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RESEARCH PAPER

Bacterial Population of Alfalfa (*Medicago sativa* L.) Plant Nodules in Koya city

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A B S T R A C T:

Global food productivity is severely hampered by soil nutrients deficiencies. The use of synthetic nitrogenous fertilizers is widespread cultivation practice to improve agricultural output. Other non-symbiotic endophytic bacteria have also been identified within the same root nodules at the same time as rhizobacteria. It is a common occurrence for non-symbiotic soil microbes to reside in leguminous nodulation. In the current study, Rhizobia and non-symbiotic commensal bacteria that promote plant development were isolated from the native leguminous Medicago sativa. According to our analysis, these non-symbiotic microbes are frequently found inside root nodules and work in concert with rhizobacteria to enhance nodulation and nitrogen fixation in legume crops. The current research work aimed to isolate identification & characterizing root nodulating species from wild alfalfa (*Medicago sativa*. L) plant nodules collected from different regions of Koya city in the Erbil district. Isolation of these species was done by culturing on YEMA (Yeast Extract Mannitol Agar) medium and incubation period of 48 hours at 30 °C. The sample identification was processed using standard microbiological and biochemical techniques as well as 16S rDNA partial sequence and *nifH* gene. Results showed that thirty nodule samples yielded a total of two Rhizobium species isolates, one of them was recorded in NCBI as a new strain. The others were found to be Pseudomonas and Enterobacter, Rahnella and Erwinia respectively. The findings suggested that lateral gene transfer (LGT) between non-symbiotic endophytic bacteria and rhizobacteria may have taken place.

KEY WORDS: alfa alfa, Rhizobium, Root nodulating bacteria, 16sRNA, nifH.
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1. INTRODUCTION :

Specified gram-negative Alphaproteobacteria and Betaproteobacteria are together referred to as “rhizobacteria” and they may develop nodules on the host roots to fix nitrogen and coexist in symbiosis with their host legumes (Lindström and Mousavi, 2020). Rhizobacteria are terrestrial bacteria that may live with legumes in a symbiotic way to fix nitrogen. This connection entails rhizobial infection of the root as well as bacterial invasion of root nodules that the plants forms in response to the presence of compatible partner. The two symbionts' complicated chemical communication is the basis of this connection (Acosta-Jurado *et al*., 2021).

Rhizobacterium is a collective term for soil dwelling bacteria that can fix nitrogen and live (a) in harmony with their host plant. These bacteria are members of Betaproteobacteria which include *Azorhizobium*,*Rhizobium*,*Ensifer* (*Sinorhizobium*), *Mesorhizobium*, *Bradyrhizobium*, *Neorhizobium*, *Phyllobacterium*, *Microvirga*, *Devosia* and members of Betaproteobacteria, and *Burkholderia* and *Cupriavidus* (Tejerizo *et al*., 2016). Nitrification in Rhizobium-legume symbiotic associations is critical for boosting soil quality and is essential for sustainable agricultural activities (Schulte *et al*., 2022). There is a dearth of information on the variety and distribution Rhizobacteria strain around the world as well as which strain thrive in particular soil habitats and environmental setting and provide the most advantages to the host plants (Wekesa *et al*.,

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2022). In legume root nodules, non-rhizobial microbes also were discovered, however, it is unclear what their ecological functions are. The efficiency and activity of their legume plants may be impacted by the relationship between these microbes and rhizobial bacteria within nodules. Even through variety of non-rhizobial microbial population do not causes nodulation and nitrogen fixation, they have been discovered that possess several plant growth promoting properties (Etesami, 2022).

The generation of plant growth stimulating hormones, the control of infections, nodulation, enhanced accessibility of micronutrients and macronutrients, an improvements of soil quality are just a few of the way that plant growth promoting bacteria stimulate. In addition to rhizobacteria, a wide range of GP (Gram-positive) and GN (Gram-negative) may co-exist in the legume nodules. Although some of these microbes are incapable of stimulating nodule formation, other can operate solely by affecting levels of plant hormones, enhancing nutrient availability, or reducing the impacts of pathogenic organism to improve host health (Martínez Alcántara *et al*., 2020).

