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Tolerance of *Rhizobium* Isolates to Certain Heavy Metals

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Abstract

In this study, five isolates of *Rhizobium* bacteria were isolated from root nodules of different leguminous plants as follows: *Ensifer meliloti* EW7 from *Medicago sativa* L., *Ensifer fredii* bv. *fredii* EW33 from *Vigna unguiculata* L., *R. leguminosarum* bv. *phaseoli* EW15 from *Phaseolus vulgaris* L., *R. leguminosarium* bv. *trifolii* EW19 from *Trifolium alexandrium* L. and *R. legumiosarium* bv. *viciae* EW30 from *viciae faba* L., which were collected from different cultural areas in Nineveh, Governorate-Iraq. Then pure *Rhizobium* colonies were obtained, and their agronomic and biochemical characteristics were studied. These isolates were a rod-shaped and negative Gram-stain. Whereas positive for TSI, BTB, CUT, Kovak's test, urase test, and for the methyl red and Voges-Proskauer tests. All of the rhizobial isolates showed positive results for the catalase test and for motility when tested on TY semi-solid medium. Tolerance of local *Rhizobium* isolates was tested against four heavy metal salts as follows: $Pb(CH_3COO)_2$, $Nicl_2$, $CdCl_2$, and $CoCl_2$. The isolates showed a varied response to tolerance or resistance to heavy metal salts. The highest mean of inhibition zone reached 21mm in isolate *Ensifer fredii bv. fredii* EW33 against heavy metal $CdCl_2$. Whereas the isolate *R. legumiosarium* bv. *viciae*, EW30 showed less affects which mean of inhibition zone was 9 mm only with 5000 concentration of heavy metal $CoCl_2$.

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1. Introduction:

Members of the *Rhizobiacea* family, which is composed of several genera including *Rhizobium*, *Shinella*, and *Ensifer*, the *Rhizobium* bacteria that are found in soil [1]. According to the growth of the *Rhizobium* in nutrient-rich medium, they are divided into two groups. First: a slowly growing alkaline producer, A normal multiplicity of these bacteria is 6-7 hours, and turbidity in the medium with 3-5 days. Second: which develops fast. One of its features is that it produces acids, which accelerate the turbidity of the medium. These bacteria reproduce every two to four hours on average [1].

All living organisms require nitrogen for the synthesis of urea, amino acids (proteins), nucleic acids (DNA and RNA), adenosine triphosphate (ATP), and nicotinamide adenine dinucleotide [2]. Prokaryotic microbes, such as *cyanobacteria* and *rhizobia*, which are referred to as diazotrophs, have fixed about 60% of the nitrogen [3]. Leguminous plants and *Rhizobium* bacteria have a symbiotic relationship that produces differentiated bacteria encased in intracellular compartments called symbioses within nodules on the root. The symbioses and nodules that are connected are designed to fix atmospheric nitrogen efficiently. This relationship is beneficial to both partners.

When rhizobia infiltrate adjacent cells, the cortex and peripheral cells start to divide one after the other, resulting in the creation of root nodules [4]. With the aid of the nitrogenase enzyme, the rhizobial bacteria that underwent bacteroid transformation changed air nitrogen into ammonia. Bacteriocins also referred to as protein toxins released from Gram-positive and Gram-negative bacteria, are known to be formed by certain genera of rhizobial bacteria [5]. Certain heavy metals, like copper, zinc, chromium, and manganese, are found in extremely minute amounts in the soil naturally, [6]. According to one

study, heavy metals can have an impact on the phases at which biofilms emerge. Exposure to harmful amounts of lead, cadmium, and zinc can cause this, as statistically significant variations in the thickness of formed biofilm Percentage of live and dead bacteria in the membrane [7].

Understanding the molecular mechanisms of heavy metal resistance may be one of the main goals in environmental biotechnology research, and also promotes the bioremediation of contaminated soils [8]. Rubio-Sanz *et al*. [9] reported that genetic determinants involved in heavy metal tolerance in rhizobia by efflux system in *Ensifer*, *Bradyrhizobium*, and *Mesorhizobium* isolates. Shen *et al*. [10] concluded that rhizobia can increase the heavy metal tolerance of nitrogen fixer plants by sequestering the heavy metals or changing their forms in the soil.

