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Molecular Identification of *Rhizobium* Isolates and The Effect of Nanoparticles on Growth and Differentiation

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ABSTRACT

In this study, five isolates of *Rhizobium* bacteria were isolated from several different agricultural regions of Nineveh Governorate. The five isolates showed their ability to dilute gelatin, as well as a positive result of the growth test on a triglyceride medium and iron showed their ability to produce catalase and urease enzyme, and the results of the molecular diagnosis showed The isolates are identical and in varying proportions to the standard isolates recorded in the GenBank, and the biological activity of silver nanoparticles showed a clear antagonistic effect on the growth of *rhizobium* bacteria, where the first concentration (250) was the least effect, while the third concentration (750) had the highest effect ratio.

Keywords- *Rhizobia*, isolation and identification, silver nanoparticles, TiO2NPs, NiONPs.

I. INTRODUCTION

Rhizobium bacteria belong to the Rhizobiaceae family, which includes several genera, including *Rhizobium*, *Ensifer*, and *Shinella*. *Rhizobium* bacteria were classified, depending on the nutrient medium, into slow-growing bacteria, as this section produces alkali and needs 3-5 days for growth, and the average multiplication of these bacteria ranges between 6-7 hours, while the second section is called fast-growing *rhizobium* bacteria, and this section produces acids that lead to an increase in turbidity in the medium in which it grows The average doubling of this group ranges between 2-4 hours (Chhetri *et al*., 2019). *Rhizobium* bacteria are found in the rhizosphere, that is, around the roots of leguminous plants. Through their presence in this region, they infect plants in the root hairs, and infection often occurs through the existing cracks. On the surface of the roots of leguminous plants, and as a result of the entry of *rhizobium* bacteria into the apex of the root hair, distortions will occur in the root hair and the formation of what is known as an infection thread (Oldroyd *et al*., 2011) the *Rhizobium* bacteria invade neighboring cells, then the pericycle and cortex cells begin to divide leading to the formation of root nodes (Roy *et al*., 2020).

Root nodules are created by the secretion of the roots of leguminous plants, a mixture of chemical compounds, including Flavonoides, Isoflavonoides, and other stimuli, which are chemical signals that attract *Rhizobia*. Root nodules are divided into limited nodules and unlimited nodules depending on the plant host and the type of nodule formed in it (Andrews and Andrews, 2017).

Rhizobium bacteria stimulate plant growth through the biological fixation of nitrogen, dissolution of phosphates and production of plant hormones (Andrews and Andrews, 2017). Capacity (Gu and Milton, 2020), that the principle of the biological nitrogen fixation process is the reduction of atmospheric nitrogen N2 to ammonia NH3 in the presence of the enzyme Nitrogenase (Yang *et al*., 2018).

The genome of *rhizobium* bacteria is large and multi-part consisting of a circular chromosome and a set of plasmids (Mazur *et al*., 2011). The genes responsible for symbiotic interactions with legumes such as knot

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formation and nitrogen fixation are (nif_fix) genes, which are carried by large symbiotic plasmids or incorporated into the chromosome as a symbiotic island (Pérez Carrascal *et al*., 2016) and the symbiotic plasmid (pSym) range in size from 150 kbp to 400 kbp (Unger *et al*., 1985).

There are several techniques for diagnosing *rhizobium* bacteria, the most important of which is the polymerase chain reaction (PCR) technique (Graham, 2008). The basis for the work of this technology depends on the manufacture of many copies of DNA pieces using a thermal polymerization device, which works to raise the temperature between 55_98, which leads to the separation of the DNA strands into two single strands (Rahman, 2013), where the PCR reactions made an important revolution in diagnosing Microorganisms by distinguishing between close members of the species up to the strain level based on distinct genetic characteristics, which have high reliability and more sensitivity compared to other traditional methods (Baginsky, 2014).

Nanotechnology includes the study of applications using materials whose dimensions do not exceed 100 nanometers and are called nanoparticles (Madkour, 2019). The importance of nanoparticles is due to the large ratio of surface area to volume, and this characteristic increases the attachment of other objects to it (Pérez Carrascal, 2016) and silver nanoparticles AgNPs are considered one of the most important elements in nanoscience, due to their medical, electrical and chemical properties, as they are used in burns and wounds (Alaqad and Saleh, 2016) and a study proved that silver nanoparticles AgNPs have harmful effects on the formation of bacterial nodules and nitrogen fixation inside a plant soybeans (Alla *et al*., 2016).

II. MATERIALS AND METHODS

2.1 Media, reagents and dyes:

2.1.1 Ready culture media:

Muller Hinton medium, Tripple Sugar Iron Agar medium, and Gelatin Agar medium were prepared according to the manufacturer's instructions.