Rhizobium species were believed to be the sole nitrogen former present in the legume nodules for long time. Yet new study from legume nodules have shown a variety of beta and alpha proteobactria, notable species including *Pantoea*, *Burkholderia*, *Serratia*, *Pseudomonas*, *Bacillus* and *Enterobacter* (Mir *et al.*, 2021; Martínez-Hidalgo and Hirsch, 2017; Lu *et al.*, 2017). In addition to their capacity for Nitrification, the majority of these nodulating rhizobacteria have been shown to possess PGP properties and productivity improvement (Gopalakrishnan *et al.*, 2015, Dobbelaere *et al.*, 2003).

The *dinitrogenase reductase* is coded by *nifH* gene, one of the *nitrogenase* complex's components, which is in charge of converting atmospheric nitrogen into ammonia (Laranjo *et al*., 2014). *Nitrogenase* enzyme catalyzes the concurrent reduce a single N_2 and two Hydrogen to yield ammonia beside hydrogen gas molecule. Enzyme makeup of dual proteins, an iron protein and a molybdenum-iron protein. The whole procedure consumes 16 mol from ATP and a source of protons and electrons happens in optimum way among legume plants plus bacteria (Yang *et al*., 2018, Kawaka, 2022).

The main objective of this study to investigate genetic biodiversity of root nodulating (Rhizobial and non-Rhizobial) bacteria associated with *Medicago sativa* from divergent area in Koya city, in addition to studying their morph-physiological characteristic & biochemical properties and resistance to different antibiotics. More than that detecting the existence of *nifH* gene in the isolates and nucleotide sequencing to find out the isolates strain via genetic approach.

2. MATERIALS AND METHODS

2.1. Field site and sample collection:

Sample from nodules of wild Alfalfa plant were collected between November and February 2022, the first rainy season from 30 locations in the Koya region and around.

2.1.1. Isolation of Root Nodulating Bacteria

Medicago sativa plant root nodules were used to recover root nodulating bacterium strains. Roots were gathered from several Koya city farming experimentation areas. To get rid of the dirt that had attached, the harvested nodules were rinsed in tap water. A selection of large, healthy nodules was made to isolate the bacteria. The root nodules were submerged in 95% alcohol for 5-10 seconds, then 2.5% sodium hypochlorite (NaCLO) for five minutes, and then thoroughly washed six times with sterilized distilled water to get rid from chemicals. A sterile glass rod was used to aseptically smash each surface sterilized nodule in a test tube containing 1 ml of sterile distilled water (Mir *et al*., 2021).

2.1.2. Growth conditions and isolation of bacteria

Petri dishes with YEMA (mannitol, 10g/l; yeast extract, 1g/l; K2HPO4, 0.5g/l; MgSO4 7H2O, 0.2g/l; NaCl, 0.1g/l; Agar) was streaked with nodules suspension using loop then incubated for 48hours at 30°C (Azib *et al.*, 2022). After incubation time a single colony corresponding to each nodule was chosen and further purified on YEMA then re-streaked on YEMA containing 0.0025% Congo red so as to

differentiate the contamination along with the rhizobial colony in the medium. For further characterization, the pure cultures were kept on YEMA and kept in refrigerator at 4 °C (Mir *et al*., 2021). All isolates were stored at -80°C, in a 25% (v/v) glycerol stock of yeast extract mannitol medium.

2.1.3. Colony Morphology

Morphological and biochemical examination remain one of the main criteria used for the initial identification and characterization of bacteria. To verify the bacterial isolates' colony features, phenotypic properties such as shape, size, margin, and growth pattern of the 31 bacterial isolates were carried out in this investigation (Banjare *et al*., 2022).