2. Research Method

2.1 Nutrient media

2.1.1 Yeast extracts mannitol Medium:

This medium was made by dissolving 1g yeast extract, 0.1gm NaCl, 10gm mannitol sugar, 0.2 gm $MgSO_4.7H_2O$, 0.5 gm, KH_2PO4, and 16gm Agar in 1.0 L of distilled water. After that, the medium was autoclave-sanitized and the pH was raised to 6.8 [11].

2.1.2 Tryptone Yeast Extracts Medium:

3g of yeast extract, 5gm of tryptone, and 0.12 gm of $CaCl_2.2H_2O$ were dissolved in a liter of distilled water to prepare this medium. After adjusting the pH to 6.8, the autoclave was used to sterilize the material [12].

2.1.3 Triple sugar iron agar Medium:

pH was adjusted to 6.8 after 65 gm of the medium was dissolved in 1.0 L of distilled water for preparation, and the autoclave was used to sanitize it [13].

2.1.4 Simmon Citrate Medium:

23 gm of the medium was prepared by dissolving it in 1.0 L of distilled water, adjusting the pH to 6.8, and autoclaving the mixture [14].

2.1.5 Muller-Hinton Medium:

35 gm of the medium was prepared by dissolving it in 1.0 L of distilled water, adjusting the pH to 6.8, and autoclaving the mixture [15].

2.1.6 Motility Test Medium:

Prepared by dissolving 1.3 gm of Tryptone yeast (TY) semi-solid, in 100 ml of distilled water then adding 0.5 gm of agar, finally adjusting the pH to 6.8 and autoclaving the mixture [16].

2.1.7 Peptone water, glucose, and phosphate medium:

After dissolving 5 gm of peptone and 5 gm of K_2HPO_4, adjust the pH to 6.8. Add 1% glucose sugar that has been autoclave-sterilized in Millipore filters with a 0.20 µm filter [17].

2.1.8 Urea medium:

Prepared by adding 50 ml of urea solvent, 40% sterilized in Millipore filters 0.20 µm, after dissolving 24g of base urea agar in 950 ml of distilled water and adjusting the pH to 6.8 in an autoclave [18].

2.2 Reagents and solvents:

2.2.1. Methyl red reagent:

Methyl red reagent is made by dissolving 0.1 gm of methyl red stain in 300 ml of ethanol at a 95% concentration and then adding distilled water to make 500 ml of volume. It was employed to identify full glucose sugar fermentation [19].

2.2.2. Catalase reagent:

Prepared by mixing 97 milliliters of pure water with 3.0 milliliters of hydrogen peroxide [20].

2.2.3. Kovac's reagent:

Dissolve 10 gm of *P*-dimethyl amino benzaldehyde in 150 milliliters of isoamyl alcohol.

In 150 milliliters of isoamyl alcohol, dissolve 10 gm of P-dimethyl amino benzaldehyde. Once thoroughly combined, add 50 milliliters of pure HCL and store it until needed [21].

2.2.4. Voges-Proskauer reagent:

This reagent is made up of two solutions: solution B (40 percent KOH dissolved in distilled water) and solution A (α-naphthol dissolved in pure alcohol) [22].

2.3 Sterilization:

The glassware was sterilized in an oven at 170 °C, and the nutrient media were sterilized using an autoclave sterilizer device at 121 °C and 15 pound/inch2 of pressure. Millipore filters with a 0.20-0.40 micrometer diameter were used to filter materials and solutions that were heat-sensitive [23].

2.4 Isolation of the *Rhizobium* bacteria:

Plant material provided from various agricultural regions in Nineveh Governorate/ Iraq, that are Al-Shamsiyat, Bartella, Zumar, Nimrod, and Yaramja. Healthy and pink root nodules were cut from roots washed under tap water and then immersed in 70% ethanol for 1 min. Then washed with distilled water, after immersion in 0.1% $HgCl_2$ for 2 min [24].

2.5 Purification of the *Rhizobium* isolates:

A loop carrier was used to purify the *Rhizobium* bacteria that were isolated from root nodules. Using the 2-4 stage planning approach, individual colonies with a mucous appearance were taken and subsequently cultivated on TY medium. At every stage, a flame is used to sterilize the loop, and the plate is thereafter incubated for a duration of 24-48 hours at 28 ^{0}C.

