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Molecular Characterization of Bacteria Isolated from Root Nodules of Lupinus Albus and Determination of its Family Specialization

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ABSTRACT

An endophytic bacteria isolated from root nodules of white lupin *(Lupinus albus*) on agar solidified yeast extract mannitol (YEM), after 48 hours, were white in color, with sticky appearance, and showed the ability of mucus production. Isolated bacteria were positive to oxidase and catalase and negative to gram stain. The bacteria were resistance to 30µg/ml metronidazole antibiotic. According to the diagnosis of the isolate at the molecular level by using DNA sequencing technique for the analysis of 16S rRNA gene with the global database, national center for biotechnology information (NCBI), the results revealed 99.26% similarity of the isolate in this study to the genus *Rhizobium* sp. were found in gene bank, and its recorded for the first times as *Rhizopium* sp. ZAZ strain in NCBI and the results showed responded of the white lupin seedling grown on nitrogen free medium (NF) to inoculated with *Rhizobium* sp. ZAZ isolate at 30 min and successful to form nodules on roots in the rate of 65% and the rate number of nodes/seedling was 2.3.

Keywords: White lupin, *Lupinus albus*, *Rhizopium* sp., 16s rRNA, endophytic bacterial.

INTRODUCTION

Lupinus albus belongs to the *Fabaceae* family (Abdulhadi and Altaii, 2028), which ranks as the third order among the flowering plant families (Covered Seed Plants) Angiosperms (Shavanov, 2021), as include more than 770 genera and 19500 species (Dalavi *et al*., 2021). With 26% of all crops produced worldwide, legumes rank second in importance only to cereals, making them a significant source of both food and revenue (Kebede, 2020). Additionally, they play a crucial role in the nitrogen cycle in both agriculture and the environment (Raza *et al*., 2020), as they establish symbioses with rhizobia, soil endosymbiotic α- and β-proteobacteria possess the ability to fix nitrogen dioxide within modified roots known as root nodules. (Hamowaki and kanartzi, 2018). A root nodule is a mutualistic relationship that occurs between nitrogen-fixing soil rhizobia and mostly leguminous plants. Through this relationship, plants can fix atmospheric nitrogen and give rhizobia carbon as a source of energy (Mahmud *et al.*, 2020).

Rhizobia and plants regulate a series of regulatory mechanisms that result in root nodule symbiosis, the nodule's development into a symbiotic organ is unquestionably the crucial event for the symbiosis' establishment (Suzaki *et al.*, 2015). Flavonoids, which are frequently released into the soil by legume roots, draw rhizobia into the rhizosphere and cause them to make lipo-chitooligosaccharides (LCOs), also known as Nod factors (NFs) (Yang *et al*., 2022). Plant LysM receptors at the plasma membrane recognize NFs, which initiates symbiotic signaling in the host and causes the creation of nodules and bacterial infection (Ratu *et al*., 2021). The curls of root hairs around the rhizobia form the infection thread, and they are caused by the Nod factors that are received by the legume root tip and aid in the movement of the rhizobia through the legume root (Kumar *et al*., 2020). In exchange for their carbon sources of energy, rhizobia give the plant ammonium (NH_4).

In consequence, this acts as a bio-fertilizers for the legume host, promoting plant development without requiring nitrogen to be provided externally (Wheatley *et al*., 2020). Most legume species can fix atmospheric nitrogen (N_2) via symbiotic bacteria (general term “rhizobia”) in root nodules, and this can give them an advantage under low soil nitrogen (N) conditions if other factors are favorable for growth (Wang *et al*., 2019). Legume nodules can typically be categorized as either determinate or indeterminate in terms of growth (Gage, 2019). Determined nodules have a temporary meristem, whereas indeterminate nodules preserve meristematic tissue (Tiwari *et al*., 2021).