2.1.2 Prohibited culture media:

2.1.2.1 Yeast Extract Manitil Medium (YEM): It was prepared according to the method (Lakzian *et al*., 2002).

2.1.2.2 Urea Test: It was prepared according to the method (Tille, 2017).

2.1.2.3 Catalace Reagent: It was prepared according to the method (Tariq *et al*., 2017).

2.1.2.4 Gram stain: It consists of 4 solutions (MacFaddin, 2000).

2.3 Isolation of rhizobium bacteria from the root nodules of leguminous:

Leguminous plants, including cowpeas, beans, alfalfa, and broad beans, were collected from several agricultural regions in different parts of Nineveh governorate, including Al-Rashidiyah, Hawi Al-Yarmjah, Al-Fadeliyah, and Al-Shamsiyat. They were isolated in the following way. To remove traces of alcohol, then immersed in sodium hypochlorite NaOCI solution for 15 minutes and then washed with distilled water several times to remove traces of NaOCI. After ensuring the efficiency of sterilization, the root nodules were ground with 1 ml of physiological solution using a glass lube and 0.1 ml of this bacterial suspension was spread on YEM solid medium. The dishes were incubated in the incubator at 28°C for 48_72 hours (Vincent, 1970).

2.4 Biochemical tests for rhizobial bacteria:

Tests were conducted according to (Cappuccino and Sherman, 2014).

2.4.1 Gram stain test:

This test is carried out by preparing a clean and sterile glass slide, by adding (1-2) drops of distilled water to the slide, then transferring a young and pure colony of bacteria to the glass slide, mixing it with water drops, and passing it over the flame three times in order to fix the smear, after which several Drops of crystal violet dye for one minute, then the slide was washed with distilled water, then an ethanol alcohol solution was added at a concentration of (95%) for half a minute, then washed with distilled water, then Safranin dye was added for one minute, and then washed with distilled water. They were dried on filter paper and examined under a light microscope under an oil lens (1000x). A positive result is indicated by the appearance of *rhizobium* bacteria cells in red.

2.4.2 Growth of triple sugar iron agar test:

This test is carried out by inoculating a triglyceride and iron medium with a young colony of *rhizobium* bacteria and incubated at a temperature of (28 ± 2) ° C for (48) hours. The positive result is indicated by the change in the color of the medium to yellow.

2.4.3 Gelatin liquification test:

This test is carried out by inoculating test tubes containing gelatin agar with young colonies of *Rhizobium* bacteria using an inoculation needle, and the tubes are incubated at a temperature of (28 ± 2) ° C for (48) hours, and the positive result is indicated by the liquefaction of gelatin due to the production of gelatinase After placing it in the refrigerator for half an hour at a temperature of (4) ° C.

2.4.4 Catalase test:

This test is carried out by placing a young colony of *rhizobium* bacteria on a clean glass slide and adding 1_2 drops of hydrogen peroxide H2O2 solution at a concentration of (3%) to it. Toxic hydrogen peroxide H2O2 to H2O and O2 and thus leads to the liberation of oxygen gas.

2.4.5 Urease test:

This test is conducted by inoculating the medium of urea acres placed in test tubes in the form of habitats with young colonies of *Rhizobium* bacteria, and the habitats were incubated at a temperature of (28 ± 2) °C per hour. The positive result is indicated by a change

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in the color of the medium from yellow to pink as an indication of the ability of bacteria to produce urease, convert urea into ammonia, and raise the pH of the medium in terms of changing the color of the indicator, phenol red.

2.5 Molecular diagnosis of Rhizobium bacteria using PCR technique:

The DNA was extracted from the bacteria based on the analysis kit prepared by Geneaid company, and the next stage was the preparation of the agarose gel and the electrophoresis process of the DNA, and then the PCR technique was performed by mixing the DNA sample and the special primer for each gene with the components of the master-mix Where the initiator that was used in the technique is Forward and Revers, then the molecular diagnosis of *Rhizobium* bacteria was carried out and the nucleotide sequences of the 16rRNA region were determined based on the DNA Sequencing technique (Rahman *et al*., 2013)

2.6 The effect of nanoparticles on rhizobial bacteria by agar disk fuge:

Three concentrations were prepared, namely 250,500,750 mg/l, by suspending the nanoparticles used in this research (AgNPs, TiO_2NPs, NiONPs) with sterile distilled water and placing them in an ultrasonic device for 10 minutes (Fan *et al*., 2014), and the tablets were prepared Using an office paper punch, then No. 1 Whatman filter papers were perforated, and the tablets were placed in a tightly closed glass vial in order to sterilize them with an autoclave and kept until use. Using a cotton swab, the liquid bacterial culture is spread on Mueller-Hinton agar medium and the plates are left to dry for 10 minutes, then the tablets are placed according to the concentrations and a control sample saturated with sterile distilled water is placed and the plates are placed in the incubator at a temperature of 28 °C (López-Oviedo *et al*., 2006). Three replicates were made for each treatment.

