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Genetic diagnosis of root nodules bacteria isolated from some leguminous Plants in Nineveh Governorate

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Abstract

Because of the importance of atmospheric nitrogen-fixing bacteria, the study aimed to isolate bacteria from the root nodules of plants from four areas within the city of Mosul during the winter planting period, for the season 2020-2021. Biochemical tests, phenotypic and agricultural characteristics were used to diagnose bacterial isolates and study their sensitivity and resistance to ten types of antibiotics. The results showed that the highest percentage of resistance was to the antibiotics Trimethoprim and Streptomycin at 100%, while it was the least resistant to the antibiotics Tetracycline and Rifampicin at 22.2%. The four bacterial isolates were resistant to cadmium chloride salts (CdCl), while the lowest growth rate was when treated with mercury chloride salt (HgCl). Polymerase Chain Reaction (PCR) technology was used to diagnose samples based on the analysis of nitrogenous bases in the 16S rRNA gene and compare the sequences generated by DNA amplification with standard isolates within NCBI to detect new isolated bacterial isolates.

Keyword: *Rhizobium*, Antibiotics, Heavy metals, PCR, 16S rRNA

التشخيص الجزيئي لبكتيريا العقد الجذرية المعزولة من بعض النباتات البقولية في محافظة نينوى

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الخلاصة:

لأهمية البكتريا المثبتة للنتروجين الجوي، فقد هدفت الدراسة إلى عزل البكتريا من العقد الجذرية للنباتات من أربع مناطق ضمن مدينة الموصل في مدة الزراعة الشتوية، للموسم 2020-2021 واستخدمت الاختبارات الكيموحيوية والخصائص المظهرية والزراعية لتشخيص العزلات البكتيرية ودراسة حساسيتها ومقاومتها لعشر أنواع من المضادات الحيوية. إذ أظهرت النتائج أن أعلى نسبة مقاومة كانت للمضادين الحيويين Trimethoprim و Streptomycin بنسبة 100٪ ، بينما كانت أقل مقاومة للمضادين الحيويين Rifampicin و Tetracycline بنسبة 22.2٪. وكانت العزلات البكتيرية الاربعة مقاومة لأملاح كلوريد الكادميوم (CdCl) بينما أقل معدل للنمو كانت

عند المعاملة بملح كلوريد الزئبق (HgCl). أستخدمت تقانة Polymerase Chain Reaction (PCR) لتشخيص العينات بناءً على تحليل القواعد النتروجينية في جين 16S rRNA ومقارنة التسلسلات الناتجة عن تضخيم الحمض النووي بالعزلات القياسية ضمن NCBI للكشف عن العزلات البكتيرية المعزولة الجديدة.

الكلمات المفتاحية : 16S rRNA، PCR، المعادن الثقيلة، المضادات الحيوية، Rhizobium

1. Introduction

The leguminous family Leguminosae is known as the Bean family, and It is a major source for humans and animals. It comes after cereal crops in terms of nutritional quality. It is a source of protein and calcium. There are 34 genera and 300 species in Iraq, in addition to 76 species grown for agricultural purposes. The legume family is considered one of the largest families of flowering plants (Angiosperms) [1]. Placed in Order Laguminalse, under three families fall: Pcaescaeae, (Papilionaceae) [2]. The members of this family are distinguished from other plant families by forming a symbiotic relationship with Rhizobia, where this relationship resulted in the formation of root nodules and the fixation of atmospheric nitrogen and its conversion into ammonia that can be utilized by plants [3]. The genera of the family Rhizobiaceae are *Rhizobium* and *Allorhizobium* and *Mesorhizobuim* and *Bradyrhizobium and Sinorhizobium* [4]. Members of this family are organotrophic, aerobic and facultative [5], is rod-shaped, don't from of spores, endemic to the soil and its members form root nodules that fix atmospheric nitrogen in the roots of legumes [6]. There are found in single or pairs and are motile by grow better growth ranges between (25-30^{o} C) and many of they are unable (37^{o} C) and they have the ability to benefit from a wide range of carbon compounds [7]. These bacteria are collected inside the ganglia to form the so-called Bacteroids, which have the ability to fix nitrogen [8]. The benefit of this symbiotic relationship is to improve the productivity of the soil in addition to its economic importance, in addition to the fact that the biological fixation of nitrogen pollutes the environment[9]. Local studies have shown the possibility of isolating different species of rhizobia from different areas of Nineveh Governorate [10][11]. More accurate and faster efficient molecular techniques have been developed to assist traditional phenotypic and morphological techniques in differentiating between different microbial genera, species and strains [12]. PCR primers for rhizobia species based on 16s rRNA sequence analysis and comparison with fixed data by DNA detection of species and shapes between strains of the same species using PCR technique to know and estimate the diversity of rhizobia and show ERIC and REP sequences are present in Rhizobia [13].The aims study to isolate the bacterial species belong to the genus *Rhizobium* from some leguminous plants in the some areas of Nineveh Governorate and to identify the isolated bacterial species using the microbiological, biochemica characteristics and their diagnosis at by using the specific PCR technique.