2.5 Microscopic examination:

On a sanitized glass slide, a tiny quantity of cultivated bacteria was applied together with a few drops of distilled water. Making sure to hold the slide over a flame, the slide was allowed to dry. Gram stain, a differential dye, was used to stain it. After that, examined at 100X magnification using an optical microscope [25].

2.6 Biochemical Test for Rhizobia bacteria:

2.6.1 Motility test:

Using a loop, young colonies of rhizobial bacteria were added to petri dishes filled with semi-solid TY medium for this test. Dishes were incubated at 28 ^{0}C for 24 to 48 hours. The spread of development away from the injection region suggests that bacteria are mobile [20].

2.6.2 Citrate Utilization Test:

Rhizobia colonies were added to tubes with Simmon citrate medium in order to perform this test. The medium's hue changing from green to blue shows that the bacteria can successfully use sodium citrate as its only carbon source [26].

2.6.3 Triple Sugar Iron Agar Test (TSI):

In order to perform this test, (TSI) medium was inoculated using the stabbing method on the inclined surface and the streaking method on the bottom of tubes containing *rhizobia* colonies. The development of the black color at the bottom of the tube indicates that the bacteria are producing hydrogen sulfide (H_2S), and the shifting color of the tube bottom indicates the fermentation of lactose, sucrose, and glucose [27].

2.6.4 Catalase test:

The presence of gas bubbles suggests that the bacteria are capable of producing the catalase enzyme and that the test findings are positive [28].

2.6.5 Urease test:

Rhizobia colonies were added to tubes containing agar-urea medium. The medium's color turning pink, a sign that bacteria are able to synthesize the urease enzyme, indicates a positive response [29].

2.6.6 Indole production test:

To test for indole, *Rhizobia* bacteria are added to tubes filled with peptone water, the tubes are incubated for 24 hours, and then 4-5 drops of Kava's reagent are added. The emergence of a crimson ring at the top of the middle, which denotes the breakdown of amino acids (tryptophan) and the generation of indole, is indicative of a positive outcome [30].

2.6.7 Methyl red test:

Rhizobial bacteria was added to the tube containing the MR/VP medium, and after 24 hours of incubation, 4-5 drops of methyl red reagent should be added. The medium's ability to turn red pinkish, indicating that the rhizobia are capable of producing acids, is a sign of a successful outcome [31].

2.6.8 Voges-Proskauer test:

After incubating for a day, tubes containing the *Rhizobia* bacteria-inoculated MR/VP media had 10 drops of the first solvent (alpha-naphthol) and 5 drops of the second solvent (KOH). After shaking the tubes, they were left for half an hour. The medium's color changing to red indicates a positive outcome and provides proof that bacteria can ferment glucose to generate acetyl methyl carbonyl [31].

2.6.9 Bromothymol blue test (BTB):

Rhizobia bacteria were added to a YEMA medium containing 0.0025% bromothymol blue dye, and the mixture was then cultured for 48 hours. I observed that the stain color changed from green to yellow due to the fast-growing rhizobia to green due to the slow-growing rhizobia [32].

2.8 Heavy metal salts resistance test of *rhizobia* isolates:

This experiment was done by making pores with 6 mm in Muller-Hinton agar medium to assess the tolerance of rhizobial isolates to heavy metal salts. Salts for various heavy metal concentrations (100, 500, 1000, 5000) µg/ml, including cobalt chloride ($CoCl_2$), cadmium chloride ($CdCl_2$), nickel chloride ($NiCl_2$), and lead $Pb(CH_3COO)_2$. Petri dishes were incubated for 24 to 48 hours at 28 0 ^{0}C. The damping diameter rates surrounding the hole were measured [33].

3 Results And Discussion

3.1. Isolation of Rhizobia:

Rhizobial bacteria were purified following their isolation from root nodules. According to Vincent [24] specificity of the strain of microsymbiont legumes demonstrates a high degree of symbiotic specialization, the isolated rhizobial bacteria as follows: *Ensifer meliloti* EW7 from root nodules produced on each *Medicago sativa* L., *Ensifer fredii* bv. *fredii* EW33 from *Vigna unguiculata* L., *R. leguminosarum* bv. *phaseoli* EW15 from *Phaseolus vulgaris* L., *R. legumiosarium* bv. *viciae* EW30 from *Viciae faba* L., and *R. leguminosarium* bv. *trifolii* EW19 from *Trifolium alexandrium* L. At 4 ºC in the refrigerator, a nutritional medium was used to store and purify these isolates.

