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Evaluation the mechanisms action of plant growth promoting rizobacteria under laboratory conditions

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Article Informations

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indole-3-acetic acid (IAA), phosphate solubilization, rhizobacteria, siderophore,

A B S T R A C T

This research was conducted to evaluate the efficiency of some mechanisms of rhizobacteria that promote plant growth and to recommend their use as biofertilizers. Therefore, atmospheric nitrogen-fixing bacteria were isolated from the root nodules of six legume species (*Vicia faba, Trifolium repens, Lens culinaris, Trigonella foenum-graecum*, *Lens culinaris subsp orientalis* and *Medicago sativa*). These isolates were positive for most biochemical tests (Urease production, Congo red capture, Bromothymol blue capture, Lactose fermentation and Growth on glucose peptone agar), as well as to test the results of the effect of direct (nitrogenase activity, phosphate solubilization and indol production) and indirect (siderophore production) mechanisms. This indicates their effectiveness in providing the plant with necessary elements. All isolates were able to produce indole acetic acid (IAA) at varying levels. The ability of the legume isolate (MA6) to grow on glucose-peptone agar may indicate that it is not a *Rhizobium*, unlike the other five isolates. After detection of the *16S rRNA* gene sequence of this isolate, it was found with 95% similarity to the global isolate *Serratia surfactantfacien* strain YD25 registered in GenBank. These results can contribute to recommend the necessity of using these isolates as well as emphasizing the use of the *S. surfactantfacien* isolate, to promote plant growth as a green and environmentally friendly biofertilizer to sustainably improve agricultural production.

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Introduction

Soil bacteria can grow rapidly, use a variety of materials as nutrient sources, often attach to soil particles, and interact with plant roots [1]. The rhizosphere is one of these well-characterized ecological niches, containing the highest percentage of bacteria dependent on root exudates for nutrition [2]. Root zone bacteria activate mineral nutrients transport and uptake by plant roots [3]. Overall, about 5% of rhizobacteria are plant growth promoting; they are one of the most important genera; *Acinetobacter, Agrobacterium, Arthobacter, Azotobacter, Azospirillum, Bradyrhizobium, Rhizobium, Serratia, Pseudomonas* and *Bacillus* [4].

A comprehensive understanding of plant growth promotion (PGP) mechanisms is essential for robust optimization of plant productivity. These mechanisms are divided into direct and indirect [5].

Direct mechanisms include ;1) Biological nitrogen fixation, the strains of *Rhizobium* bacteria that have the property of fixing nitrogen are classified symbiotically with legumes and have the specificity of infecting roots to produce nodules. Atmospheric nitrogen fixed by bacteria is not only used for its own benefit, but also absorbed by plants, making it more available for nitrogen uptake [6]. Nitrogen fixation is therefore an important feature of plant growth promotion rhizobacteria or PGPRs because it provides nitrogen directly to the plant. So property was exploited 20 years ago to commercialize nitrogen-fixing *Rhizobium* strains as Biofertilizers to promote sustainable agriculture [7]. 2) Several strains of this genus have gained importance because, they help plants grow by producing phytohormones, which have led to significant increases in crop yields [8]. The plant responds to any phytohormone produced by rhizobacteria that can mediate the processes of root cell hypertrophy, division, and elongation [9;10]. Long-term treatment of plants with IAA has been shown to result in the formation of highly developed roots, which in turn allow the plant to better absorb nutrients and ultimately improve overall plant growth [11]. 3) Phosphorus is the most limiting nutrient for plants, and may not be available in a soluble form suitable for plant uptake, so microorganisms secrete acids to dissolve complex phosphates into mono- and diphosphates [12]. .

Indirect mechanisms include siderophore production, as iron is an essential nutrient and its deficiency in the plant leads to severe metabolic changes [13]. Iron is often unavailable to plants in the soil, but it can be made available by some rhizobacteria can release siderophores that attract iron to the rhizosphere where it can be absorbed by the plant [14].

Aims of this study:

This research was conducted to enrich sustainable agriculture by evaluating the effectiveness of some bacterial root nodules and recommending their use as biofertilizers.

Material and Mehtodes

Collection of samples

Samples were collected from root nodules of six legumes (*Vicia faba, Trifolium repens, Lens culinaris, Trigonella foenum-graecum, Lens culinaris subsp. Orientalis,* and *Medigago sativa*) grown in different areas of fields in Mosul city.