2. Research Method

Isolation of Rhizobia bacteria from the root nodules of leguminous plants

The nodules were separated and washed with distilled water. These nodules were immersed in ethanol (70%) for 2-4 minutes and then washed several times with sterile distilled water, then the root nodules was immersed in a 3% NaOCl solution 15 minutes [14], were washed with distilled water 3 times and placed on sterile filter papers to dry and to ensure the efficiency of marking the isolated root nodules were used [15]. Bacteria were cultured on Solid YEMA medium [16].

Identification of bacteria (*Rhizobium*):

The phenotypic characteristics were studied using Gram stain Kit using a compound light microscope (100X)[15]. The isolated bacteria were classified according to their plant host into nine cases, and each culture was coded according to the Leguminous plant as in the following (Table-1).

Table (1) The code of groups isolated bacteria were classified according to their plant host

Vigna sp. (Bean	*Pisum sativum* (Pea)	*Phaseolus vulgaris* (Common bean)	*Lens* sp. (Lentil)	*Cicer arietinum* (Chickpea)	*Trigonella foenum-graecum* (Fenugreek)	*Vigna unguiculata* (Cowpea)	*Trifolium* spp (Clover)	*Medicago sativa* (Alfalfa)
RhV	RhP	RhPh	RhC	RhA	RhG	RhR	RhM	RhS

The family specialization test was conducted for the bacteria isolates under a study by taking of pure colony for each isolates to prepare 10 ml of liquid YEM medium and placed for 48 hours in the shaking incubator with 150 cycles/ minute at 28°C±1 for 48 hours. The plant seeds were planted on NF medium after sterilization It was washed several times with running water and immersed in ethanol 70% concentrate for 2 minutes, then washed sterile distilled water for 3 times and then placed in 3% sodium hypochlorite solution (NaOCl) for 15 minutes and then washed with sterile distilled water for 3 times and dried on several times., then the root total of plants inoculated after four to six days of growth using the inoculum (10^8x3) cells /ml the seedlings of the plants under study mentioned in table [1] was soaked with bacterial inoculum for 15 minutes and one method was used for surface sterilization of the seedlings mentioned above .To prepare the bacterial cutler for 4 days, according to the method of [17], and growing on a medium free of nitrogen (NF). The purpose of this is to ascertain the purity of the bacterial isolate and to make sure its ability to form root nodules on its host [18].

The Heavy metals medium:

Salts of heavy metals (mercury chloride, copper chloride, cadmium chloride, nickel chloride) to the sterilized YEMA media and cooled to a temperature 45-50°C, each separately according to the method [19].

The medium for selecting antibiotics:

The appropriate antibiotic was added to the sterilized and cooled YEMA medium at a temperature of 45-50°C with final concentrations of micrograms per liter mentioned in Table -2. The stock solutions of antibiotics were prepared according to the methods [20] [21].

Table 2- Stock and Final Concentrations of Antibiotics Solvent

Antibiotic	Code	Stockpile conc. (µg/ml)	Final conc. (µg/ml)	Solvent
Tetracycline	Tet	5	10	Ethanol 70%
Ampicillin	Amp	5	50	Sterile distilled water
Amoxicillin	Amo	5	50	Ethanol 70%
Streptomycin	Str	50	20	Sterile distilled water
Rifampicin	Rif	5	50	Methanol
Nystatin	Nst	5	50	Ethanol 70%
Erythromycin	Ery	10	15	Absolute ethanol
Gentamycin	Gen	40	25	Sterile distilled water
Trimethoprim	Tri	5	50	Ethanol 70%
Cefixime	Cef	5	50	Sterile distilled water

Biochemical tests for *Rhizobium* Bacterial Isolates:

Some biochemical tests were used on the genera of Rhizobia isolated from the root nodules of the group of leguminous plant under study. The Citrate Utilization test was conducted according to the by a method of [22]. The urease product test was prepared according to the method [23], and the fluorescence test was performed according to the method [17]. The catalase test was conducted according to [24]. A gelatin liquefied test was carried out [17]. The Cytochrome Oxidase Test was performed by the method [23]. The Voges-Proskuar Test was prepared according to the method [25]. The Methyl Red Test was prepared according to the method [26]. Motility test was carried out according to the method [27]. The Indol Production Test was performed according to the method [28].Test (Bromothymol Blue Test BTB), The test was conducted according to the method [29]. The Macconkey Agar Medium test was prepared according to the method [30].

Isolatio and purification of the genomic DNA cotent from *Rhizobium*

Purification DNA Kit supplied by (Geneaid) was used to extract DNA from bacteria samples of the genus *Rhizobium*, by transferring pre-purified colonies of the root ganglion bacteria under study grown on YEMB liquid medium in a volume of 10ml and incubated in a Shaking Incubator at (150 rpm) at a temperature of 28±1°C for 48-72 hours and according to its protocolTo detect the purified DNA, the samples were transferred to a 1% agarose gel in Run Tank with a transducer using (X1) TBE buffer, and then the gel was imaged by Gel Documentation to be able to view the genomic DNA bundles as well as the PCR reaction product [31].