2.1.4. Gram stain and Biochemical tests

Gram staining was carried out to determine if the cultures were Gram negative or positive. Staining was done the method described by (Elbehiry *et al*., 2022). The bacterial isolates were subjected to conventional Gram stain, and they were viewed at X1000 under light microscopy. Catalase, Oxidase, Urease, TSI, IMVC and motility tests were conducted on all bacterial isolates.

2.1.5. Antibiotics sensitivity test

On Muller-Hinton agar, a single colony of each pure culture was streaked out using a swab of sterile cotton. After placing antibiotics aseptically on the plates, the diameter was measured after 24 hours of incubation at 30°C (Nusrat *et al*., 2017). Antibiotic discs were used with the following concentrations: Doxycycline (10µg), Ceftazidime (30 µg), Imipenem (10 µg), Amikacin (10 µg), amoxicillin/clavulanic acid (30 µg), Levofloxacin (5 µg). The results were compared to that of the CLSI (Rasmi *et al*., 2022).

2.2. Molecular method.

2.2.1. DNA extraction

The total DNA of bacterial colonies was isolated by Genomic DNA Extraction Kit (FAVORGEN, Taiwan. FABGK100) following the guidelines provided by the manufacturer. The quality of DNA was verified by electrophoresis in 1% agarose gel. Bands were visualized by a UV transilluminator Gel Documentation (InGenius LHR, Syngene, UK), and the quantity of DNA was determined using a nanodrop spectrophotometer (NanoVue plus, UK.). The extracted DNA was stored at -20 °C until further analysis.

2.2.2. Plasmid extraction

The bacteria were grown in yeast extract mannitol broth on a rotary shaker 150rpm for 16 to 24h at 30°C until the late log phase. The plasmid was extracted by plasmid DNA extraction kit (FAVORGEN, Taiwan. FAPDE001) following the guidelines provided by the manufacturer. The quality of DNA was verified by electrophoresis in 1% agarose gel. Bands were visualized by a UV transilluminator Gel Documentation, and the quantity of DNA was determined using a nanodrop spectrophotometer. The extracted plasmid DNA was stored at −20°C until further analysis

Table 1. Primer sequence, product size for *16s rRNA, V4* and *nifH* genes in Root nodulating bacteria

Targeted gene	Primer sequence	Size
16S rRNA	P1F: TGAAGAGTTTGATCATGGCTCAG	**1442bp**
	P1R :TTCCCCTACGGTTACCTTGT	
(*V4*	P2F: GTAATACGGAGGGTGCAAGC	**300bp**
	P2R: TCTAATCCTGTTTGCTCCCCA	
nifH	nifHF: TCCAAGCTCATCCACTTCGT nifHR : AGCATGTCCTCCAGTTCCTC	**194bp**

2.2.3. PCR amplification of 16S rDNA

The 16S ribosomal DNA (rDNA) was sequenced in order to support findings of the phenotypical and biochemical tests. The PCR was done using a pair of primers for the 16S rDNA, which produces a 1442 bp product amplicon the amplification was performed in a Thermal Cycler (Biorad, USA) with 30µl reaction containing 100ng of genomic DNA 15µl AddStart Taq DNA *polymerase* (2X) from (ADDBIO- Sweden) and of 1µl (0.05 pmol) of each primer and 12µl of *nuclease* free water. The reaction conditions of the PCR consist of the initial denaturation step at 95°C for 5min followed by 20 cycles with denaturation at 95°C for 45s, primer annealing at 59°C for 40s, and extension at 72°C for 1 min, and the final extension at 72°C for 5 min, and hold at 4°C (Kawaka *et al*., 2018). the PCR products were electrophoresed on 1% of agarose (GeneDirex, USA) in Tris- Boric acid-EDTA at 80V for 1h, the expected size of the

product 1442bp, were visualised using UV transilluminator Gel Documentation (InGenius LHR, Syngene, UK).