Isolation of bacteria from root nodules:

Bacteria were isolated from root nodules after washing with running water to remove the soil. Surface sterilization of these nodules with a portion of the root was performed by immersing them in 70% ethanol for 30 seconds and then in 3% sodium hypochlorite solution for 1 minute, taking care to wash each time with sterile distilled water [15], placed on the surface of sterile filter paper to remove suspended water and crushed with a sterile glass rod in approximately 100 µL of liquid Yeast Extract Mannitol (YEM) medium, 10 µL of bacterial suspension was spread on YEM medium with a heat-sterilized ring and incubated at 28°C for 24-48 hours. They were stored at 4°C in screw-capped test tubes containing slant YEMA medium, with care taken to renew them every month.

Microscopic and culturing characteristics of isolated bacteria

Pure young isolates grown in YEMA were smeared on clean glass slides, stained with Gram stain and examined by compound light microscopy at 100X magnification [16]. All isolates were characterized in terms of colony size, shape, borders, height, color, mucosa, transparency, and ability to produce exopolysaccharides (Somasegaran and Hoben, 1994).

Biochemical tests

The urea-base agar [17] slant was inoculated with a loopful of pure culture of the isolated bacteria and the test tubes were incubated at 28°C for 18 to 24 hours. The hydrolysis of urea by the enzyme urease produces ammonia and carbon dioxide. A positive result is indicated by the change in color of the phenol red indicator from bright orange to pink as a result of the change in pH from 6.8 to pH 8.1 over 24 hours.

Isolate capture test for Congo red dye (25 mg/L) added to YEMA medium, production of these isolates from acids or bases was determined in YEMA medium supplemented with 25 mg/L bromothymol blue (BTB) dye as indicater to determined the formation of acid or alkali [18]. In

both cases, positive results are indicated by the bacteria absorbing the red dye and the medium changing color from green to yellow [19].

The isolated bacteria were grown on glucose-peptone agar and incubated at 28°C for 24-48 hours. This medium was used to distinguish *Rhizobium* from other soil colonising bacteria [20].

MacConkey agar media [21] were used to demonstrate the ability of isolated bacteria to ferment lactose.

Tests of direct and indirect mechanisms for PGPR

Direct mechanisms tests

The acetylene reductase activity (ARA) method was used to measure the nitrogenase activity of bacteria isolated from root nodules. The nodules were separated from the roots of various legume plants and placed in a small glass vial containing a quantity of acetylene gas, and after 30 minutes ethylene formation was measured using a Shimadzu GC-8A gas chromatograph (Shimadzu, Kyoto, Japan) [22].

For detecting phosphate solubilization, dishes containing Pikovskaya's agar medium were inoculated with the isolated bacteria and then incubated at 28±2°C for a period of 24-48 hours. Note that the appearance of a transparent halo around the colony indicates a positive test and vice versa for a negative test.

For detecting of indole-3-acetic acid (IAA), test tubes containing a medium of peptone water, phosphate, and glucose were inoculated with a colony of isolated and pure bacteria, then incubated at 28 degrees for 48 hours, then 3-5 drops of Kovac's reagent were added to the culture. The appearance of a red ring in the liquid medium indicates the positivity of the test, while its absence indicates the test is negative.

For quantification, isolates were grown in nutrient broth supplemented with 5 mM tryptophan and incubated at 28°C for 2 to 7 days [23]. The medium was centrifuged and the supernatant was mixed 1:5 with Salkovsky's reagent and left for 15 min at room temperature; pink colour indicates IAA production. The concentration was calculated by projecting the absorbance values at 530 nm onto the standard curve of IAA at different concentrations (0-100 µg/ml) and YEM broth not inoculated with bacteria was used as a negative control.oth not inoculated with bacteria was used as a negative control.

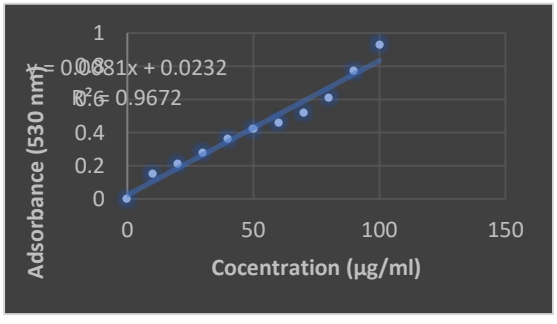

Figure 1: IAA Standard Curve

Indirect mechanisms tests

For the siderophore test, nutrient agar supplemented with 200 mM 2,2'-Dipyridyl was used as the iron-restricted agar medium for the culture of all isolated bacteria. Any bacterial growth was considered a positive result, indicating the ability of the bacteria to produce siderophores [24].