Table 1. Types of isolated rhizobia from nodules developed on host plants, and areas of collection.

Isolate No.	*Rhizobium* species	Host plant	Collection area
Ew7	*Ensifer meliloti*	*Medicago sativa* L.	Nimrod
Ew15	*R. leguminosarum bv. phaseoli*	*Phaseolus vulgaris* L.	Yaramja
Ew19	*R. leguminosarium bv. trifolii*	*Trifolium alexandrium* L.	Al-Shamsiyat
Ew30	*R. legumiosarium bv. viciae*	*Viciae faba* L.	Bartella
Ew33	*Ensifer fredii bv. fredii*	*Vigna unguiculata* L.	Zumar

3.2. Morphological and microscopic diagnosis:

After 48 hours of incubation at 28 ^{0}C, all isolated rhizobial strains from root nodule plans showed good growth on the YEMA solid medium [34]. Bacteria of *Rhizobium* genera were smoothly growing and rod-shaped, found singly or clustered, and negative to Gram stain [35].

3.3. Biochemical characterization of rhizobial bacteria:

The outcomes of the biochemical tests (Fig. 1), demonstrated that every isolate is capable of producing urease enzymes. The catalase enzyme, which breaks down harmful hydrogen peroxide and releases water and oxygen, is an additional indicator that bacteria are capable of producing it. This shows that they can create gas bubbles on the glass slide. The motility test yielded positive results. The ability of these isolates to manufacture the urease enzyme and break down urea into ammonium is demonstrated by the medium's color changing from yellow to pink as a result of the pH increase [36].

The indole test revealed that every isolate possessed the tryptophanase enzyme, which is responsible for converting tryptophan to indole as indicated by the development of a red ring at the tube's top [37]. This investigation revealed that all isolates failed the methyl red test, the medium's color stayed yellow, and the Voges-Proskauer test revealed no scarlet color [38]. The study's

findings demonstrated that every isolate tested was positive for BTB (Table 2), moreover, the medium's color changed to yellow [39].

The medium's color turned yellow upon receiving a positive reaction from the TSI test [40]. The findings demonstrated that by simply turning the medium's hue from green to citrate, all isolates are capable of using it as their exclusive source of carbon. [41].

Table 2. Biochemical tests to diagnose the isolated rhizobial bacteria.

samples	Indole production	Catalase test	Methyl red	Triple sugar iron	BTB	Motility test	Urease enzyme	Citrate utilization	Voges-Proskauer
Ew7	+	+	-	+	+	+	+	+	-
Ew15	+	+	-	+	+	+	+	+	-
Ew19	+	+	-	+	+	+	+	+	-
Ew30	+	+	-	+	+	+	+	+	-
Ew33	+	+	-	+	+	+	+	+	-