2.2.4. PCR Amplification of nifH Genes

The PCR amplification of the *nifH* gene was performed in a Thermal Cycler with 20µl reaction containing 100ng of plasmid DNA 10µl AddStart Taq DNA *polymerase* (2X) and 1µl (0.05 pmol) of each primer and 4µl of *nuclease* free water. the temperature profile was as follows: initial denaturation at 95°C for 5min; 40 cycles of denaturation at 94°C for 50s, annealing at 55°C for a few strains at 57°C for 1min, and extension at 72°C for 50s; and final extension at 72°C for 5 min. the PCR products were electrophoresed on 2% of agarose in Tris- Boric acid- EDTA at 80V for 1h.

2.2.5. Gel Electrophoresis Analysis

A 3 µl of PCR reaction products were separated by electrophoresis on a 1% agarose gel including ethidium bromide (0.5 µg/ml) alongside a 100 bp DNA marker (GeneDirex,Marker 100bp) and ran in 1X TBE buffer at 80 V for one hour to verify that the targeted genes were amplified to the right size. Following running, the DNA amplicons were viewed and captured employing (UV Gel Imager SynGene) (Koskey *et al*., 2018). Except for *nifH* genes were electrophoresed on 2% agarose.

2.2.6. Sequencing of the *16S rRNA* Gene Amplicons

After size confirmation, the produced PCR products were forwarded for sequencing. Sequencing was performed for all 31 samples that were amplified. The sequencing was carried out by (Macrogen Genome Center, Republic of Korea. Sanger sequencing/ ABI3500). using P2R reverse primers for partial 16S rDNA gene sequencing.

2.2.7. Sequence Quality and Length

DNA Baser Assembler was used for sequence analysis and editing. When there were more than 80% excellent bases in window of 20 bases, the beginning and end of the sequences were trimmed to achieve quality trimming. When less than 90% of the bases with fewer than 25 quality values were found in the sample after trimming, it was judged to be of low quality. When more than 90% of the bases have quality value of above 25, the sequence samples are judged to be excellent. The sample was rejected if the length of the final DNA fragment after trimming was less than 600bp.

2.2.8. Novel Species Identification

Using the EzTaxon database, the *16S rRNA* genes query sequence was aligned with previously discovered bacterial nucleotide sequences to identify each isolated bacterial sample (Yoon *et al.,* 2017). The following parameters were used to identify new Rhizobia strains: >99% similarity was judged to be the same species, possibility for uncultured species (similarity threshold between 98.7% and 99.0%), genus (95.3-90.0%) or family (<90.0%). (Stackebrandt and Ebers, 2006; Schlaberg et al., 2012). Using the website http://www.ncbi.nlm.nih.gov/BankIt data were submitted to the GenBank database for strain that lacked any comparable sequences.

3. RESULTS

3.1. Sample Collection, Bacterial Isolation

In total, 31 bacterial species were identified from 30 root nodules of wild alfalfa plants. The Samples were collected from different areas of Koyesnjaq and around. The wild alfalfa plants were used as the host plants. The isolated bacteria were carried out from 30 different locations during November and February (2021-2022). In general, the isolated bacteria colonies show colour on YEMA, as white, creamy, milky, dense, transparent, velvety, sticky, solid, or dry. Most of the isolates had a mucoid texture due to the production of excess EPSs. All isolates had circular colony shapes and an entire colony margin but the colony elevation varied consistently with convex and raised colonies being observed on YEMA media after 48h of incubation (Figure.1). Most of the isolates did not absorb congo red dye when incubated in the dark on YEMA-CR medium, only isolates (6sh,7sh) absorbed the red dye and (8sh) partially absorbed congo red dye.