Spcific Amplification of DNA-PCR:

The purity and concentration of the DNA samples of the bacterial isolates understudy were measured by using Nanodrop to obtain the concentration required to perform the PCR reactions and it was (50) ng/microliter for each sample. Prepare the master reaction mixture for each PCR reaction by mixing the DNA sample and the special primer shown in Table (3) for each gene with the components of the master-mix inside a 0.2 ml Eppendorf tube supplied by the English company Biolabs and complete the reaction volume to 20 µl with distilled water, and then The mixture was discarded in the Microfuge for a period between (5-3) seconds to ensure that the reaction components did not remain on the walls of the reaction tubes. The reaction tubes were inserted into the Thermocycler to conduct the multiplication reaction using the special program for each reaction shown in Table (4):

Table (3) Special primers used in the PCR reaction

Primer	Name	Sequence
Forward	pA*	AGAGTTTGATCCTGGCTCAG
Revers	pH*	AAGGAGGTGATCCAGCCGCA

Table (4) The cycle of PCR program

No.	Stage	Temp.	Time (min.)	Cycle
1	**First Denaturation**	95	6	1
2	**Denaturation**	95	0.45	
3	**Annealing**	55	1	35
4	**Extension**	72	1	
5	**Final extension**	72	5	1

Detection of nucleotide sequences for amplified DNA segments using DNA sequencing:

Determining the sequence of nitrogenous bases to the bacterial samples understudy, as the PCR products of the 16SrRNA region were sent to the products of samples with the primers of the resulting. The sequence was read for the genes based on the 3130 Genetic Analyzer device supplied by the Japanese company Hitachi, and use the National Center Biotechnology (NCBI) and results were analyzed using Mole-Balast and Blast software.

3. Results And Discussion

Diagnostic test for Rhizobia bacteria isolated:

After 8 to 10 days the plant roots were periodically examined under a compound light microscope, and it was noticed that there was a deformation in the root hairs of leguminous root plants Figure -1). The researcher Selami and others were described through their study of the shape of the nodules and their anatomy in the plant *Retama monosperm*a the country of Algeria that the shapes of the nodules are elongated[32].

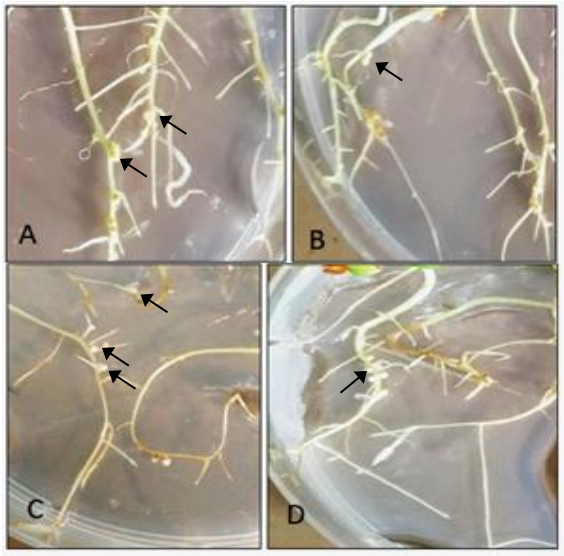

Figure 1-shows the root nodules and their shapes on the roots of some plants understudy
A: The root nodules formed on the roots of a Pea plant (indicator Part)
B: The root nodules formed on the roots of a bean plant (indicator Part)
C: The root nodules formed on the roots of a chickpea plant (indicator Part)
D: The root nodules formed on the roots of a ring plant (indicator Part)

Morphological and Cultural Characters for Isolated Rhizobial Bacteria

When bacterial colonies were grown in YEMA medium, they appear of different colors between ivory and cream, circular and sticky, with smooth edges. It was observed that these bacterial colonies produce in rich media on this technology with carbon exopolysaccharides, and after several days of their growth in YEMA medium, the colonies sticky substance on the cover of the dish even if it is stored at a temperature of 4^{o}C. A single colony was taken from the culture and stained by the gram, then they were examined by a compound light microscope (100X), they appear gram–negative, this was noted by the researchers [1].