Molecular identification of alfalfa root nodule bacteria

The growth of bacteria isolated from the root nodules of the alfalfa (*Medicago sativa*) plant on the Pepton-glucose agar medium, which is used to distinguish *Rhizobium* bacteria from other soil-endemic genera, indicates that these bacteria do not belong to the genus *Rhizobium*, so they were molecularly characterized to identify them at the genus and species level.

DNA Extraction

DNA was extracted from alfalfa nodule bacteria by transferring the bacterial sample to an Eppendorf tube containing 200 µL Chelex®100 (Bio-Rad Company, USA) and 100 µL TE buffer. The tube was then placed in a water bath at 95°C for 10 minutes. The mixture was centrifuged at 13,000 rpm for 10 minutes. The upper aqueous layer containing the DNA was carefully removed, transferred to an Eppendorf tube and stored at -4°C in the refrigerator.

Diagnostic by PCR and DNA sequencing

The total DNA extracted from bacteria isolated from the alfalfa root nodules was used as a template in the polymerase chain reaction (PCR), the final volume of the reaction was 25 µl and contained the basic components of 12. 5 µL of Master mix and 0.5 µL of the forward primer (F: GTG TAG CGG TGA AAT GCG), 0.5 µL of the reverse primer (R: ACG GGC GGT GTG TAC AA) of the *16S rRNA* gene, 6. 5 µL distilled water and 5 µL of genomic DNA. The main stages of the PCR cycle are shown in Table 1 below. As well as using the volumetric guide to measure the resulting band size of the amplified *16S rRNA* gene. PCR reaction products were separated on a 2% agarose gel. containing ethidium bromide and the resulting band was visualized under UV light.

To diagnose the isolated bacteria, regions of similarity and dissimilarity between nitrogenous base sequences were identified using the BLAST program [25], which compares local nitrogenous base sequences with all conserved sequences in databases.

Table 1. Main stages of PCR cycles.

PCR Steps	cycle	Temperature	Time
Pre Denaturation	1	95	5 min
Denaturation		95	30 sec
Annealing	35	58	1 min
Extension		72	30 sec
Final extension	1	72	5 min
Hold		4	

Results and discussion

The results show that the six bacterial species isolated were bacilliform and Gram-negative. All colonies were malignant, convex, smooth and mucoid. Their color on the medium was creamy white. The results of biochemical tests showed that 100% of the isolates were positive for urease, BTB and fermentation to lactose, 80% were positive for Congo red dye and 16% were positive for growth on glucose- peptone agar. In terms of their plant growth-promoting mechanisms, they showed their effectiveness in fixing atmospheric nitrogen, as indicated by nitrogenase activity estimated indirectly by acetylene reductase assay, and in their production of the growth hormone IAA, while their phosphate solubilization rate was 66%, but they were 100% producers of sidophore (Table, 2 and Figure,2).

These findings are consistent with, and different from, those of researchers working in the field. Pervin *et al.*, [20] found that the isolates were Gram-negative, short bacilli and did not form spores. Most isolates produced clear, white to milky, convex and mucoid colonies. While Al-Mansor and Thaher, [26] indicated cultivation characteristics, the shapes of their colonies were all were: vicious, convex, smooth and mucous, 100%.

On the other hand, the bacteria isolated from the first five root nodules (MA1-MA5) were confirmed to be *Rhizobium* bacteria by not growing on GPA medium, which is in agreement with what Pervin *et al.*, [20] showed that this medium has the property of distinguishing between *Rhizobium* bacteria, which cannot grow on it, and other types of bacteria endemic to the soil, such as *Agrobacterium*, which can succeed on it and change the pH of the medium. The effect of Congo Red and BTB dyes on the growth of ten isolates of Rhizobium bacteria was 20% [26]. Al-Barhawee and Ahmed [27] indicated that the seven isolates from bacterial root nodules were positive for indole, catalase, motility and ONPG and could ferment the following sugars: Glucose, Mannitol, Galactose, Mannose and Xylose and were negative for Methyl red, Voges Proskauer, Citrate test, Urease and Gelatinase tests.

Phosphate solubilizing microorganisms are typical plant growth promoting rhizobacteria (PGPR) that can convert insoluble phosphate into soluble form HPO_4^{2-} and $H_2PO_4^-$, which are known as available phosphorus, through various mechanisms including secretion of phytase and phosphatase enzymes, organic and inorganic acids, siderophores and extracellular polysaccharides chelation to produce dissolved phosphate that can be easily and directly absorbed by plants, increasing crop yield and maintaining sustainable agriculture [28;29].This is because phosphor is a vital element that plays a critical role in the synthesis of various compounds such as nucleic acids and phospholipid in plants. [30]. Siderophores, which are low molecular weight substances produced by bacterial cells in soil or culture medium under conditions of iron deficiency [31]. On the other hand, siderophore chelates can offer environmentally safe, harmless, and biodegradable properties, making them a preferred alternative to synthetic chelates that are not biodegradable [32].

