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Effect of Some Environmental Factors and Antibiotics on the Growth of Different Species of Rhizobium

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Keywords: Antibiotic, Herbicide, Rhizobia, and Isolation.

Article History

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Abstract:

Six native rhizobial isolates from various cultural zones in Ninavah-Governate, Iraq, were used in this investigation. The following rhizobial strains were isolated from leguminous plant root nodules: Rhododendron japonicum SM29 from Glycin max L., Rhizobium leguminosarum bv. trifolii SM35 from Trifolium alexanrinum L., Rhizobium leguminosarum bv. viciae SM10 from Vicia faba L., Ensifer ferdii bv. Fredii SM13 from Vigna unguiclata L., Ensifer meliloti SM28 from Medicago sativa L., and Rhizobium leguminosarum bv. phaseoli SM42 from Phaseolus vulgaris L. Rhizobial bacteria were identified by the spherical, clear colonies seen after cultural investigation. Methyl red and Voges-Proskuar biochemical tests yielded negative results, but urease, catalase, indole, starch, Congo red, citrate, and motility tests yielded positive results. A high tolerance was found by the KNO_3 tolerance test.

Keywords: Antibiotic, Herbicide, Rhizobia, Isolation.

دراسة تأثير بعض العوامل البيئية والمضادات الحيوية على نمو اجناس مختلفة من *Rhizobium*

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الخلاصة:

تم في هذه الدراسة عزل ستة عزلات من الرايزوبيا المحلية من مناطق مختلفة من محافظة نينوى-العراق. تم عزل هذه العزلات من العقد الجذرية من النباتات البقولية وكما يأتي:

Rhizobium leguminosarum bv. viciae SM10 من نبات الباقلاء, *Ensifer ferdii bv. Fredii SM13* من نبات اللوبيا, *Ensifer meliloti SM28* من نبات الجت, *Rhododendron japonicum SM29* من نبات فول الصويا, *Rhizobium leguminosarum bv. trifolii SM35* من نبات البرسيم والعزلة *Rhizobium leguminosarum bv. phaseoli SM42* من نبات الفاصوليا. أظهر الفحص الزرعي مستعمرات دائرية شفافة دلالة على انها بكتريا الرايزوبيوم. أظهرت الفحوصات الكيموحيوية نتيجة سالبة لاختباري احمر المثيل وفوكس-بروسكر, في حين أظهرت اختبارات اليوريز والكتاليز والأندول وتحلل النشأ و احمر الكونغو والسترات والحركة نتيجة موجبة ولجميع العزلات. اظهر اختبار تحمل ملح نترات البوتاسيوم درجة تحمل عالية لعزلات الرايزوبيا المحلية، حيث وصل الى ١٠٪ (وزن/حجم) وللعزلتين *SM10* و*SM28*. كانت جميع العزلات مقاومة للأمبسلين ٢٥ مايكروغرام/مل باستثناء العزلة *SM42* حيث أظهرت حساسية لهذا المضاد الحيوي. أعطت جميع العزلات مقاومة عالية للمبيد العشبي الغليفوسات بتركيز ٥٠ ملغم/مل.

الكلمات المفتاحية: عزل، رايزوبيا، مضادات حيوية، مبيدات عشبية.

1. Introduction:

To manage and lessen weed growth on agricultural grounds, chemicals have been employed extensively in agriculture [1]. Roughly one-third of agricultural land is treated with herbicides these days [2-4]. Because so few herbicides reach the intended organisms, the destiny of these chemicals has come under scrutiny [5, 6]. Thus, herbicides have an impact on the ecosystem's ability to operate, either directly or indirectly [7]. Glyphosate is a pre-emergence herbicide that is non-selective and is often used to eradicate undesired plants of all kinds, with a focus on weeds [8]. Since the recommended rate of glyphosate applied to the soil is based on the recommended rate for a particular location, the herbicide application must be done appropriately and by the required concentration [9]. Farmers use a lot of the chemical glyphosate to get rid of weeds. The pesticide's hazardous ingredient has the potential to infect Rhizobium bacteria, which fix nitrogen in the soil [10]. Additionally, using it in excessive doses can harm microbes, plants, and animals, which eventually deteriorates the food chain and results in nutritional deficiencies.