Biochemical Tests:

Table -5A-B of biochemical tests were shown the results, where all the isolates understudy were positive for the motility test, and these results were almost identical to the results of [33]. As for the

production test, all samples under study and all sugars were positive, and the results were similar to those of [34]. As for the citrate consumption test, all samples were negative, and these results were similar to the results of [35]. Red methylation and catalase test for all isolates were positive and the results converged with [36],while the results of the indole test showed that all isolates were positive and the results converged with [37]. As for The Voges-proskaur test, all isolates were negative. It matched with the results of [38]. As for the urease production test, all isolates under study were positive,it matched with the results of [37]. As for the gelatin test, all isolates were negative and it was similar to the results of [35]. The results of the oxidase test for collecting isolates were positive. The results matched with [39]. The table[5] also shows that when cultured groups of bacteria RhP, RhR. RPh, RhM, RhV, RhC, RhS,RhA, and RG isolated from nodules *Pisum sativum, Vigna unguiculata, Phaseolus vulgaris, Trifolium spp, Vicia faba, Lens culinaris, Medicago sativa, Cicer arietinum L. and Trigomella faenum-graeum* Plants respectively on Kings Medium.All these isolates had the ability to photoluminescence Fluoresce by exposure to UV rays at a wavelength of 320nm and this result is almost identical to what was observed by researchers [35]. In this study of several strains of *Rhizobium*,the results showed that the selection of the medium of MacConky Agar on the previously mentioned isolates was positive and these results match what was indicated by both researchers [39]. As for the BTB test and the Congo red test, all isolates under study were positive, and this was indicated [40].

Table 5-A Shows the biochemical tests for isolates of *Rhizobium* bacteria.

. **Group of *Rhizobium***	**RhS**				**RhV**				**RhC**				**RhA**			
Biochem **Test**	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Voges Proskaur	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MacConky Agar	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BTB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Congo-rad	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fluoresce	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Urease	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate Utilization	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin liquefaction	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indol production	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Methyl Red	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid from glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid from maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid from ramenose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid from lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid from Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid from xylose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Test negative result (−) Test positive result (+)

Table 5-B Shows the biochemical tests for isolates of *Rhizobium* bacteria.

. **Group of *Rhizobium***	**RhG**				**RhM**				**Rhph**				**RhP**				**RhR**			
Biochem **Test**	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Voges Proskaur	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

MacConky Agar	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BTB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Congo-rad	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fluoresce	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Urease	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate Utilization	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin liquefaction	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indol production	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Methyl Red	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid from glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid from maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid from ramenose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid from lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid from Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid from xylose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Test negative result (−) Test positive result (+)

Resistance and Sensitivity Test of Bacteria Isolated from Root Nodules to Antibiotics

Ten antibiotics were selected to study their effects in terms of resistance and sensitivity of *Rhizobium* bacteria, Table 4 of these results was shown there are differences in sensitivity to antibiotics and can make groups of bacteria isolated from the root nodules of leguminous plants. Where it was noted that all bacteria isolates were 100% resistant to Trimethoprim and Streptomycin, as for the antibiotics Rifampicin and Tetracycline, the resistance was less than it was 22.2 % among the groups of isolates understudy, while the percentage differences in resistance to other antibiotics of the bacterial isolates in the study amounted to 88.8 % in Erythromycin and Amoxicillin, and in Nystatin the percentage reached 77.7%, and the percentage in Ampicillin reached 77.7%, while the percentage in Gentamycin was 33.3%, and the percentage in Cefixime was 88.8%. The results of this study were the same as what the researcher discovered [41]. In terms of the resistance of most of the isolates to the antibiotic Amoxicillin, where the results of *Rhizobium* resistance to Tetracycline,Streptomycin, Amoxicillin, and Ampicillin converged with many studies that indicated *Rhizobium* possessing resistance to these antibiotics and several isolates of *Sinorhizobium meliloti* and *R. Leguminosarum*, [42] [43]. There is also a difference in the percentage of antibiotic resistance in other groups of *Rhizobium* bacteria isolated from different regions, which are understudy.

Table 6 Resistance and sensitivity of *Rhizobium* bacteria isolated from leguminous plant nodules to antibiotics.

Antibiotics			**Tet**	**Cef**	**Gen**	**Amo**	**Tri**	**Rif**	**Amp**	**Str**	**Nst**	**Ery**
Final conc.µg/ml			**10**	**50**	**25**	**50**	**50**	**50**	**50**	**20**	**50**	**10**
***Rhizobium* bacteria groups used in the study**	**RhS**	1	S	R	R	R	R	S	R	R	R	R
		2	S	R	R	R	R	S	R	R	R	R
		3	S	R	R	R	R	S	R	R	R	R
		4	S	R	R	R	R	S	R	R	R	R
	RhV	1	R	R	S	R	R	R	R	R	R	R
		2	R	R	S	R	R	R	R	R	R	R
		3	R	R	S	R	R	R	R	R	R	R
		4	R	R	S	R	R	R	R	R	R	R
	RhC	1	S	R	S	R	R	S	R	R	R	R

