fungal and bacterial pathogens such as *F. oxysparum*, *Aspergillus flavus*, *Botrytis cinerea*, *Candida albicans*, *Neurospora crassiza* , *Rhobiumra diobacter* and *Erwinia amylovora*(Hallaj-Nezhadi etal.,2022 ; Salem etal.,2024) indicated that the inoculation of the spelt plant with the bacteria *Bacillus rugosus* led to an increase in wet weight and dry weight, reaching 61.8 and 77.2, respectively, as well as an increase in leg length, root length and chlorophyll content. Many researchers have pointed out the vital ability of many species of the genus *Bacillus sp*. Against pathogenic soil fungi of varying efficiency, which makes it necessary to choose a strain that is characterized by the stability of its effectiveness and can produce more than one type of antimicrobial compounds in addition to adhering to the walls of fungal cells and releasing hydrolyzed enzymes (chitinase, protease) and others that cause deformities in the fungal filaments, puncture in the walls of cells and leakage of protoplast (Radhakrishnan & Abd_Allah, 2017; ValanArasu &Al-Dhabi, 2023). The Maximum Likelihood Genetic Asset Tree (Fig. 2) was constructed from the nucleotide molecular sequence of the its region of tested bacterial isolates *Bacillus rugosus isolated* from soil of cucumbers-grown greenhouses with their equivalent counterparts retrieved from the gene bank. The SDTv1.2 program was used in the creation of the colored lollipop (Muhire etal.,2014), which shows the percentage of match between the bacteria Bacillus rugosus with the equivalent nucleotide sequences deposited in the NCBI gene bank and showed a 97% match with the Saudi isolation registered under the entry number PP096823. Fig 3.

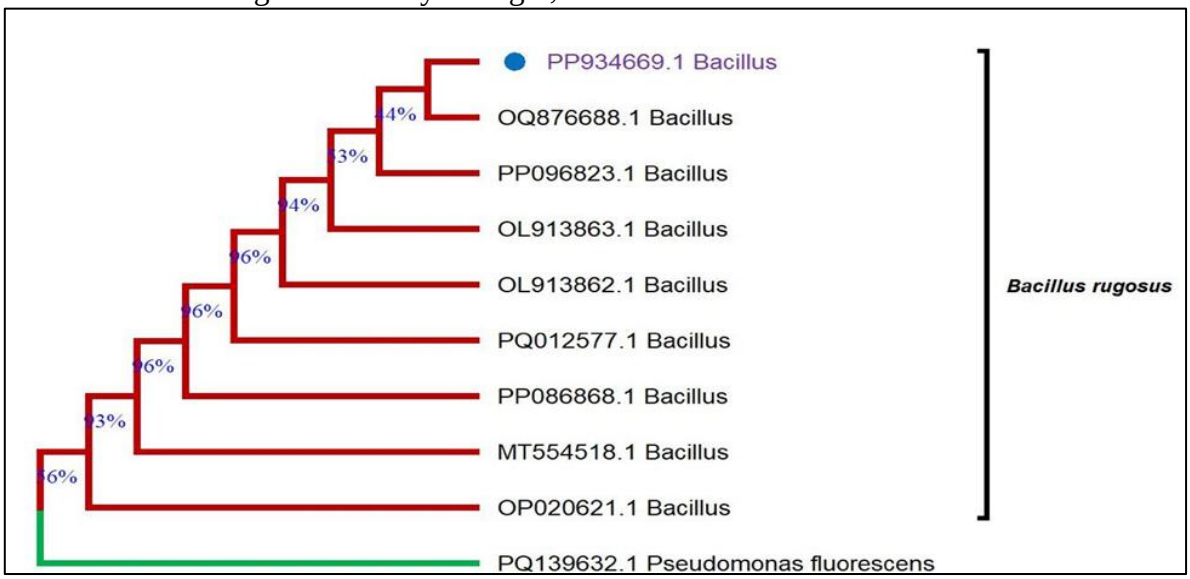

Figure 2. Genetic origins tree of the sequence of the its region of bacterial isolates *Bacillus rugosus* with its equivalent counterparts in the gene bank.