Colonies of rhizobia grow on solid media with mannitol sugar, yeast extract, and a small amount of inorganic salt, and they are in form of sticky white colonies, carbohydrates and casein are used as a source of energy and urea, nitrates and amino acids as a source of nitrogen (Yang *et al*., 2018). *Rhizobium* members have a lipid layer outside the membrane, the outer layer of which consists of lipopolysaccharide, and the inner layer is from phospholipids, it's also characterized by free living (Jordan, 1984). They are gram-negative, do not develop endospores, and can move by using one polar flagellum or two to six peritrichous flagella. Optimal growth of most strain occurs at a temperature range of 25-30°C and a pH 6.0-7.0 (Somasegaran and Hoben, 2012). Capable of prolonged independent exist in soil (Rupela and Saxena, 1987).

Different species of *Rhizobium* show a great degree of specificity. Hence, they can be used as biofertilizers only for the specific crops: *Rhizobium meliloti* (Medic-*Rhizobium*): Luceren and Fengugreek, *Rhizobium trofoli* (Clover_*Rhizobium*) Egyption clover and clover, *Rhizobium lupin* (Lupin-*Rhizobium*): Lupines and white lupines. Which characterized by the presence of symbiotic plasmid (pSym) was range in size 150-400 kbps, and its principle when the enzyme *nitrogenase* is present, atmospheric nitrogen (N_2) is reduced to ammonia (NH_3) (Yang *et al*., 2018). During which 60% of the total nitrogen is fixed, and this explain paramount important of this mechanism (Hamowaki and kanartzi, 2018). The processes of biological atmospheric nitrogen fixation include the conversion of molecular nitrogen to ammonia at the presence of the *Nitrogenase* enzyme.

$$N_2 + 8H^+ + 8e- + 16ATP \xrightarrow{Nitrogenase} 2NH_3 + H_2 + 16ADP + 16Pi$$

(Hoffman *et al*., 2000)

White lupin *(Lupinus albus*) is a species of the genus lupines, tribe genisteae, family *Fabaceae*, lupinus species mainly nodulated by slow-growing bacteria that belong to genus *Bradyrhizobium* (Sujak *et al*., 2006).

This study aimed to isolation of bacteria symbiotic with *Lupinus albus* root nodules and its microbiological, biochemical, molecular biology detection with re-inoculation of seedling root, to test its family specific.

MATERIALS AND METHODS

Bacterial isolation from the root nodules of *Lupinus albus* plant

Seeds of *Lupinus albus* plant provided from the local markets of Mosul city and cultured in the house garden (Alzohur neighborhood). After seven days of seeds germination, the root nodules began formed, Fig. (1) the root nodules isolated from plants after 21 days of grown, and washed several times in tap water to remove the rest soil , then the pink nodules up taken and surface sterilized by soaking in 96% ethyl alcohol for two minutes, followed by 15 minutes in a 3% sodium hypochlorite (NaOCl) solution, then three times in sterile distilled water washing (one/minute), and dried on sterile filter paper (Qaddawi and Mohammed, 2021).

The sterile nodules were placed on a surface of 20 ml solid Nutrient Agar (NA) medium and incubated at 28±2°C for 72 hours to insure its surface sterilization efficiency (Aneja, 2003). Ten nodules were selected and crushed well in 3 ml of liquid yeast extract mannitol (YEM) using sterile glass rod. Six decimal dilutions were prepared from suspension, 0.1 ml of the last three dilutions were taken and spread on the surface of agar solidified YEM medium separately by using L shape sterile glass, and incubated for 48 hours at 28±2°C. After growth, one colony was taken for each dilution and cultured on the surface of agar solidified YEM medium in 0.9 cm plastic dishes by plotting method (Prajapati *et al*., 2018) to obtain single, pure colonies were incubated at the same condition above. The plates were transferred and kept in the refrigerator at 4°C until used.

Fig. 1: Nodules on the roots of *Lupius albus* plant after 20 days.

Characterization of isolated bacteria

Morphological characters

The colony characteristics were determined by observing the colonies on YEM plates that grow for 48 hours at 28°C. Microscopic observation of the isolated bacteria was done using gram stain technique (Nagalingam *et al*., 2020).