	2	S	R	S	R	R	S	R	R	R	R
	3	S	R	S	R	R	S	R	R	R	R
	4	S	R	S	R	R	S	R	R	R	R
	1	S	R	S	R	R	S	R	R	R	R
RhA	2	S	R	S	R	R	S	R	R	R	R
	3	S	R	S	R	R	S	R	R	R	R
	4	S	R	S	R	R	S	R	R	R	R
	1	S	S	S	R	R	S	S	R	S	S
RhG	2	S	S	S	R	R	S	S	R	S	S
	3	S	S	S	R	R	S	S	R	S	S
	4	S	S	S	R	R	S	S	R	S	S
	1	R	R	S	S	R	S	R	R	S	R
RhM	2	S	R	S	S	R	S	R	R	S	R
	3	S	R	S	S	R	S	R	R	S	R
	4	S	R	S	S	R	S	R	R	S	R
	1	S	R	R	R	R	S	R	R	R	R
Rhph	2	S	R	R	R	R	S	R	R	R	R
	3	S	R	R	R	R	S	R	R	R	R
	4	S	R	R	R	R	S	R	R	R	R
	1	S	R	S	S	R	S	R	R	R	R
RhP	2	S	R	S	S	R	S	R	R	R	R
	3	S	R	S	S	R	S	R	R	R	R
	4	S	R	S	S	R	S	R	R	R	R
	1	R	R	R	R	R	R	S	R	R	R
RhR	2	R	R	R	R	R	R	S	R	R	R
	3	R	R	R	R	R	R	S	R	R	R
	4	R	R	R	R	R	R	S	R	R	R

The emergence of the characteristic of resistance (R) The emergence of the characteristic of sensitivity (S)

Resistance and Sensitivity Test of Bacteria Isolated from Root Nodules to Heavy Metal:

By looking at the table-7 groups of *Rhizobium* bacteria that isolated from the root nodules of leguminous plants showed that among isolates were resistant to cobalt chloride ($CoCl_2$) and ($CdCl_2$) at 94.4%.As for the percentage of the resistance of these aggregates to the heavy metal $HgCl_2$was 77,7% while the percentage of resistance of these aggregates to heavy metal nickel chloride ($NiCl_2$) was 55.5%. The results showed that the isolates in (RhV) had a very high resistance that recodes 100% of the percentage of all heavy metals used in the study, While the remaining bacterial groups were RhA, RhS, RhG, RhC, RhPh, RhM, RhP, RhR which isolates from the nodes, The roots of plants: *Vigna unguiculata, Pisum sativum, Trifolium spp, Phaseolus vulgaris,Lens culinaris,Trigonella foenum- graeum, Medica go sativa and Cicer arietinum,* respectively, the resistance to heavy metals was disparate The bioaccumulation of heavy metals and their toxicity in the environment affect the life of living organisms tremendously, as heavy metals cannot be broken down through chemical and biological processes, which is the opposite of organic pollutants, but it can turn into lower toxic types. to withstand heavy metals from several polluted industrial areas. Minerals affected their protein profiles and most of the changes were offset by a decrease in the expression of polypeptides. This study indicated that there is a relationship between root tolerance and soil contamination with heavy metals and change in protein pool as a result, analysis of protein changes appears to be a good indicator for estimating the level of stress imposed on *Rhizobium* groups exposed to

contamination. with heavy metals. The long-term deposition of minerals in the soil resulted in high concentrations of minerals, which negatively affects the microorganisms in the soil [44]

Table 7-Resistance and sensitivity of *Rhizobium* bacteria isolated from nodules of leguminous plants to heavy metals.

Heavy Metals Final conc.µg/ml		$NiCl_2$ 25	$HgCl_2$ 25	$CoCl_2$ 25	$CoCl_2$ 25
***Rhizobium* bacteria groups used in the study**					
RhS	1	++	++	+++	++
	2	++	++	+++	++
	3	++	++	+++	++
	4	++	++	+++	++
RhV	1	+++	+++	+++	+++
	2	+++	+++	+++	+++
	3	+++	+++	+++	+++
	4	+++	+++	+++	+++
RhC	1	+	++	+	++
	2	+	++	+	++
	3	+	++	++	++
	4	+	++	++	+++
RhA	1	+++	+	++	+++
	2	+++	+	++	+++
	3	+++	+	++	+++
	4	+++	+	++	+++
RhG	1	+	++	+++	++
	2	+	++	+++	++
	3	+	++	+++	++
	4	+	++	+++	++
RhM	1	+	+++	++	++
	2	+	+++	++	++
	3	+	+++	++	++
	4	+	+++	++	++
Rhph	1	++	++	+++	+++
	2	++	++	+++	+++
	3	++	++	+++	+++
	4	++	++	+++	+++
RhP	1	++	+	++	++
	2	++	+	++	++
	3	++	+	++	+
	4	++	+	++	+
RhR	1	+	++	+++	+++
	2	+	++	+++	+++
	3	+	++	+++	+++
	4	+	++	+++	+++

High resistance (+++) Medium resistance (++) Weak resistance (+)[45]

The researchers conducted [44] a study in which *Rhizobium leguminosarum biovar viciae* was isolated from areas was different contents of minerals and their variances were apportioned pool in

Rhizobium groups were also evaluated. Physical and chemical parameters were determined and mineral concentrations were analyzed in soil by ICP-AES-isolates were screened for tolerance in YEMA supplemented with different heavy metals (Zn, Pb, Co, Cd, Ni, and Cr). The proteins were extracted and separated by SDS-PAGE soil (EI and EL Engineering industries) Presented the highest concentration of minerals and thus the soil was more contaminated. The isolates showed different growth responses (Control) and M (Mines) were less tolerant than isolates of EI_1, EI_2, and C_1 (Chemical industries). The change in protein pool as a consequence analysis of protein changes appears to be a good indicator for estimating the level of stress imposed on Rhizobia populations exposed to heavy metal contamination.