2.7 The effect of nanoparticles on rhizobial bacteria by turbidity:

Also, three concentrations were prepared, as mentioned in the previous paragraph, and YEM liquid medium was prepared and placed in test tubes with an amount of 9.8 ml, 0.1 ml of liquid bacterial culture, and 0.1 ml of nano-solution, and mixed well with the Vortex device, and the tubes were placed in the shaking incubator Shaker for 48 hours at a temperature of 28 °C, after which optical density was measured with a spectrophotometer at a wavelength of 600nm, compared to a control sample (Paul, 2021).

III. RESULTS AND DISCUSSION

3.1 Isolation of rhizobium bacteria from the root nodules of leguminous plants:

After following the method for isolating and purifying the *rhizobium* bacteria, an isolate was obtained from the cowpea plant Vigna unguiculata L. It was given the letter HA5 from the Hawi Al-Yarmjah area, and two isolates from the bean plant *Phaseolus vulgarise* L were given the letters HA10, HA 13 from the Rashidiya and Al-Fadiliya regions, and an isolate from The plant Medicago sativa L. was given the letter HA15 from the area of sunflowers, and an isolate from the bean plant was given the letter HA23 from the area of Hawi Al-Yarmjah.

3.2 Biochemical tests for rhizobium bacteria:

The results of the chemical tests of the five isolates, when examined under a microscope, showed that they were sticky in shape, pink in color, and mobile. They showed a positive result for the catalase test and urea analysis to produce the urease enzyme. These five isolates also showed that they were positive for the growth test on a medium of trisaccharide and iron, and positive for the gelatin liquefaction test. These results are consistent the findings with Findings of the researcher (Bhattacharjee and Banerjee, 2018).

3.3 Molecular diagnosis of rhizobium bacteria using the PCR technique:

The genomic interaction of the pure DNA extracted from the five isolates was carried out using the primer of the 16S rRNA gene of the *Rhizobium* bacterium. The results of the analysis using the Blast program for sequential analysis of the PCR products showed a similarity between the isolate HA5 with the standard isolate registered in the International GenBank with the number NR. 118339.1, with a rate of up to 98%. In light of the observation of the results of the analysis of the isolate (HA10) using the DNA Blast site, it showed that there is a similarity of up to 80% between the sequences of the nitrogenous bases of isolate HA10 with the sequences of the nitrogenous bases of the standard isolate registered in the Genome Bank with the number NR_115801.1 and through Note the results of the analysis for isolate (HA13) using the DNA Blast website It appeared that there was a great similarity of up to 100% between the sequences of the nitrogenous bases of isolate HA13 with the sequences of the nitrogenous bases of the standard isolate registered in the GenBank with the number NR_113895.1. The nitrogenous base sequences of isolate HA15 with the nitrogenous base sequences of the standard isolate registered in GenBank with the number KJ128057.1. By observing the results of the analysis for isolate (HA23) using the DNA Blast website, there was a great similarity of up to 82% between the nitrogenous base sequences of isolate HA23 with the base sequences aureus of the standard isolate registered in the GenBank with the number NR-117539.

3.4 Effect of nanoparticles on rhizobium bacteria by disc diffusion method:

These results showed that there was an inhibitory effect towards each of the five isolates, except for isolate A15, which was not affected by any concentration and was resistant to the influence of nanoparticles. Sedimentation is a result of the electromagnetic attraction forces resulting from the