Biochemical tests

Catalase test

On a glass slide, a fresh bacterial colony is situated, with a drop of 3% hydrogen peroxide solution H_2O_2, and the appearance of gas bubbles indicates the positive result of the test and the bacteria's capacity to generate the enzyme *catalase* that breaks down H_2O_2 into H_2O and O_2, thus releasing oxygen gas (Chhetri *et al*., 2019).

Oxidase test

A fresh, pure bacterial colony was placed on a piece of filter paper that had been dampened with oxidase reagent drops. The ability of the bacteria to manufacture the enzyme is shown by the presence of the violet or purple color. *Cytochrom oxidase*, which oxidizes the reagent to the product indophenol (Wadhwa *et al*., 2017).

Antibiotic sensitivity test

The sensitivity of bacteria isolated was tested with different types of antibiotics in 9 cm petri dishes containing YEM solid medium and the antibiotics discs were added to the surface of medium. The antibiotics selected in this study are listed (Table 1).

Table 1: The antibiotics used and their final concentrations.

Antibiotic	Final concentration (µg ml^{-1})
Metronidazole	30
Ampicillin sulbactum	10
Piperacillin	30
Tetracycline	10
Gentamicin	10
Ticarcillin clavunnic acid	10
Ceftriaxone	10
Levofloxacin	5

Separately by spreading on every medium, 0.1 milliliter of the suspension of bacteria using a sterile L. shape the antibiotics with different concentrations provided from (CLSI, 2018) on the discs form were added to the surface medium after being sterilized; the dishes were closed with their lids and placed in the incubator at 28C° in dark conditions for 48 hours (Soriful *et al*., 2010), then the samples were observed to identify the response of the bacteria towards antibiotics tested.

Preparation of the bacterial suspension

The bacterial suspension was prepared by transferring one full lobe of the isolated bacteria grown on the solid YEM medium to flask containing 20 ml of liquid YEM, and incubated in the shaker incubator at 28°C for 48 hours and rotation speed of 130 cycle.min^{-1}. Then bacterial suspension was harvested in a refrigerated centrifuge for 10 minutes at 500 rpm^{-1}, the filtrate was excluded and one ml of liquid YEM medium was add to precipitated bacteria to obtain the bacterial inoculum. 1.5 ml of culture were taken in Eppendorf tubes and another containing 1.5 ml of liquid YEM medium alone (control), and the optical density samples was measured at wavelength of 600 nm using spectrophotometry (Godschalx *et al*., 2017).

Identification of isolated bacteria by 16s rRNA gene amplification

Genomic DNA extraction of bacteria was isolated using a genomic DNA purification kit (Geneaid, Korea) according to the manufacturer's instructions. Nanodrop (a spectrophotometer made in England by Biodrop) was used to estimate its concentration (Dhahi *et al*., 2011). Isolated DNA samples were electrophoresed in 1% agarose gel using Safe red stain (Safe red stain Dye, Korea) while being shocked with 80 volts for an hour.

Molecular identification of the bacteria

PCR amplification of the 16s rRNA gene universal primers:

27 F: AGAGTTTGATCMTGGCTCAG

1522 R: AAGGAGGTGATCCARCCGCA

(Abellan-Schneyder *et al*., 2021)

PCR mixture, amplification condition and PCR products sequencing were conducted according to (Wang *et al*., 1996). Safe red stain was used to visualize the amplification products on a 1.0% w/v agarose gel.

Determination of nucleotide sequencing of amplified pieces using DNA sequencing technique

The sequence of the nitrogenous bases of the amplified DNA was determined by sending the PCR products with 16S rRNA gene primers to read the sequence of the gene using the 3130 genetic analyzer devices (supplied by the Japanese company Hitachi) located in Erbil city. Using BLAST software, the sequences of Gene-specific were compared to those listed in the (NCBI).