Particularly vitamins and minerals may also be hazardous to the body as a whole. [10] Glyphosate also has an impact on the functional characterization of plants, where beneficial bacteria are essential for the synthesis of iron and auxin siderophores, as well as for solubilizing zinc and phosphate uptake. [10] Glyphosate has a major effect on soil biology and is poisonous

to beneficial species such as earthworms and microflora. Herbicides containing glyphosate penetrate the soil through the surface of the soil or seep into it, influencing the populations of rhizobia [11]. Although the effect varies throughout species, there are multiple findings suggesting glyphosate herbicides can lower Rhizobium species populations [12]. Numerous studies have demonstrated that the nitrogen fixation process is adversely affected by nonselective glyphosate spraying, as it reduces the amount of rhizobia.

Antibiotics are small bioactive molecules that are naturally produced by microorganisms like bacteria and fungi during their secondary metabolism [13,14]. Antibiotic resistance is an ancient and naturally occurring phenomenon widespread in the environment. The rhizosphere contains a mixture of metabolically active microbial populations that compete in this environment about size, diversity, and biochemical activity. Production of antibiotics by some soil harbouring microorganisms, mainly bacteria and fungi, has been largely documented [15].

2. Materials and Methods

2.1 Extracting Rhizobial Bacteria from Root Nodules: According to Vincent [16], local rhizobial bacteria were identified from nodules produced on the hair roots of plants growing in several places in Nineveh Governorate/Iraq. Isolates began to be collected at the beginning of September for two months, and the plants were three to four weeks old. Leguminous plants come in six varieties: phaseolus, cowpea, alfalfa, soybeans, and clover. Rhizobia bacteria have a specialized symbiotic relationship with leguminous plants, as bacteria are only associated with a specific type of legume plants. The plant roots developed nodules and were washed with distilled water. After being immersed in 70% ethyl alcohol for three minutes, they were thoroughly cleaned three times with sterile distilled water. Subsequently, they were submerged in a sodium hypochlorite NaOCL solution with a 3:1 v/v water/sodium hypochlorite concentration.

After being submerged in it for fifteen minutes, the sample was thoroughly cleaned three times using sterile distilled water. It was then moved to a Petri plate with sterile filter paper inside to remove any remaining water from the nodes. Nodules were transferred to Yeast Extract Mannitol Agar (YEMA) and incubated for a duration of 24 to 48 hours at 28°C to guarantee sterilizing effectiveness. Using a sterile glass rod, sterilized nodules were crushed and then arranged in stripes on a Petri plate filled with YEMA media. The dish was then incubated for 24 to 48 hours at 28°C.

2.2 Cultural diagnosis of isolates from the area: Under the oil lens (x100) of a compound light microscope (**Figure 1**), a growth sample of the colonies was inspected for colony appearance and tested for Gram stain using a Stain Gram kit [17].

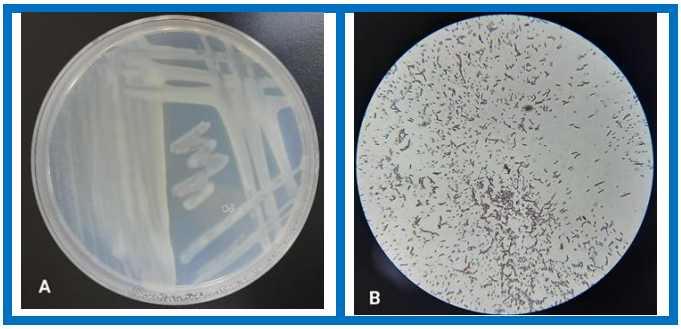


Figure 1: Isolated rhizobia colonies and cells
A: Colonies of rhizobia bacteria isolated from SM35
B: a smear of bacteria isolated from the root nodules of SM28, stained with gram stain under the oil lens

2.3 Tests using biochemistry to isolate Rhizobium bacteria: Bacterial strains obtained from the root nodules of the chosen plants were subjected to biochemical assays. The assays included Voges-Proskaur [18], Bromothymol blue [19], Congo red [16], motility [20, 21], urease, methyl red, starch degradation, catalase, Kovacs (indole) and citrate utilization [17].

2.4 Tolerance of the isolates to salt: By transferring a single culture of young rhizobial bacteria and spreading them out by plotting on YEMA medium that contains different concentrations of potassium nitrate KNO3 as follows: 2, 4, 6, 8, and 10%, and then incubating at 28°C for a while of 24–48 hours, this test was carried out to determine the ability of rhizobial isolates to grow in the presence of different concentrations of potassium nitrate KNO_3 [22].