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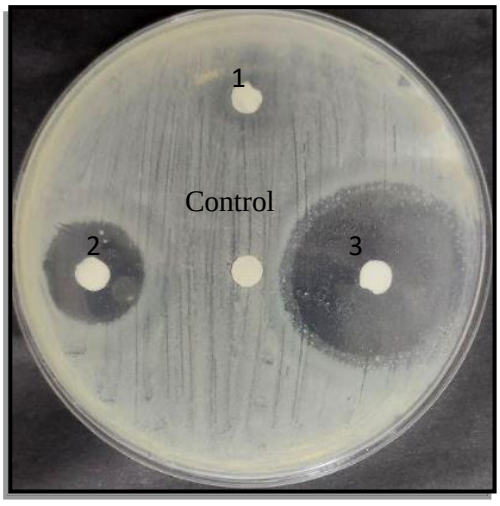
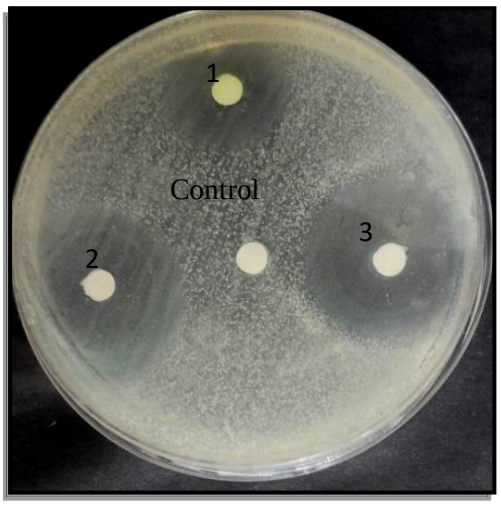
charges present on its surface, and this led to the difficulty of maintaining the spread of nanoparticles and their non-contact with bacteria due to the lack of required diffusion, or perhaps another reason is the low concentration of nanoparticles in the prepared solutions or the shape of the nanoparticles (Khaled *et a*l .,2020) As for the rest of the isolates, the effect varied according to the concentrations, and in general, the first concentration 250 was the least effective and the third concentration 750 was the most effective, as shown in the results in Table (1), where it is considered that AgNPs are toxic to bacterial cultures and have a superior ability to penetrate the plasma membrane of the bacterial cell Where the silver nanoparticles work to stick to the surface of the bacteria and change the properties of the membrane, where the peptidoglycan layer of the *rhizobium*-negative bacteria is a thin layer, and the silver nanoparticles work to replace the multiple lipid sugar molecules and accumulate inside the membrane by forming pits Which causes a significant increase in the permeability of the membrane, and the penetration of silver nanoparticles into the bacterial cell causes DNA destruction, and the dissolution of silver nanoparticles with the liberation of antibacterial silver ions. These results converged with the results of the researcher (Mohddam *et al*., 2017).

Table 1: Biological activity of silver nanoparticles against *Rhizobium* bacterial isolates, according to the average inhibition zone (mm).

Isolation name	250 mg/L	500 mg/L	750 mg/L
Ensifer fredii bv. *fredii* HA5	10.6667 1.15470	15.6667 1.15470	31.3333 2.30940
R. leguminosarum bv. *phaseoli* HA10	8.0000 1.00000	14.3333 1.15470	24.3333 1.15470
R. leguminosarum bv. *phaseoli* HA13	9.0000 1.00000	12.3333 0.57735	29.0000 1.00000
Ensifer meliloti HA15	0.0000 0.00000	0.0000 0.00000	0.0000 0.00000
Rhizobium leguminosarum bv. *Viciae* HA23	0.00000	19.6667 0.57735	1.15470 27.0000
control group	0.00	0.00	0.00



HA5



HA10

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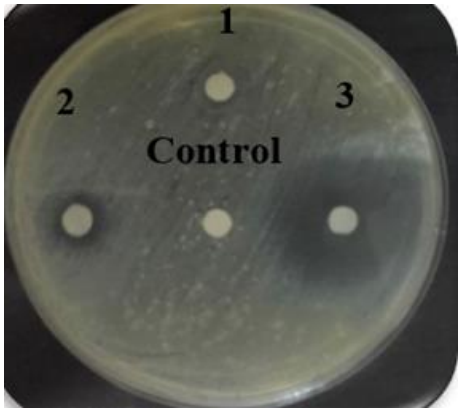
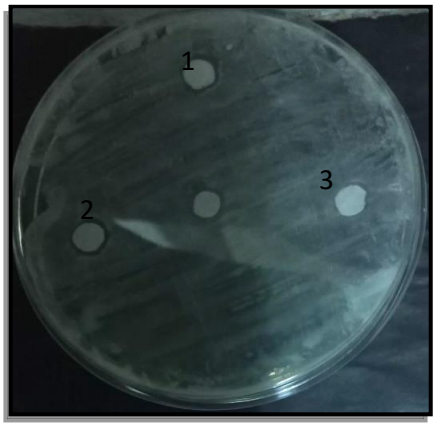
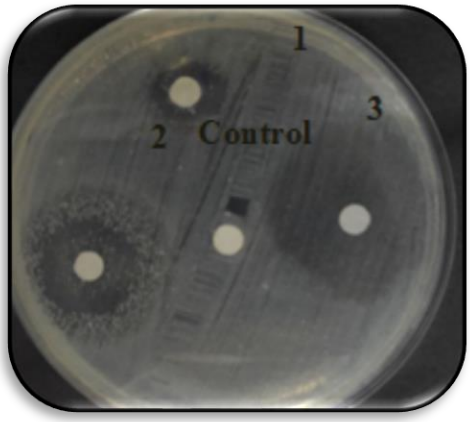


HA13



HA15



HA23

Picture 1: show the biological activity of silver nanoparticles AgNPs at three concentrations against rhizobial isolates compared to the control medium containing a physiological solution as a negative control.

As for the effect of titanium dioxide nanoparticles TiO_2NPs as well, the results showed different inhibition diameters according to the concentrations, as shown in the analysis table (2). The third concentration showed the highest inhibition diameters relative to the second and first concentrations, and all isolates showed an inhibitory effect of TiO_2NPs except for isolate HA13 *R. leguminosarum* bv. *phaseoli* for the aforementioned reasons.