Surface sterilization of *Lupinus albus* seeds and aseptic seedling production

lupinus albus seeds were provided from local markets in Erbil city, after that, flowing water was used to wash them. To get rid of dust and dirt, and it was superficially sterilized by immersing it in a 96% ethyl alcohol for two minutes, and in commercial bleach diluted with sterile distilled water, minor: Distilled water, 2:1, volume: Volume for twenty minutes. After that, it was rinsed with sterile distilled water three times per minute to remove any remaining sterile material, and it was dried using sterile filter paper. After being moved at a pace of two seeds per bottle to bottles containing only NF solid medium, all samples were maintained in a growth incubator at a temperature of 25±2℃ in a dark environment (Sinha *et al*., 2003). It was moved to the same environment with 16 hours of light after it germinated, and the ratio was calculated to reach 100%.

Inoculation of the root system of *Lupinus albus* with bacterial suspension

Sterilized *Lupinus albus* seedling were incubated on the NF medium by removing them from the medium and immersing their roots in flask contain 20 ml of bacterial suspension at a rate of 40 seedlings /treatment at 15 and 30 minutes individually, then transferred to a surface of 20 ml of solid NF medium in 9 cm at plastic petri dishes with 2-3 seedling/dish, samples were sealed with covers, coated with parafilm and kept vertically (the root were down) in the incubator at 24±2℃ and 600 lux/8hours dark, with the root total covered with black sticky (Ali *et al*., 2023).

RESULTS AND DISCUSSION

Characterization of isolated bacterial colonies

The single colonies of isolated bacteria which grow on agar solidified medium appeared to have sticky texture, produce mucus substance after two days of incubation, Fig. (2) this result agreed with findings of (Singh *et al.*, 2013) that reported the characters of bacterial colonies belonging to the *Rhizobium* sp.

Fig. 2: Cultural characteristics of isolated bacteria from the *Lupinus albus* root nodules grown on the YEM medium after 48 hours of incubation.

Biochemical characters of isolated bacteria

The results of biochemical tests of isolated bacteria showed positive response to catalase test, as indicated by the formation of gas bubbles on the glass slide used in the examination after adding

young colonies to a 3% solution of hydrogen peroxide H_2O_2, which indicates its ability to produce the *catalase* enzyme that reduces H_2O_2 and releases oxygen. Positive for the oxidase test, as the appearance of the violet color indicates the ability of the bacteria to produce the *Cytochrome Oxidase* enzyme after transferring a young colony of bacteria to the surface of a filter paper saturated with the oxidase reagent. While it was negative reaction to Gram's staining, Fig. (3).

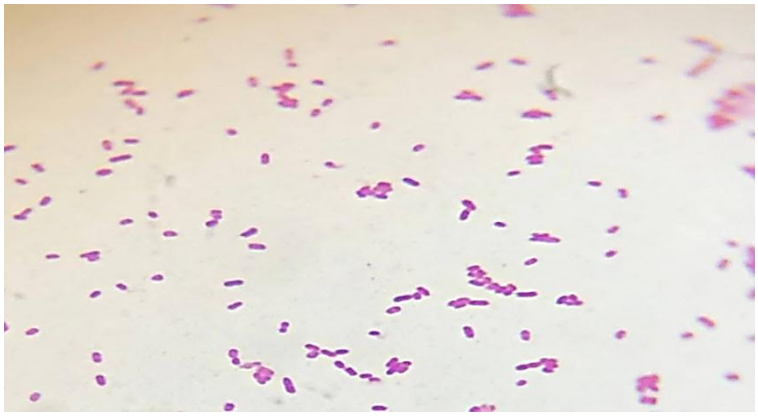

Fig. 3: Gram stain result of isolated bacteria under the light microscope 40X.

Antibiotics susceptibility test

The results of isolated bacteria grown on solid YEM medium contain different types of antibiotics showed that the bacteria were resistant to Metronidazole 30 $\mu g.ml^{-1}$ only Fig. (4), and sensitive to Ampicilin sulbactum 10$\mu g.ml^{-1}$, Piperacillin 30$\mu g.ml^{-1}$, Tetracycline 10$\mu g.ml^{-1}$, Gentamicin 10$\mu g.ml^{-1}$, Ticarcillin clavunnic acid 10$\mu g.ml^{-1}$, Ceftriaxone 10$\mu g.\ ml^{-1}$ and Levofloxacin 5 $\mu g.ml^{-1}$ (Table 2).