Study and characterization of the genetic content of rhizobia bacteria understudy

Extraction of DNA content from groups of Rhizobia bacteria isolated from leguminous plants. The process of electrophoresis of the extracted genomic DNA samples was carried out using agarose gel a with concentration (1%) using UV rays and Gel Documentation, gel imaging was performed to trace and detect the genomic DNA bands. Figure 2 - shows the results of the detection, also shows that the genomic DNA bundles appeared in equal and larger sizes as a result of their proximity to the etching of the agarose gel.

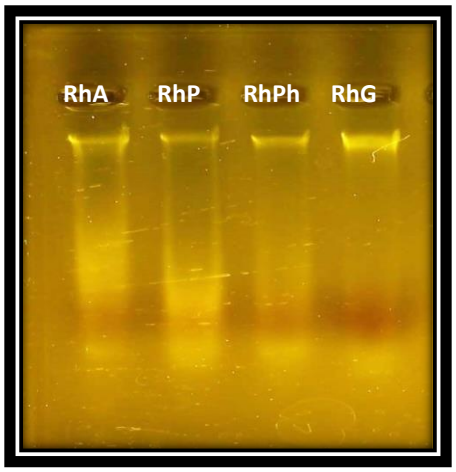

Figure 2-The Electrophoresis in agarose at a concentration of 0.7% of the genomic DNA.

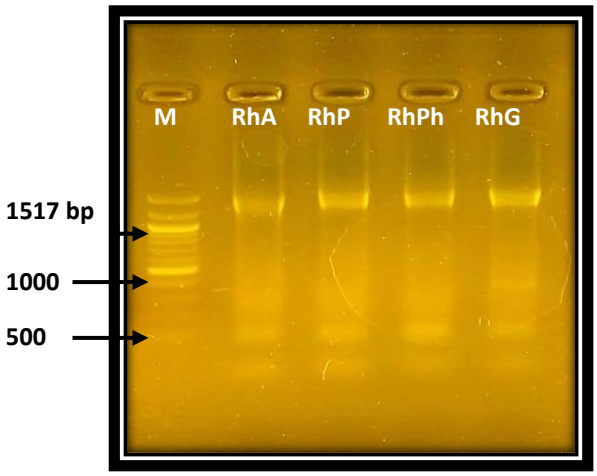

Figure 3-The Electrophoresis in agarose at a concentration of 0.1% of the PCR Products to 16S rRNA of Rhizobium isolates

16S rRNA partial sequencing of the gene:

Figure -3. it was present concluded that there are four amplified bundles of the genomic DNA site, prepared from the isolates of Rhizobia bacteria under study (RhA, RhP, RhPh, RhG), appear as results of gel electrophoresis of PCR product to all isolates which were equal and large range about of 1500bp. The reason for the emergence of these bundles of pure DNA is the result of the presence of a common sequence of the nucleotide present in the DNA of these bacterial groups, where this similarity enabled them to complement the nitrogenous bases in the specialized primers, the completion reaction and the production of DNA bundles of large and equal sizes this was similar were found in the researches were reported by researchers through their study on 25 selected isolates of (*Phaseolus vulgaris* L.), where PCR amplification of 16S rRNA genes produced a single 1500bp sequences. Sequences were deposited in a bank and their access numbers were determined. The newly obtained 16S rRNA fragment with known bacterial sequences in the genebank database using BLASTN analysis showed sequence similarity to nitrogenous bases with a percentage of 99.2% [46] and [47]. Genetic analysis was conducted for four randomly selected isolates using the sequences obtained from DNA sequencing technology by using the

Mole-Blast program through the link NIH.Govto find the phylogenic tree of the genotypes that shows the genetic relationship between the *Rhizobium* isolates under study and the standard strains registered in the gene bank, The results of this study are close to the findings of the research [10] when they studied the genetic diversity often isolates of *Rhizobium leguminosarum* bacteria in Egypt.