It is noted from Table (2) that the antibacterial titanium dioxide nanoparticles increase their effectiveness in relation to the large surface area and the size of the small nanoparticles, which leads to a high penetrating power of the nanomaterials towards the *Rhizobium* bacteria. It is known that the size of the nanoparticles is the main factor in the toxic effect, as it causes an attack to bacteria due to free radicals, and these results converged with the results of the researcher (Fenoglio *et al*., 2009) in the effectiveness of nanoscale titanium dioxide against bacteria, and these results also agreed with the results of the researcher (Vedam *et al*., 2004) In his results, he indicated a decrease or complete killing of bacteria whenever the concentration of TiO_2NPs increased, while these results differed with the results of the researcher (Feizi *et al.*, 2012), as he indicated in their study that the high concentration led to enhanced growth and that the anti-TiO_2NPs activity may be attributed to Its crystal structure, size, shape, and surface area (Azam *et al*., 2012; Khezerlou *et al*., 2018) and TiO2NPs are widely used due to its strong antimicrobial effect (Sirelkhatim *et al*., 2015).

Table 2: Biological activity of TiO_2NPs against *Rhizobium* bacteria isolates, according to the average inhibition zone (mm).

Isolation name	250 mg/L	500 mg/L	750 mg/L
Ensifer fredii bv. *fredii* HA5	14.33 1.155	21.00 1.000	23.00 1.000
R. leguminosarum bv. *Phaseoli* HA10	13.67 1.155	18.67 1.155	26.33 1.155

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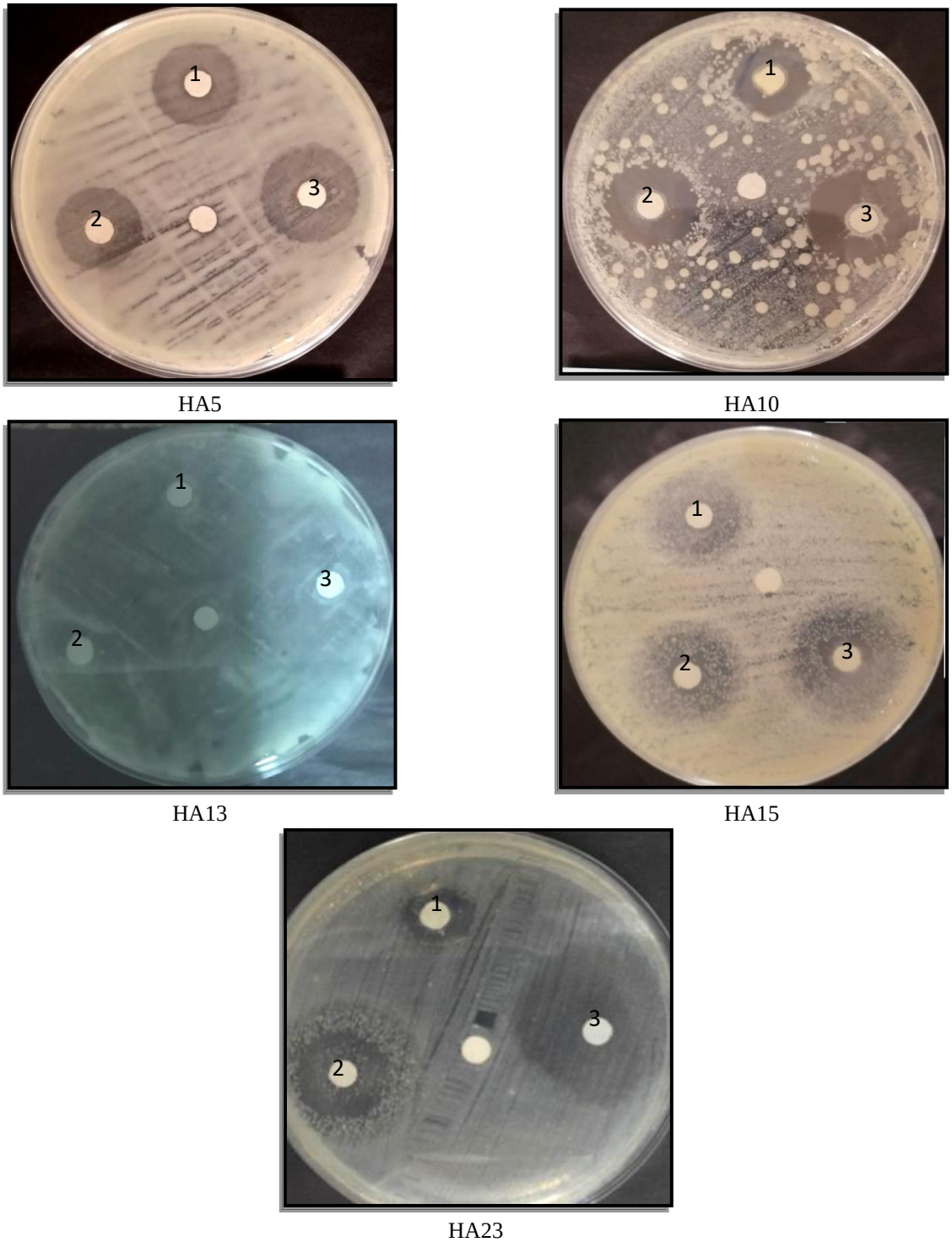
R. leguminosarum bv. *phaseoli* HA13	0.00 0.000	0.00 0.000	0.00 0.000
Ensifer meliloti HA15	17.00 1.732	22.67 1.155	27.00 1.732
Rhizobium leguminosarum bv. *Viciae* HA23	20.67 1.155	25.67 1.155	33.33 2.082
control group	0.00	0.00	0.00