2.5 Testing for antibiotic resistance in rhizobial isolates: To determine the growth of rhizobial isolates in the presence of the following antibiotics (µg/ml): Ampicillin, 25, Rifampin, 5, Tetracycline, 10, and Neomycin, 10. These antibiotics were produced by the Turkey Bioanalyase Company.. Using a cotton swab, tested rhizobial isolates were distributed over Mueller-Hinton agar (M.H.A.) medium before the antibiotic discs were moved in. Using sterile forceps, vital cells were extracted, spread out on a medium, and cultured for twenty-four hours at 28°C. The presence of a clear halo surrounding the antibiotic tablets was used to identify isolates of rhizobial bacteria that were sensitive and resistant to different antibiotics [23, 24].

2.6 Glyphosate's Impact on the Rhizobial Isolates' Growth: The purpose of the experiment was to show how tolerant rhizobial isolates were to glyphosate, a pesticide. YEMA medium was used to create the calculated amount of herbicide at concentrations of 0, 5, 25, and 100 mg/ml. Sterile Petri dishes were filled with the medium. Herbicide-free YEMA media was made, transferred onto sterile Petri plates, and regarded as a control. The herbicide-containing Petri dishes were used to cultivate the bacteria. For two days, plates were kept in the incubator at 28°C. The efficiency of the herbicide against rhizobial isolates was assessed by its effect on growth [25].

3. Results:

3.1 Analyzing culture and conducting biochemical testing: Following the separation of the bacteria from the plant's root nodules, convex, mucoid, semitransparent, and white colonies with smooth edges began to grow on the solid YEM medium. Under a light microscope, rhizobial cells were examined. Negative, bacilli-shaped Gram bacilli with no spores were found. **Table 1** provides a summary of the biochemical test results. The findings showed that the Vogus-Proskaour and methyl red tests yielded negative results for every isolated bacterium. On the other hand, positive results were obtained from the motility, urease, catalase, indole starch, Congo red, and bromothymol blue tests **Figure 2**.

Table 1: Biochemical tests for local rhizobial isolates.

	SM10	SM13	SM28	SM29	SM35	SM42
Methyl red	-	-	-	-	-	-
Urease	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Indole	+	+	+	+	+	+
Starch	+	+	+	+	+	+
Congo red	+	+	+	+	+	+
BTB	+	+	+	+	+	+
V-P	-	-	-	-	-	-
Citrate	+	+	+	+	+	+
Motility	+	+	+	+	+	+

BTB: Bromothymol blue; V-P: Voges-Proskaur

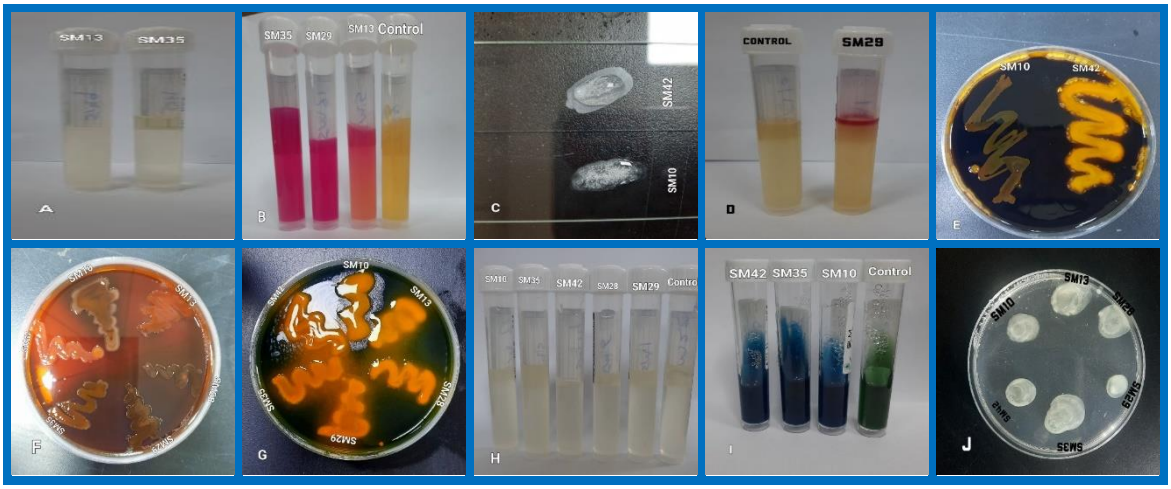


Figure 2: Biochemical test.

A- Methyl red test, B- Urease test, C- Catalase test,D- Indole test , E- Starch test, F- Congo red test , G- BTB test, H- V-P test,I- Citrate test, J- Motility test.