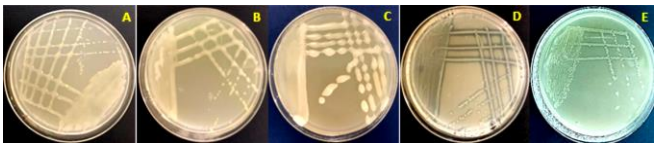

Figure 1. Morphology of the isolated bacteria from root nodules of the Alfalfa plant. *The isolates were grown on*

YEMA medium ***(A)*** Rhizobium sp. (**B)** Pseudomonas sp. **(C)** Enterobacter sp**. (D)** Rahnella sp. **(E)** Erwinia sp**.**

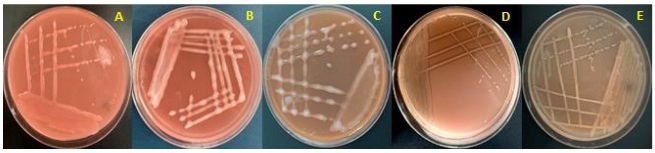

Figure 2. Morphology of the isolated bacteria was grown on (CR-YEMA) with congo red medium **(A)** *Rhizobium sp. (B) Pseudomonas sp. (C) Enterobacter sp. (D) Rahnella sp. (E) Erwinia sp.*

3.1.2. Microscopic characterization and Biochemical test

All isolates were found Gram-negative rods and short-rods shaped when examined by light microscope at X1000 magnification. According to the biochemical tests that were carried out to identify the isolated strains, all the isolates were found to be positive for catalase and motility and all isolates were positive for methyl red except (15wsh). and only (8sh and 25sh) were weakly positive for urease test and negative for indole and Voges Proskauer test and isolates (1sh, 2sh, 6sh, 7sh, 8sh,10sh, 11sh, 14sh, 22sh, 24sh, 25sh, 27sh, 29sh) were negative for citrate utilization and (1sh, 2sh, 4sh, 9sh, 10sh, 11sh, 12sh, 26sh, 27sh) were positive for oxidase. And only (14sh, and 16sh) were positive for the TSI test. The results of the biochemical tests were presented in (Table 2).

Table 2. Biochemical characteristics of all isolated bacteria :**(-), negative**; **(+) positive.**

Sample No.	Enzymes			IMVC test				Triple sugar iron (TSI)	motility	congo red
	Oxidase	Catalase	Urease	Indole	Methyle red	Voges Proskauer	Simmons citrate			
1,2,3,5,13,15w,17,18,19,20,21,23,28,30	+	+	–	–	+	–	–	alkaline alkaline	+	–
4	+	+	–	–	+	–	+	alkaline alkaline	+	–
6,7,14	–	+	–	–	+	–	–	alkaline alkaline	+	+
8	–	+	+	–	+	–	–	alkaline alkaline	+	+
9,12	+	+	–	–	+	–	+	alkaline alkaline	+	–
10	+	+	–	–	+	–	–	alkaline alkaline	+	–
11	+	+	–	–	+	–	–	alkaline alkaline	+	–
15p	–	+	–	–	–	–	+	alkaline alkaline	+	–
16	–	+	–	–	+	–	+	alkaline alkaline	+	+
22,24,29	–	+	–	–	+	–	–	alkaline alkaline	+	–
25	–	+	+	–	+	–	–	alkaline alkaline	+	–
26	+	+	–	–	+	–	+	alkaline alkaline	+	–
27	+	+	–	–	+	–	–	alkaline alkaline	+	–

3.2. Antibiotics sensitivity test

The results of antibiotic sensitivity are presented in (Table 3) showed that all the isolates were susceptible to (Levofloxacin 5µg) except (14sh and 18sh) which were intermediate. And all isolates were susceptible to (Imipenem 10µg) except (2sh) was intermediate. And also, all of them were resistant to (Amoxicillin clave 30µg) except (8sh, and14sh) were sensitive and (25sh) was intermediate. About (Ceftazidime 10µg) (7sh, 13sh, 16sh, and 22sh) was intermediate, (11sh, 15wsh, and 19sh) were sensitive and the remains were resistant. The isolates (2sh, 12sh, 19sh) were intermediate to (Doxycycline 10µg) and the remaining were sensitive. Isolates (25sh, 8sh, and 14sh) were resistant to (Amikacin 10 µg) and (1sh, 3sh, 4sh, 7sh, 15wsh, 17sh, 22sh, and 24sh) were sensitive and the remaining were intermediate.