Picture 2: show the biological activity of silver nanoparticles TiO_2NPs at three concentrations against rhizobial isolates compared to the control medium containing a physiological solution as a negative control.

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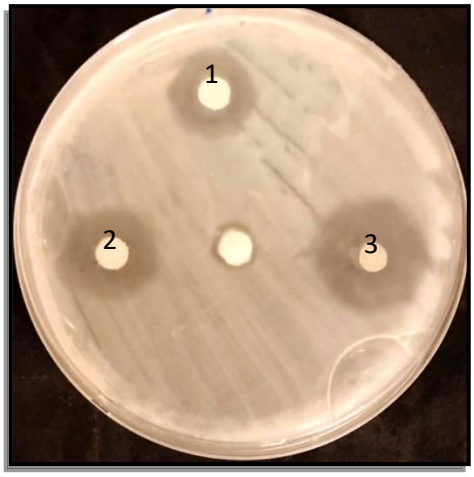
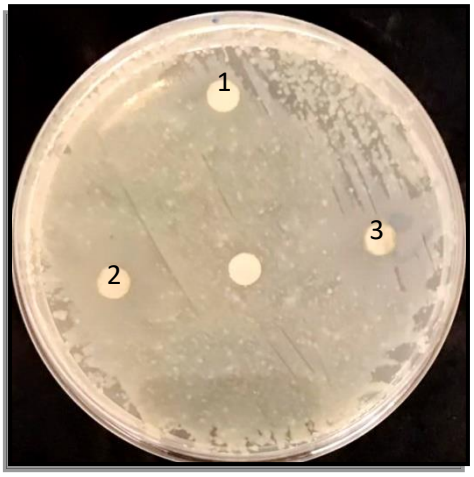
As for the results of the biological activity of NiONPs, the isolates were affected by the anti-NiONPs activity, except for *R. leguminosarum* bv. *phaseoli* HA10. The results shown in Table (3) also showed an increase in the rate of inhibition of bacterial growth as the concentration of NiONPs increased, meaning that the third concentration gave the highest inhibition rate compared to the first and second concentrations, and this effect can be attributed to the nickel ions emitted from the NiO nanoparticles, as the ions The liberated cells, in turn, may increase the permeability of the membrane and promote oxidation, which in turn activates the cell death pathway (Kim *et al*., 2007; Jin *et al*., 2009), and the results of this study converged with the results of the researcher's study (Luzala, 2022).

Table 3: Biological activity of NiONPs against *rhizobium* isolates, according to the average inhibition zone (mm).

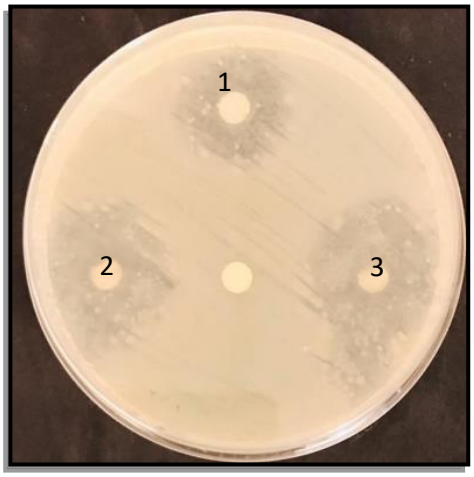
Isolation name	250 mg/L	500 mg/L	750 mg/L
Ensifer fredii bv. *fredii* HA5	12.0000 1.73205	15.6667 1.15470	19.6667 0.57735
R. leguminosarum bv. *phaseoli* HA10	0.00 0.000	0.00 0.000	0.00 0.000
R. leguminosarum bv. *phaseoli* HA13	9.0000 1.73205	15.3333 0.57735	21.3333 1.52753
Ensifer meliloti HA15	12.0000 1.00000	15.3333 0.57735	18.3333 1.15470
Rhizobium leguminosarum bv. *Viciae* HA23	14.3333 1.15470	25.3333 0.57735	29.0000 1.00000
control group	0.00	0.00	0.00