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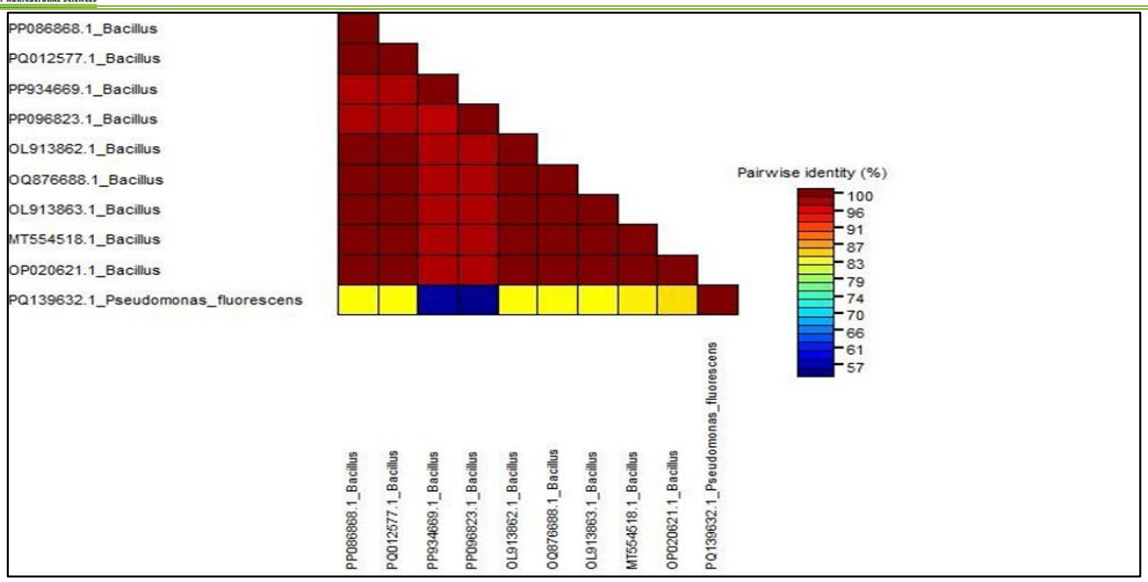

Figure 3. Laminated chromatography showing the proportion of match between the bacterium *Bacillus rugosus* with the equivalent nucleotide sequences deposited in the NCBI gene bank.

CONCLUSION

Isolation and identification revealed were 12 bacterial isolates (B1-B12) from healthy cucumber plant roots in greenhouse soil. Bacteria vary by genus and kind depending on the sampling location and environmental circumstances. The results showed that all bacterial isolates inhibited *R.solani* growth well. Compared to the control treatment, bacterial isolates had distinct growth rates and inhibition percentages. Fungus colony growth rates ranged from 1.17 to 6.63 cm, while pathogenic fungus inhibition rates were 26.3-87.0 %. Polymerase chain reaction was used to identify three isolates of bacteria: Bi7, Bi11, and Bi12. Bi7 was Bacillus cereus, while Bi11 and Bi12 were Bacillus thuringiensis and rugosus, respectively. The gene bank collected bacterial isolate nitrogenous base sequences under the joining codes PP935479.1, PP935461.1, and PP934669.1.

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كفاءة بعض انواع البكتريا المرافقة لجذور الخيار ضد الفطر*Rhizoctonia solani*

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المستخلص

أجريت هذه الدراسة في مختبرات قسم وقاية النبات التابع لوزارة الزراعة بهدف عزل بكتيريا مرافقة لجذور الخيار من بيوت بلاستيكية في بغداد، وصلاح الدين، والسليمانية، والبصرة، وتقييم مقدرتها على تثبيط *Rhizoctonia solani* المُسبب لمرض تعفن جذور الخيار على وسط بطاطا دكستروز اجار(PDA) تم الحصول على 12 عزلة بكتيرية من تربة مرافقة لجذور خيار سليمة. اختلفت هذه العزلات في أنواعها اعتمادًا على موقع الجمع والظروف البيئية. أظهرت جميع العزلات نشاطًا تثبيطيًا عاليًا ضد *R. solani*، أذ تراوحت أقطار مستعمرات الفطر بين 1.17 و6.63 سم، ونسب التثبيط بين 26.3% و87.0% مقارنة بمعاملة السيطرة. تم إجراء التشخيص الجزيئي باستخدام تفاعل البلمرة المتسلسل (PCR) لثلاث عزلات: *Bacillus cereus* و *Bacillus thuringiensis* و *Bacillus rugosus*. وقد تم إيداع تسلسلات القواعد النيتروجينية الخاصة بها في قاعدة بيانات بنك الجينات تحت أرقام الانضمام PP935479.1، PP935461.1 وPP934669.1 على التتابع. وتُبرز هذه النتائج إمكانية استخدام العزلات البكتيرية المحلية كعوامل مكافحة حيوية ضد تعفن جذور الخيار.

الكلمات المفتاحية: *Bacillus* sp ،المكافحة الحيوية، بكتيريا المحفزة لنمو النبات، مرض تعفن الجذور.