Table 3. Antibiotic sensitivity test.

sample No	DO10µg	LEV 5µg	AMC 30µg	AK 10µg	IPM 10µg	CAZ 30µg
1sh, 3sh, 4sh, 17sh, 24sh	S	S	R	S	S	R
2sh	I	S	R	I	I	R
5sh, 6sh, 9sh, 10sh, 15psh, 20sh, 21sh, 23sh, 26sh, 27sh, 28sh, 29sh, 30sh	S	S	R	I	S	R
7sh, 22sh	S	S	R	S	S	I
8sh	S	S	S	R	S	R
11sh	S	S	R	I	S	S
12sh	I	S	R	I	S	R
13sh, 16sh	S	S	R	I	S	I
14sh	S	I	S	R	S	R
15wsh	S	S	R	S	S	S
18sh	S	I	R	I	S	R
19sh	I	S	R	I	S	S
25sh	S	S	I	R	S	R

S= Sensitive**.** R= **Resistant.** I= **Intermediate. (**Do=**Do**xycycline**;** **LEV=** Levofloxacin**;** A**MC=** Amoxicillin clave**; IPM**=Imipenem**;** **AK=** Amikacin**; CAZ=** Ceftazidime**)**

3.4. Genomic characterization

The recovered DNA template from 31 different bacterial isolates gave a specific size of the DNA fragment (1442) when they were used in the PCR reaction. There is no DNA amplicon found in the negative control (figure.3). The 16S rDNA PCR amplicons of each bacterial isolate were shipped for sequencing using the reverse primer P2R to identify each isolate to its precise taxa.

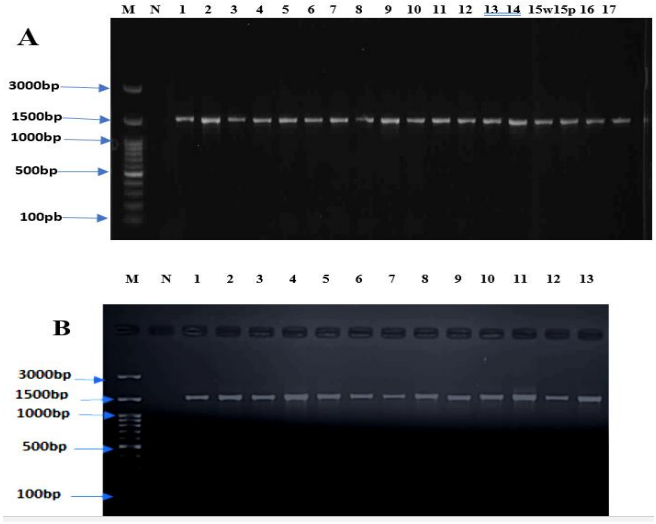

Figure 3. PCR amplified *16S rRNA* of the isolates in 1% agarose gel. Lane M 100bp DNA ladder (GeneDirex). Lane N is negative control that has been run without any DNA template, respectively. The bands are showing 1442bp of PCR amplicons. Panel A Lanes 1 to 17 contain PCR products from 1sh, 2sh, 3sh, 4sh, 5sh, 6sh, 7sh, 8sh, 9sh, 10sh, 11sh, 12sh, 13sh, 14sh, 15wsh, 15psh, 16sh and 17sh, respectively. Panel B 1 to 13 contain PCR products from 18sh, 19sh, 20sh, 21sh, 22sh, 23sh, 24sh, 25sh, 26sh, 27sh, 28sh, 29sh, and 30sh, respectively.