BTB: Bromothymol Blue (+): Positive response; (-): Negative response

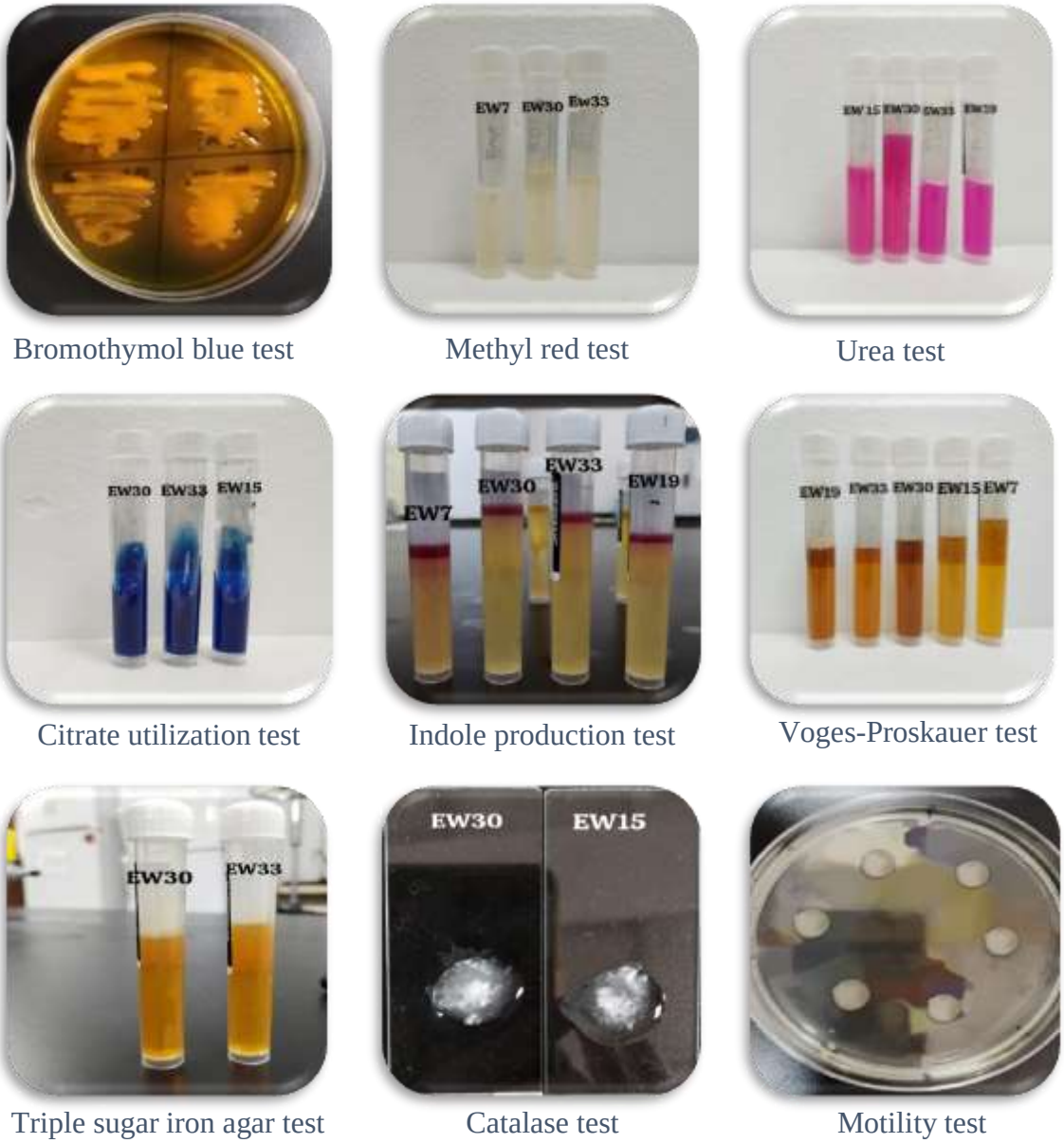


Figure 1. Biochemical tests for isolated strains of rhizobia.

3.4. Tolerance of rhizobial isolates to heavy metals:

Data demonstrated that the *Rhizobium* isolates tolerated each concentration the 100, 500, 1000, and 5000 µg/mL of heavy metal salts. The findings demonstrated a high level of tolerance to $NiCl_2$ (nickel chloride) at all doses examined, except the *Ensifer fredii* bv. *fredii* EW33, which expressed an inhibitory zone that reached 13 mm with a concentration of 5000 µg/ml. By pumping heavy metal ions out of the cell in a process regulated by house-keeping genes like *atpD*, *recA*, and *rpoB*, reducing heavy metal ions and converting them to less toxic forms, and forming complexes with heavy metal ions inside the cell, rhizobium bacteria protect themselves from the toxic effects of heavy metal salts [42].

The local isolate *R. leguminosarum* bv. *phaseoli* EW15 demonstrated good tolerance to all tested concentrations of cadmium chloride, according to the results of the cadmium chloride tolerance assay. On the other hand, *Ensifer meliloti* isolate EW7 and *Ensifer fredii* bv. *fredii* isolate EW33 showed elevated tolerance levels up to 1000 µg/ml. Table 3 shows that *R. legumiosarium bv. viciae* EW30 was sensitive to different concentrations of cadmium chloride and could tolerate 100 µg/ml.

The harmful effects of heavy metals cause the microorganisms in the soil to become resistant. Furthermore, the presence of plasmids in Gram-negative bacteria can cause this development. Additionally, plasmids aid in bacterial adaptation to pollution and their acquisition of novel genetic traits [43].