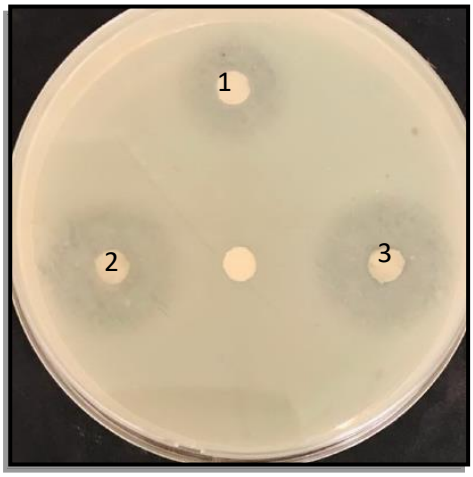
HA5



HA10



HA13



HA15

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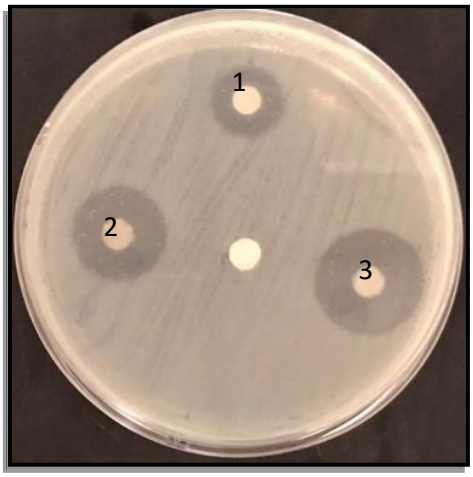

HA23

Picture 3: show the biological activity of silver nanoparticles NiONPs at three concentrations against rhizobial isolates compared to the control medium containing a physiological solution as a negative control.

Through the results that appeared and the tables, it was found that the highest rates of inhibition were due to the effect of silver nanoparticles, as AgNPs are considered the most toxic nanomaterials and have antibacterial properties and have a wide spectrum of antagonistic activity and a superior ability to penetrate the plasma membranes of bacterial cells (Feng *et al*., 2013). , and that the high effectiveness of AgNPs may be due to two sources, one of them: the silver ions emitted from the AgNPs, which seem to have the main effect, and the second source is the chemical reactions on the surface of silver nanoparticles, including free radicals and reactive oxygen species (ROS) that are released from surface of AgNPs (Kim *et al.*, 2007).

3.5 Effect of nanoparticles on Rhizobium bacteria by turbidity method:

Through the results that appeared to us, it was found that bacterial growth decreased at the third and high concentration of silver nanoparticles, as the bacterial cultures did not grow significantly when exposed to the nano silver solution, while the bacterial cultures (control samples) grew exponentially during the same time period and by comparing the effect of nano silver on The rhizobial samples in the liquid medium show the effect of nanotechnology on bacterial cultures directly, where it was found that the effect of nanotechnology on bacterial culture is stronger due to the ease of the effect of toxic silver ions on bacteria, penetration of the plasma membrane and inhibition of growth. These results were close to the results of (Paul, 2021) as shown in the table. (4).

Table 4: A table showing the results of the turbidity method of AgNPs against *Rhizobium* bacteria

Isolation name	control	250	500	750
Ensifer fredii bv. *fredii* HA5	1.73 0.002	6.70 0.002	0.59 0.002	0.53 0.002
R. leguminosarum bv. *phaseoli* HA10	0.001 1.11	0.002 0.85	0.002 0.67	0.001 0.45
R. leguminosarum bv. *phaseoli* HA13	1.71 0.002	0.71 0.002	0.64 0.002	0.63 0.001
Ensifer meliloti HA15	1.67 0.002	0.85 0.002	0.71 0.002	0.65 0.001
Rhizobium leguminosarum bv. *Viciae* HA23	1.00 0.003	0.67 0.003	0.64 0.002	0.54 0.002

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The results of Table (5) also showed that there were significant differences between the studied treatments, and the upper limit of inhibition was at a concentration of 750 mg/L, and the results of turbidity for the rhizobia isolates showed that the highest limit of turbidity was at a concentration of 250 mg/L. The results agreed with the results of study (Burke *et al*., 2015), as the bacteria had a good growth rate in low concentrations.