The KNO_3 tolerance results are displayed in **Table 3**. Different rhizobial isolates responded differently to this stress in the YEMA medium with varying KNO_3 concentrations. Except for isolates SM35 and SM42, which displayed a decline in growth at a concentration of 6 and 10% (w/v), rhizobial isolates had very robust growth at KNO_3 concentrations of 2, 4, 6, and 8%

(W/V). Two of the six rhizobial isolates, SM10 and SM28, demonstrated robust growth at a concentration of 10% (w/v).

Table 2: Effect of potassium nitrate on growth of Rhizobium

	KNO_3 Concentrations % (w/v)				
Isolate No.	2	4	6	8	10
SM10	+++	+++	+++	+++	++
SM13	+++	+++	+++	+++	+
SM28	+++	+++	+++	++	++
SM29	+++	+++	+++	+++	+
SM35	+++	+++	++	+	+
SM42	+++	+++	++	+	+

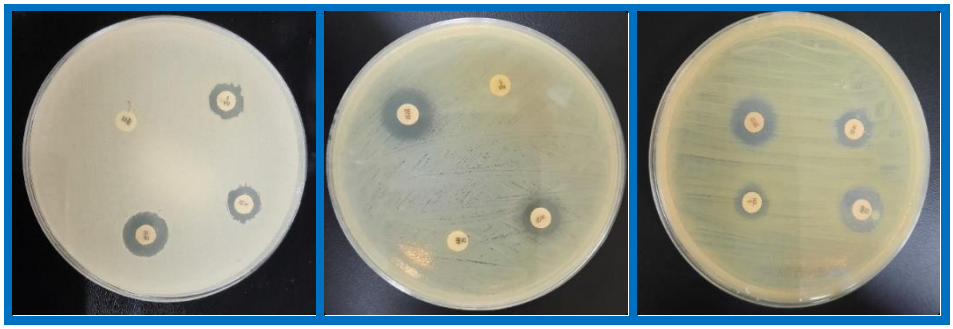
(+++) very good growth, (++) good growth, (+) medium growth, (-) No growth

About the antibiotic sensitivity test, isolate SM42 demonstrated inhibition zones 13, 9, 13, and 11 mm to ampicillin (25µg/ml), rifampin (5µg/ml), tetracycline (10µg/ml), and neomycin (10µg/ml), respectively. All other isolates demonstrated resistance to ampicillin (25µg/ml). Tetracycline (10µg/ml) and Neomycin (10µg/ml) did not affect the isolates SM28 and SM29. While the remaining isolates displayed varying levels of sensitivity to various antibiotics, strain SM35 demonstrated resistance to Rifampin (5 µg/ml), isolates SM29 and SM35 were more susceptible to antibiotics, while isolate SM42 was more sensitive to these antibiotics **Figure 3**.

Table 3: Local rhizobial isolates' susceptibilities and resistance to the drugs under study.

	Antibiotic concentrations µg/ml			
Isolate No.	Ampicillin 25	Rifampin 5	Tetracycline 10	Neomycin 10
SM10	R	13*	15	12
SM13	R	10	20	10
SM28	R	10	13	R
SM29	R	18	R	11
SM35	R	R	17	13
SM42	13	9	13	11

*: Millimeters (mm) represent the inhibition zone; R stands for resistant.



A B C

Figure 3: shows antibiotic testing on rhizobia isolates.
A-SM10, B-SM35 and C-SM42.

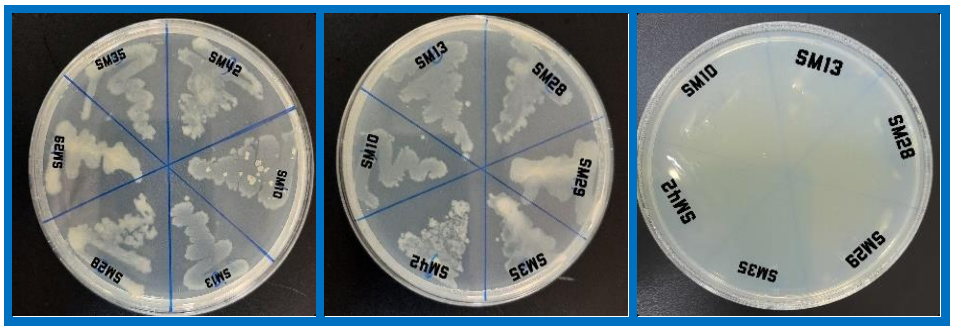
Table 5 displays the findings of the rhizobial isolates' tolerance to various glyphosate concentrations. Following a 72-hour incubation period, rhizobial isolates demonstrated their

capacity to develop at glyphosate doses of 0, 25, and 50 mg/ml (Figure 4). On YEMA plates treated with Glyophsate at a concentration of 100 mg/mL, none of the rhizobial isolates grew.