Table 3. Tolerance of local rhizobial isolates to cadmium chloride.

Isolate No.	$CdCl_2$ µg/ml			
	100	500	1000	5000
EW7	00	00	00	*15
EW15	00	00	00	00
EW19	8	10	13	16
EW30	00	10	12	15
EW33	00	00	00	21

* Inhibition zone (mm)

The isolates of *R. leguminosarium* bv. *trifolii* EW19 and *Ensifer fredii* bv. *fredii* EW33 were shown to be tolerant of cobalt chloride for all concentrations that were investigated (Table 4). Table 4 data shows that the isolate of *R. legumiosarium* bv. *viciae* EW30 had tolerances up to 1000 µg/ml (Fig.3).

Table 4. Tolerance of local rhizobial isolates to cobalt chloride.

Isolate No.	$CoCl_2$ µg/ml			
	100	500	1000	5000
EW7	00	00	*10	12
EW15	00	00	10	11
EW19	00	00	00	00
EW30	00	00	00	9
EW33	00	00	00	00

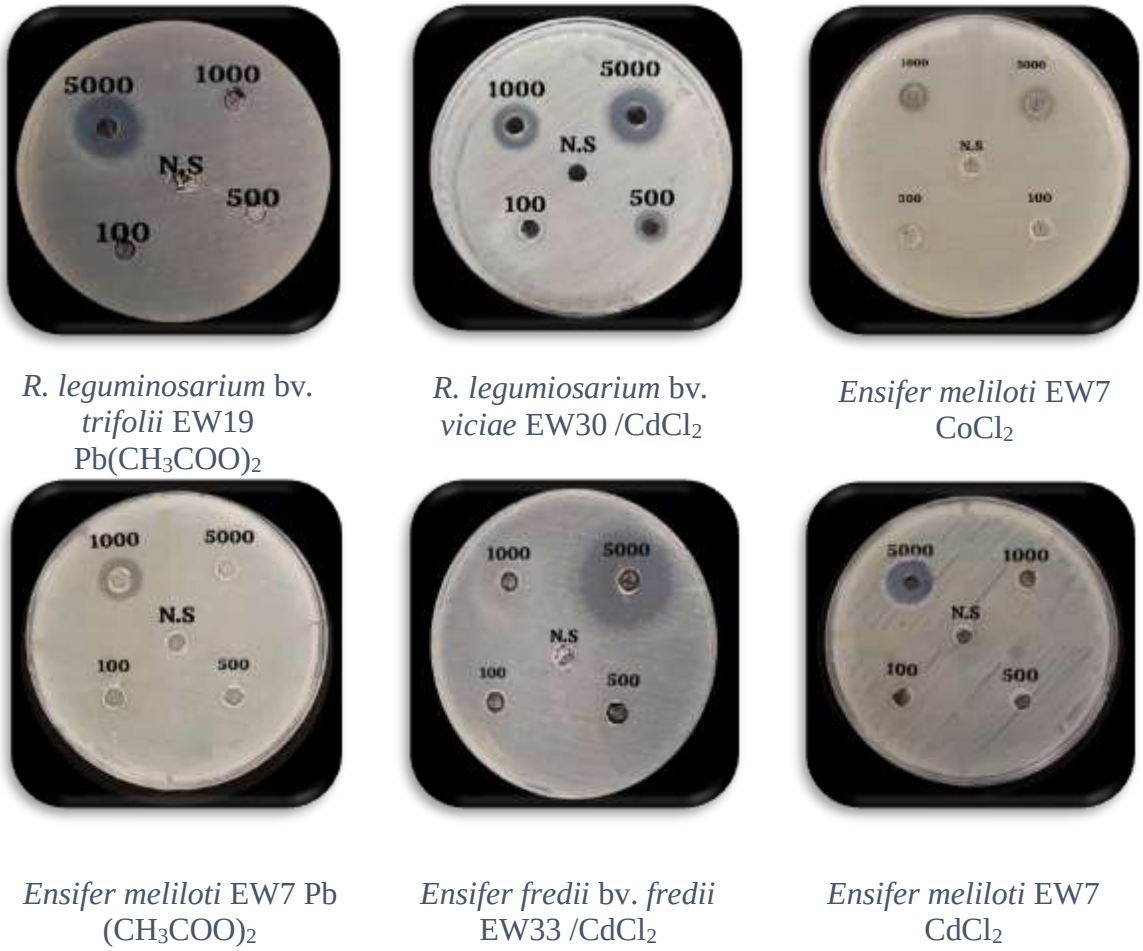
* Inhibition zone (mm)