3.4.1. Partial Sequencing of 16S rRNA Gene

All 31 samples were taken into consideration for additional analysis depending on the precision of the DNA sequencing. The retrieved sequences were analyzed using basic local alignment search tool (BLAST) in EzBiocloud database to determine the percentage of similarities in the sequence and potential species identification. The 16S rDNA gene sequence analysis of probable isolates revealed that bacterial isolates (8sh, and 25sh) fall in genus *Rhizobium* the (8sh) strain exhibited 98.7% similarity with *Rhizobium oryzihabitans* which recorded in NCBI as new strain Gene bank under accession number (OP454444). and strain (25sh) showed 100% similarity with *Rhizobium oryzihabitans.* And strain (27sh) showed 100% homology with *Enterobacter quasiroggenkampii.* Bacterial isolates (1sh, 18sh) showed 100% homology with *Pseudomonas granadensis*. Isolates (2sh, 11sh, 12sh, 19sh, and 30sh) were showed 100% homology with *Pseudomonas atacamensis* (except 10sh) and showed 99.7% similarity. While strains (3sh, 22sh, and 29sh) were showed maximum similarities with *Pseudomonas frederiksbergensis.* Isolates (4sh, 5sh, 7sh, 17sh, 20sh, and 23sh) all showed 99.8%

similarities with *Pseudomonas bijieensis* except (7sh) was 99.4%. And the isolate (14sh) showed 100% homology with *Rahnella woolbedingensis*. Strain (16sh) showed 95% similarity with *Erwinia endophytica*. strains (13sh, and 15psh) showed 100% similarities with *Pseudomonas piscium*. And strain (15wsh) showed 99.5% similarities with *Pseudomonas viridiflava*. Isolates (9sh, 21sh, 24sh, 26sh, and 28sh) showed similarity with *Pseudomonas florescence*. Isolated (9sh) showed 99.7% similarity, isolate (21sh) showed 100% similarity, and isolate (24sh) 99.8% similarity. While isolates (26sh) showed 99.7% and (28sh) showed 99.8% similarity.

3.4.2. Plasmid extraction and PCR amplification *nifH* gene

After extraction of plasmid DNA from all 31 strains. PCR was performed for amplification of the *nifH* gene via the use of a specified primer, and gel electrophoresis was carried out to check the presence of the *nifH* gene in the plasmid of all the isolates. The *dinitrogenase reductase* subunit of the *nitrogenase* enzyme, which is in charge of biological nitrogen fixation, is encoded by *nifH* gene. Out of 31 strains only 22 strains exhibited positive PCR products, the product size of the *nifH* gene was 194bp. The isolates (1sh, 2sh, 3sh, 6sh, 7sh, 8sh, 9sh, 10sh, 11sh, 12sh, 14sh, 15wsh, 15psh, 16sh, 21sh, 22sh, 23sh, 24sh, 25sh, 26sh, 27sh, and 29sh,) were positive for *nifH* gene the results showed in (figure 4).

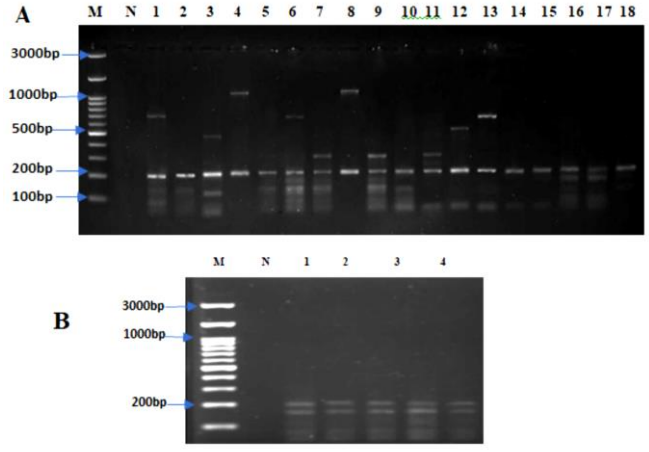

Figure 4. PCR amplified the ***nifH*** gene of the isolates in 2% agarose gel. Lane **M** 100bp DNA ladder (GeneDirex). Lane **N** negative control that has been run without any plasmid DNA template. The bands are showing 194bp of PCR amplicon of the nifH gene. **Panel A** lanes 1 to 18 contain PCR product from 1sh, 2sh, 3sh, 6sh, 7sh, 8sh, 9sh, 10sh, 11sh, 12sh, 13sh, 14sh, 15wsh, 15psh, 16sh, 21sh, 22sh and 23sh, respectively. **Panel B** lanes 1 to 5 contain PCR product from 24sh, 25sh, 26sh, 27sh and 29sh, respectively.