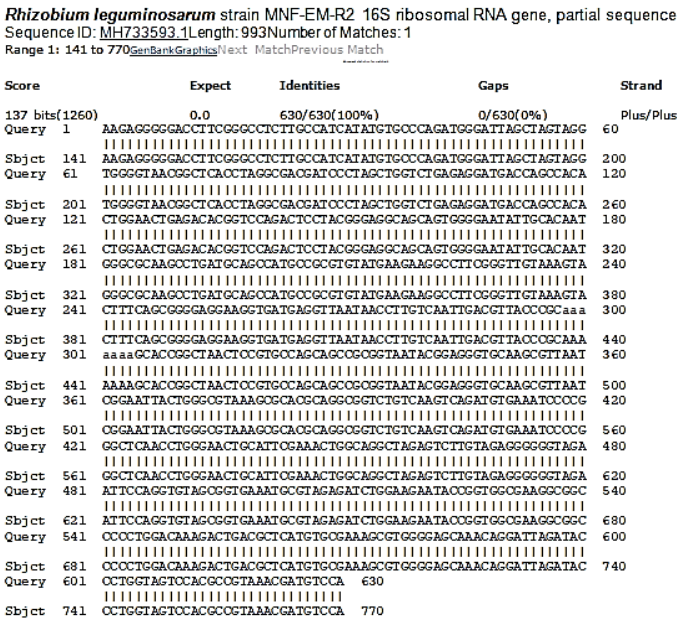

Fig 4- Comparison of the nitrogenous base sequences of the local isolate (RhG) and the major strain : MH733593.1

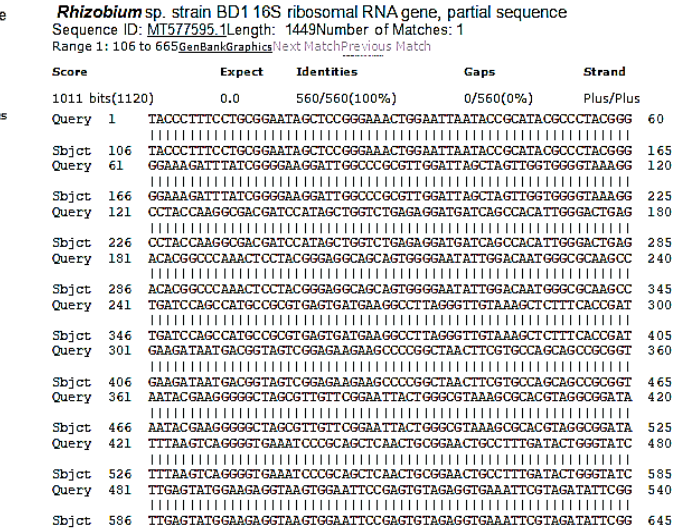

Fig 5- Comparison of the nitrogenous base sequences of the local isolate (RhA) and the major strain : MT577595.1

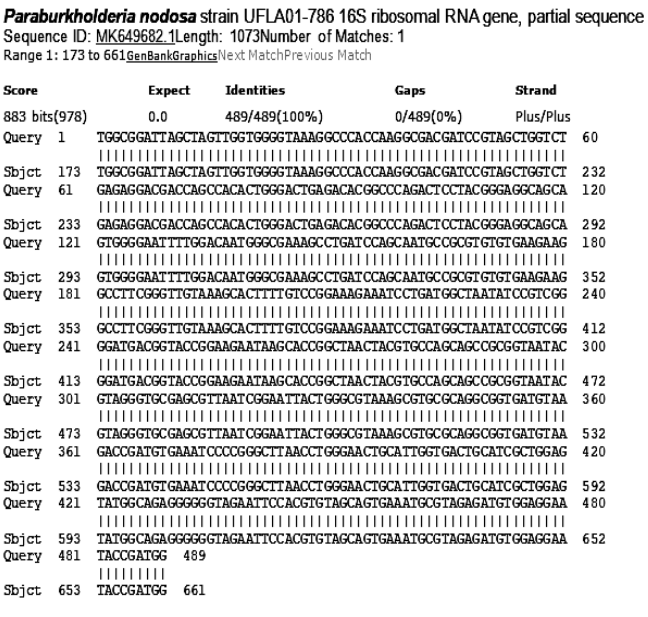

Fig 6- Comparison of the nitrogenous base sequences of the local isolate (RhPh) and the major strain : MK649682.1

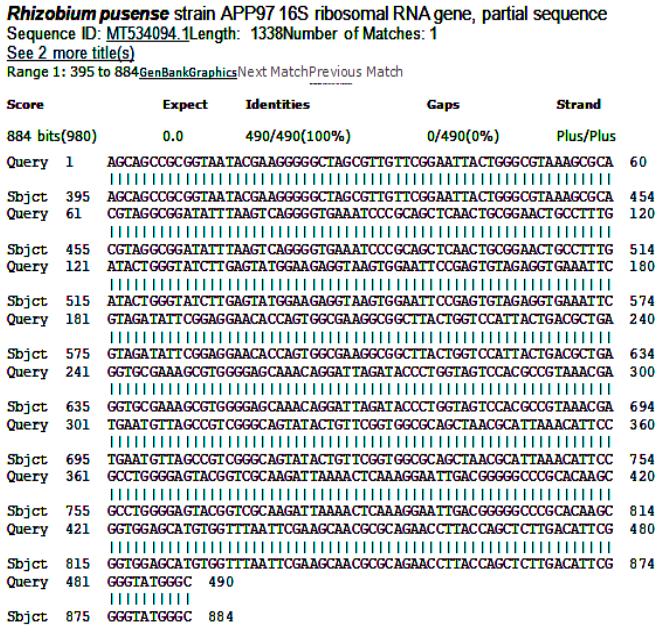

Fig 7- Comparison of the nitrogenous base sequences of the local isolate (RhP) and the major strain : MT534094.1