All other rhizobial isolates (Table 5) demonstrated tolerance to lead acetate up to 1000 µg/ml (Fig. 3), the *R. legumiosarium* bv. *viciae* EW30 isolate demonstrated a high tolerance up to 5000 µg/ml. By securing the metals or altering their forms in the soil, *rhizobia* can boost a leguminous plant's resistance to heavy metals, such as alfalfa [44]. By raising soil fertility, rhizobia's nitrogen fixation also strengthens the plant's defense against metal stress [6].

Table 5. Lead acetate tolerance of local rhizobial isolates.

Isolate No.	Pb $(CH_3COO)_2$ µg/ml			
	100	500	1000	5000
EW7	00	00	00	*12
EW15	00	00	00	15
EW19	00	00	00	16
EW30	00	00	00	00
EW33	00	00	00	10

* Inhibition zone (mm)



R. leguminosarium bv. *trifolii* EW19 $Pb(CH_3COO)_2$

R. legumiosarium bv. *viciae* EW30 /$CdCl_2$

Ensifer meliloti EW7 $CoCl_2$

Ensifer meliloti EW7 Pb $(CH_3COO)_2$

Ensifer fredii bv. *fredii* EW33 /$CdCl_2$

Ensifer meliloti EW7 $CdCl_2$

Figure 2: Tolerance of local rhizobial isolates to $Pb(CH3COO)_2$, $CoCl_2$ and $CdCl_2$.

4. Conclusion

The main conclusion is that rhizobia isolates *Ensifer fredii bv. fredii* EW33 showed high resistance to the heavy nickel chloride and cobalt chloride up to 5000 µg/ml and cadmium chloride and lead acetate up to 5000 µg/ml, according to this study. There are components in this strain that are that are significant for scientific study and agriculture.

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6. Conflict to interest

There is no conflict of interest.

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تحمل عزلات الرايزوبيوم لبعض المعادن الثقيلة

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المستخلص:

في هذه الدراسة تم عزل خمسة عزلات من بكتريا الرايزوبيا المحلية من عقد جذرية لنباتات بقولية وهي: *Ensifer meliloti* EW7 من العقد الجذرية لنبات الجت والعزلة *Ensifer fredii* bv. *fredii* EW33 من العقد الجذرية لنبات اللوبيا والعزلة *R. leguminosarum* bv. *phaseoli* EW15 من العقد الجذرية لنبات الفاصوليا والعزلة *R. leguminosarium* bv. *trifolii* EW19 من العقد الجذرية لنبات الباقلاء, جمعت من مناطق زراعية مختلفة في نينوى/العراق. تم تنقية وزراعة مستعمرات بكتريا الرايزوبيا وايضا دراستها وتشخيصها كيوحيوياً. هذه العزلات كانت عصوية الشكل وسالبة لصبغة كرام. في حين كانت موجبة للاختبارات TSI , BTB , CUT , اختبار كوفاكس, اختبار اليوريز , ولأختباري أحمر المثيل وفوكس-بروسكاور. جميع عزلات الرايزوبيوم اظهرت نتائج أيجابية لاختبار الكاتاليز والحركة عند اختبارها في وسط TY شبه الصلب. تم دراسة تأثير اربعة املاح من المعادن الثقيلة على عزلات بكتريا الرايزوبيا شملت: $Pb(CH3COO)_2$ و $NiCl_2$ و $CdCl_2$ و $CoCl_2$. اظهرت العزلات المحلية نتائج متباينة في مقاومة املاح المعادن الثقيلة، حيث سجل المعدل الاعلى من قطر التثبيط 21 ملم للعزلة *Ensifer fredii bv. fredii* EW33 تجاه المعدن الثقيل $CdCl_2$. في حين ان العزلة *R. legumiosarium bv. viciae* EW30 أظهرت اقل تأثراً, حيث كان معدل قطر التثبيط 9 ملم فقط مع التركيز 5000 مايكروغرام/ مل للمعدن الثقيل $CoCl_2$.