Table 2. Characterization of plant growth-promoting bacteria isolated from root nodules of six leguminous plants.

Morphological characteristics	Tests	Isolates from					
		MA 1	MA 2	MA 3	MA 4	MA 5	MA 6
	Shape	Rod	Rod	Rod	Rod	Rod	Rod
	Gram stain	-	-	-	-	-	-
Biochemical tests	Urease production	+	+	+	+	+	+
	Congo red capture	+	+	+	+	-	-
	Bromothymol blue capture	+	+	+	+	+	+
	Lactose fermentation	+	+	+	+	+	+
	Growth on *Glucose Peptone Agar*	-	-	-	-	-	+
Direct mechanics	Nitrogenase activity	+	+	+	+	+	+
	Phosphate solubilization	+	+	+	+	-	-
	Indol production	+	+	+	+	+	+
Indirect mechanics	Siderophore production	+	+	+	+	+	+

MA 1= *Vicia faba*, MA 2= *Trifolium repens* , MA 3= *Lens culinaris*, MA 4=*Trigonella foenum-graecum* , MA 5= *Lens culinaris subsp orientalis*, MA 6=*Medigago sativa*, + = Positive, - = negative.

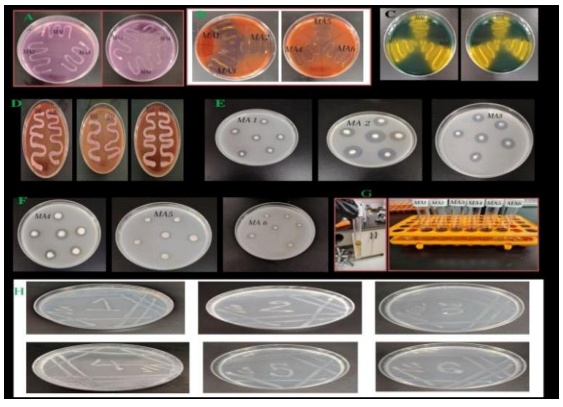

Figure 2. Results of biochemical tests, direct and in direct mechanism for PGPR isolated from six nodules of leguminous plants.

In general, Figure 3 shows that all bacterial isolates were IAA producers, production increased in the presence of the amino acid tryptophan, and the production gradient of bacterial isolates was as follows: MA6, MA4, MA5, MA2, MA3, and MA1 after 24 hours and at 28°C of incubation.

A study showed that three isolates of *Bradyrhizobium japonicum* from soybean root nodules were able to grow well and produce IAA in YEM broth in the presence of 0.5 mM tryptophan using the Salkovsky method. The amount of IAA produced varied depending on the bacterial isolate, with isolate BJ 11 (19) producing the highest amount of IAA after 4 days of incubation compared to the other isolates studied. [33]. In another recent study (Lata *et al.,* 2024), IAA-producing bacteria were isolated and characterized from rhizosphere of chickpea (*Cicer arietinum* L.) using the Salkowski colorimetric assay, the optimal conditions for IAA production were found to be 500 µg/ml of tryptophan, 35°C temperature, and pH 7.0 after incubation for 48 hours. Twenty-four PGPR were isolated and seventeen of these isolates were found to produce more than 10 µg/ml IAA [34].

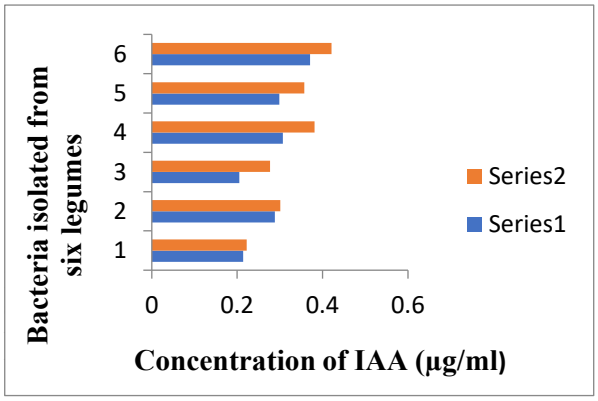

Figure 3. The concentration of IAA produced by bacteria isolated from the six legumes in media unsupplemented (series1, blue) and supplemented (series2, red) with tryptophan.