Table 4: Effect of herbicide Glyphosate on rhizobial isolates

	Concentration of Glyphosate mg/ml			
Isolate No.	0	25	50	100
SM10	+++	+++	+++	-
SM13	+++	+++	+++	-
SM28	+++	+++	+++	-
SM29	+++	+++	+++	-
SM35	+++	+++	+++	-
SM42	+++	+++	+++	-

(+++) very good growth, (++) good growth, (+) medium growth, (-) No growth



A:25 mg/ml B:50 mg/ml C:100 mg/ml

Figure 4 shows the growth of the following rhizobial isolates after two days of incubation on YMA medium supplemented with glyphosate herbicide (mg/ml): A-25, B-50, and C-100.

4. Discussion:

Table 5 lists the isolated local strains, the names of leguminous plants in Table 5 were based on the scientific names according to plant taxonomists. The isolate numbers are the name of the model that the researchers chose for this isolate.

Table (5): Rhizobial isolates, plant sources, and rhizobial species occupied its nodules.

Isolate No.	Plant source	Genus and species of rhizobia
SM10	*Vicia faba* L.	*Rhizobium leguminosarum* bv. *viciae*
SM13	*Vigna unguiclata* L.	*Ensifer ferdii* bv. *fredii*
SM28	*Medico sativa* L.	*Ensifer meliloti*
SM29	*Glycin max* L.	*R. japonicum*
SM35	*Trifolium alexanrinum* L.	*R. leguminosarum* bv. *trifolii*
SM42	*Phaseolus vulgaris* L.	*R. leguminosarum* bv. *phaseole*

Rhizobial isolates from the root nodules of leguminous plants used in this investigation had morphological traits common to Rhizobial genera. These isolates' traits included being bacilli and Gram stain-negative **[17, 26, 27]**. The tests for urease, catalase, indole, starch hydrolysis, motility, citrate consumption, Congo dye pick-up, and BTB were likewise positive for all isolates. In contrast, the isolates' findings from the Voges-Proskaur and methyl red tests were negative. According to this study, there were some variations in the biochemical examination

of the rhizobial isolates compared to previous investigations [28]. Additionally, the results of the capacity of local rhizobial isolates to grow at various KNO_3 concentrations varied. This outcome is consistent with the findings of Bed and Naglot [22], who were able to identify *Rhizobium legusarum* bv. *trifolii*.

Variations in antibiotic sensitivity or resistance were shown by the test results. Results of this study were consistent with other researchers' results regarding resistance to Ampicillin, and sensitivity to Rifampin, Tetracycline, and Neomycin [29, 30, 31, 32]. There are many mechanisms in the resistance of rhizobial isolates to antibiotics and these mechanisms were considered to be active. Rhizobial bacteria have specific protein carriers on the outer membrane. Another mechanism is a modification of biosynthetic pathways inside rhizobial bacteria as well as the production of enzymes acting against the target which is an antibiotic. Permeability of the cell wall of rhizobial bacteria is another defined choice for rhizobia to avoid antibiotic effects [33]. Studying of resistance and sensitivity of rhizobial isolates to antibiotics has double benefits in genetic and academic studies [34]. Secondly, such studies provide insight into how rhizobia as a non-pathogenic bacteria, becomes antibiotic-resistant in soil. The explanation for this statement is that rhizobia can receive R plasmids from different bacterial species through a mechanism known as horizontal gene transfer which occurs between pathogenic and non-pathogenic bacterial conjugation [35].

The herbicide experiment performed on various rhizobium isolates also revealed that while the isolates were sensitive to the herbicide and displayed growth inhibition at a concentration of 100 mg/ml, all isolates demonstrated their ability to tolerate a concentration of 25 and 50 mg/ml of the herbicide [16]. The isolates grew best at doses of 25 and 50 mg/ml, with 100 mg/ml showing the greatest growth effect [22]. Therefore, the impact of the herbicide, its concentration, and the local meteorological circumstances all affected the proliferation of rhizobial bacteria and their capacity to fix nitrogen [36].

5. Conclusion:

Different processes enable rhizobial bacteria to withstand high salt concentrations and pesticides. Poison-resistant plasmids and the production of efflux pumps are two examples of these methods. To completely comprehend the mechanisms underlying Rhizobium's tolerance capacities and investigate the possible uses of these bacteria under these circumstances, more research is required.

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