Table 5: A table showing the results of the turbidity method of TiO_2NPS against *rhizobium* bacteria

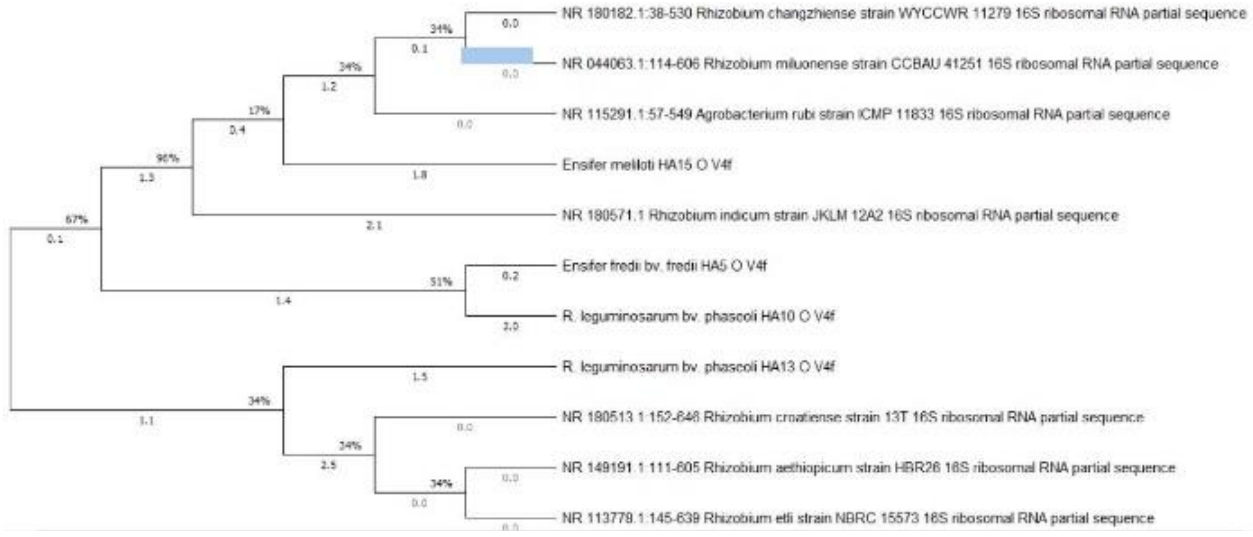
Isolation name	control	250	500	750
Ensifer fredii bv. *fredii* HA5	1.73 0.002	0.7533 0.00153	0.6147 0.00153	0.5163 0.00379
R. leguminosarum bv. *phaseoli* HA10	0.001 1.11	0.8613 0.00115	0.5867 0.00153	0.4213 0.00153
R. leguminosarum bv. *phaseoli* HA13	1.71 0.002	0.7437 0.00153	0.6413 0.00153	0.5813 0.00153
Ensifer meliloti HA15	1.67 0.002	0.7147 0.00153	0.6257 0.00208	0.4360 0.00100
Rhizobium leguminosarum bv. *Viciae* HA23	1.00 0.003	0.7813 0.00153	0.6963 0.00153	0.5347 0.00252

As for the results of the turbidity table (6), the effect of NiONPs on the rhizobial isolates showed that the highest peaks of turbidity were at the first concentration of 250 mg/L, and the lowest peaks of turbidity, i.e. the highest inhibitory concentration, was at the third concentration of 750 mg/L. The results converged with the results of the study. where the high concentration was the minimum for turbidity (Luzala et al., 2022).

Table 6: A table showing the results of the turbidity method of NiONPS against *rhizobium* bacteria

Isolation name	control	250	500	750
Ensifer fredii bv. *fredii* HA5	1.73 0.002	0.7940 0.00173	0.6430 0.00173	0.5513 0.00153
R. leguminosarum bv. *phaseoli* HA10	0.001 1.11	0.7810 0.00173	0.6463 0.00153	0.4973 0.00208
R. leguminosarum bv. *phaseoli* HA13	1.71 0.002	0.8663 0.00231	0.686 0.00100	0.6117 0.00153
Ensifer meliloti HA15	1.67 0.002	0.8120 0. 00200	0.6917 0.00058	0.6317 0. 00153
Rhizobium leguminosarum bv. *Viciae* HA23	1.00 0.003	0.8343 0.00153	0.6660 0. 00173	0.5630 0. 01058

3.6 Diagram showing the phylogenetic tree:



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The evolutionary history was concluded using the Neighbor-Joining method (Saitou and Nei,1987). The optimal phylogenetic tree is displayed. The evolutionary distances were calculated using the Maximum Composite Likelihood procedure (Tamura and Kumar,2004) and are in the units of the number of base substitutions per site. The proportion of sites where at least 1 specific base is present in at least 1 sequence for each descendent clade is exhibited next to each inner node in the phylogenetic tree. This analysis calculation involved eleven nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All vague or ambiguous positions were terminated for each sequence pair (pairwise omission choice). There were a total of 1469 positions in the ultimate dataset.

IV. CONCLUSIONS

1. Possibility of obtaining other bacterial isolates.
2. Experimenting with the effect of other nanomaterials.
3. Studying the effect of nanoparticles on the gene expression of bacteria.

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