The growth of bacteria isolated from the root nodules of alfalfa legumes (MA6) on glucose-peptone agar (GPA) medium, on which bacteria isolated from the other five legume species did not grow, suggested that these bacteria did not belong to the genus *Rhizobium* [20].This was confirmed by molecular characterization using the *16S rRNA* gene primer as well as sequencing of its nitrogen bases and comparison with those of bacteria registered in the gene bank. Accordingly, the genome size of this bacterium was found to be 500 bp (Figure, 4), and it belongs to the genus *Serratia surfaceactantfacien*, and it is 95% identical to the global isolate *S. surfaceactantfacien* strain YD25 because it has deleted and inserted nitrogenous bases at different sites as showed in Figure (5), We conclude that deleting and inserting DNA is likely to be involved in recurrent mutations leading to complex evolution [35]. Sequencing of the *16S rRNA* gene identified two species of the genus *S., marcescens* and *nematodiphila,* on the surface of healthy mangoes [36]. Their nitrogen fixation in these nodules could be explained by one of these possibilities; Plant-associated *Serratia* consists of both endophytic and free-living species in the rhizosphere [37]. the genus *Serratia* contains genes involved in the major nitrogen cycle [38], that these bacteria symbiotically contribute to nitrogen fixation and thus enhance the growth of plants inoculated with them [39]. In the natural environment, bacterial genomes can undergo various developmental processes leading to Significant genetic diversity within homogeneous groups, genome rearrangement by recombination, and the introduction of foreign genes by horizontal gene transfer are important factors contributing to evolution, and resulting in variation in basic genetic traits and their encoding into specific phenotypes [40].

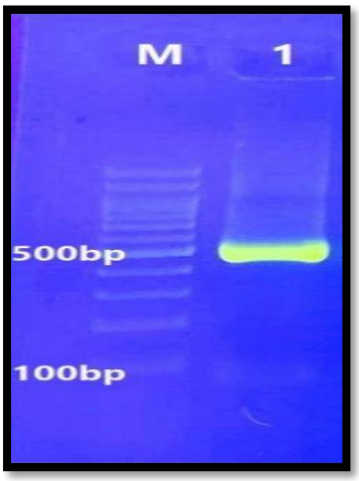

Figure 4. PCR results for the *16S rRNA* gene in bacteria isolated from alfalfa (MA6) root nodules.

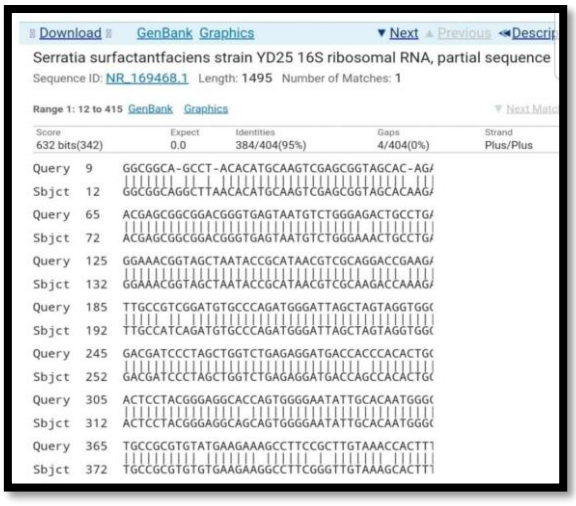

Figure 5. Sequence of the *16S rRNA* gene of the bacterium MA6 identified as *Serratia surfaceactantfacien*.

Conclusion

We conclude from the above that PGPR present in legume nodules has a significant role in influencing soil nutrient levels and providing them in the form needed by plants, therefore we recommend their use as biofertilizers, in particular, the new bacterium *S.*

surfaceactantfacien, first isolated from alfalfa plant root nodules, and the results of their direct and indirect mechanism in promoting plant growth as it is considered a green and environmentally friendly technology that would reduce the use of chemical fertilizers, thus reducing production costs and protecting the environment. Future research should determine how well these mechanisms promote the growth of different plants in the field. This study is the first to characterize the presence of *S. surfaceactantfacien* in the root nodules of alfalfa plants, contrary to the fact that this legume is specially infected by *Sinorhizobium meliloti*. Their fixation of atmospheric nitrogen and other mechanisms made them PGPRs, and more new strains are expected to be discovered as their plant growth-promoting functions are evaluated.

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Competing Interests

Conflict of Interest The authors declare no conflict of interest.

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