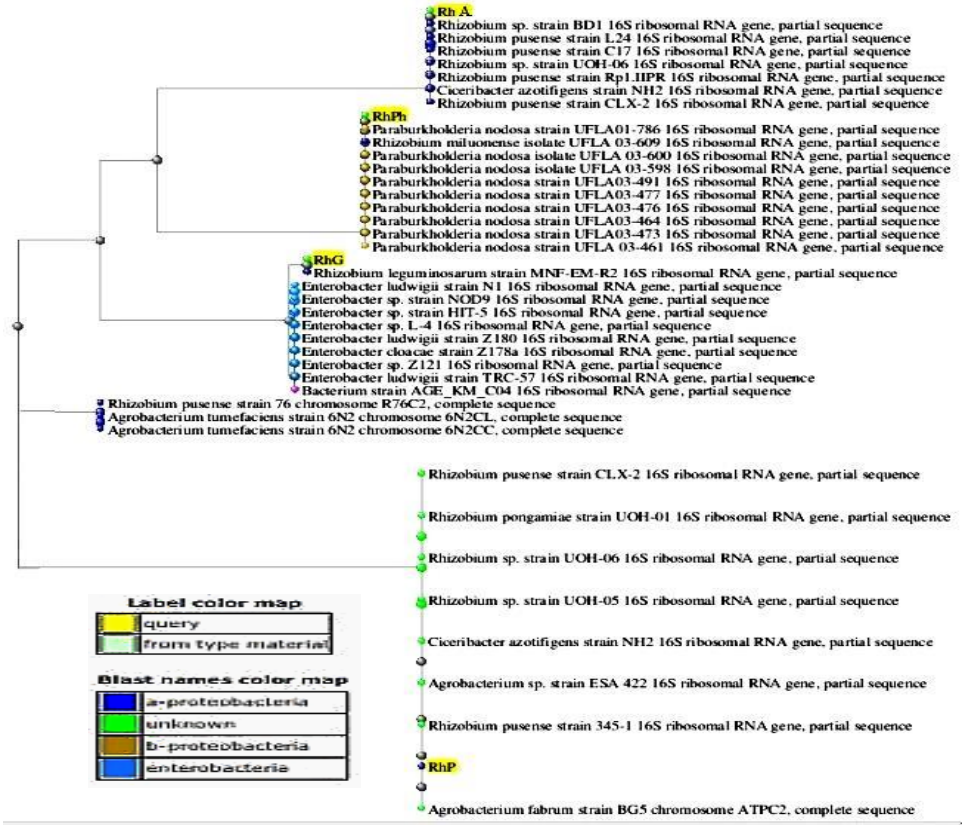

Figure 8- shows the Phylogenic tree of the genotypes using the results of the sequence analysis of four *Rhizobium* isolates under study and using the program Mole/ Blast

4. Conclusion

By observing the results of the analysis using the DNA program, it showed that there is a great similarity of up to 100% between the sequences of the bacterial isolates under study (RhA, RhP,RhPh,RhG). With the sequences of the nitrogenous bases of the standard bacterial isolates Rhizobium sp. strain BD1, *Rhizobium* presence strain APP97, Paraburkholderia nodosa strain UFLA01-786, and Rhizobium leguminosarum strain MNF-EM-R2 (MT577595.1, MT534094.1, MK649682.1, and MH733593.1) respectively and recorded in the NCBI GenBank.Figure 8, shows the DNA Blast/ NCBI program for nitrogen base analysis of bacterial isolation, the isolates in this study (RhA) were recorded as standard strain in The GenBank (NCBI) and given the accession number LC635720.1. Rhizobium pusense SAM-MA. Through the results obtained, it is clear that the possibility of genetic transfer of genes related to the process of fixing atmospheric nitrogen is the transfer between members of bacterial species through horizontal transfer of genetic traits between bacterial cells, and this is what the researchers also noticed by isolating Rhizobium bacteria in Egypt By studying the genetic variance of the genetic sequence of the 16S rRNA site and making a comparison between bacterial species [10].

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6. Conclusion:

We concluded from our current study that there is the possibility of studying the genetic similarity between genetic isolates and isolates from different regions by using PCR technique by studying the

sequence of nitrogenous bases of the 16S rRNA gene, which helps in diagnosing local isolates and the possibility of using them in finding isolates that were recorded for the first time in Nineveh Governorate as standard strain in The GenBank (NCBI) and given the accession number LC635720.1 *Rhizobium pusense* SAM-MA.

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