4. DISCUSSION

Nitrogen is necessary for plants to synthesize enzymes, proteins, DNA, RNA and chlorophyll, thus it's important for plant growth and reproduction. Biological nitrogen fixation could aid to enrich agricultural productivity and assure food security (Schulte *et al*., 2022). In the current study, all 31 isolates of root nodulating bacteria were isolated from nodules of fodder legume (*Medicago sativa*), from different regions of Koya city/Erbil. The pure colonies showed growth in 2 days. Some absorbed the colour of the medium containing Congo red, the colonies characterized by large size (2-5mm in diameter), mucilaginous, circular, glistening and translucent or white on YEM medium (Mohammed and Sultan, 2021). Microscopic examination revealed that the isolates were rod shaped and gram negative (Banjare *et al*., 2022)

Additionally, it has been shown that other nitrogen-fixing organisms are present in legume nodules in addition to rhizobia (Lu *et al.*, 2017; Martínez Hidalgo and Hirsch, 2017, Martínez Hidalgo and Hirsch, 2022). In the present study, we isolated a set of rhizobial and non-rhizobial endophytic bacteria from root nodules of the alfalfa plant. Previous studies have also documented the isolation of non-rhizobial bacteria from the root nodules of alfalfa (Lai *et al.*, 2015) and other legumes (Korir *et al.*, 2017; Leite *et al.*, 2017). In this study, the best non-rhizobial isolates and rhizobial isolates were identified depending on morphological, biochemical and molecular characteristics, which were similar to *Rhizobia sp*., *Pseudomonas spp*., *Enterobacter sp*. and *Erwinia sp*., respectively.

Sequence analysis of the *16S rRNA* gene revealed that the strains belonging to *Rhizobia sp*., *Pseudomonas spp*., *Enterobacter sp*. and *Erwinia sp*., respectively (Xu *et al.*, 2014). Seven endophytic non-rhizobial bacteria including *Enterobacter cloacae*, *Chryseobacterium indologenes*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterobacter ludwigii* and *Klebsiella variicola*, were proven has *nif*H gene (Dhole *et al.*, 2016).

Martínez-Hidalgo and Hirsch (2017) likewise specified that certain non-rhizobial strains within nodule are able to do nitrogen fixation process. *Klebsiella sp*., being as an endophytic bacterium,

similarly segregated from Vicia and Glycine, improvement in crop plants development via these bacteria by nitrogen fixation, phosphate, and solubilization IAA production (Martínez-Hidalgo and Hirsch, 2017). Deng *et al.*, (2011) also mentioned amplification of *nifH* gene was done from illustrative *Bacillus cereus* strain as well as *Bacillus amyloliquefaciens* which were isolated from soybean (*Glycine max* L.) root nodules which had the activity of nitrogen fixing (Deng *et al*., 2011).

Rhizobium 's ability to adapt to and survive in unfavorable soil and environmental conditions is likely aided by formation of mucoid. When bacteria that produce mucus are present in the soil, alters the solubility of bacterial cells, strengthening strains' resistance to biological factors that affect competition (Batista *et al.*, 2007).

5. CONCLUSIONS

Conclusively, the results of this research make it very evident that alfalfa plants cultivated in Koya city demonstrated with diverse non-symbiotic bacteria in addition to symbiotic Rhizobia related to this plant's stimulation of growth and the nodules' widespread occurrence. The relationships between symbiotic bacteria, non-symbiotic bacteria, and their environments are discussed in great detail by these studies, despite the fact that many of these nodule non-rhizobial bacteria were unable to fix nitrogen.

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