Table 2: Antibiotics susceptibility test for isolated bacteria.

Antibiotic	Final concentration ($\mu g.ml^{-1}$)	Resistance and sensitivity
Metronidazole	30	R
Ampicillin sulbactum	10	S
Piperacillin	30	S
Tetracycline	10	S
Ticarcillin clavunnic acid	10	S
Ceftriaxone	10	S
Levofloxacin	5	S

Fig. 4: Antibiotics susceptibility test for the isolated bacteria on agar solidified YEM media.

The reason for the resistance of bacteria to antibiotics may be due to their ability to adapt to live in the presence of many antibiotics in the soil that are produced by fungi and actinomycins, which led to stimulating these bacteria to increase their ability to resist antibiotics through the development of specialized self-mechanisms to maintain their growth and reproduction (Zhang *et al.*, 2018). The sensitivity of bacteria to other antibiotics may explain the process of stopping the inhibition of the protein stimulated by these antibiotics inside the bacterial cell, or the effect on the construction of nucleic acids and the cell wall (Weldrick *et al.*, 2021).

Molecular diagnosis of bacteria isolated from the root nodules of *Lupins albus* using PCR technique

This divided to:

A. Concentration and purity of chromosomal DNA

Findings indicated that the amount of extracted chromosomal DNA was 265 ngµl^{-1} and purity (1.8), UV rays of the gel piece revealed that chromosomal DNA was electrophoresed. The molecular weight was large in terms of its proximity to the agarose gel pits in which the sample was placed before migration, and it was pure in terms of its uninterrupted Fig. (5).

Fig. 5: Electrophoresis of the chromosomal DNA isolated in 1.0% agarose.

B. Polymerase chain reaction technique specialized in sequencing the chromosomal DNA based on the 16s rRNA gene

The results of the electrophoresis in 1% agarose gel of the chromosomal DNA amplified product by PCR using the specific primer of 16s rRNA gene showed the separation of a single band with a size of 1495 bp Fig. (6) comparable to the particular primer's molecular weight employed in this investigation.

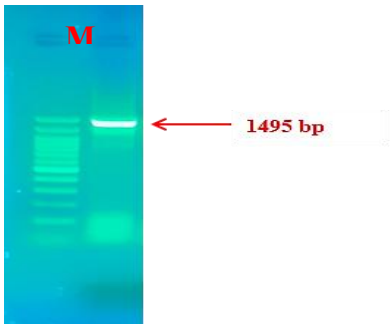

Fig. 6: Electrophoresis of the DNA amplified for the 16s rRNA gene by PCR and isolated from the studied bacteria in 1.0% agarose. (M=Ladder, S= Sample).

The appearance of the band with this size corresponds to the value of identical sequences in the sequence of nitrogenous bases present in the template chromosomal DNA, which can be duplicated when using its primer (Bustin, 2002).

Results of 16S rRNA gene sequence of nucleotides with DNA Blast program showed there is a 99.26% similarity between this sequence and the sequences of *Rhizopium* sp. Genus registered

with the Gene bank in NCBI. After sending the results of nitrogenous bases sequences analysis to the gene bank at the NCBI, the study was able to register it for the first time as a new strain and was given name *Rhizopium* sp. ZAZ Fig. (7). It's possible to find genetic variation between the studied isolates through the technique of studying nitrogenous bases sequences conducted by the researchers (Grison *et al*., 2015) to isolate 56 isolates of rhizobia bacteria from the leguminous host *Anthyllis vulneraria* in southern France, where the researchers used the technology, DNA sequencing for 16S rRNA region.

Rhizobium sp. strain ZAZ 16S ribosomal RNA gene, partial sequence

GenBank: OQ254797.1

FASTA Graphics

Go to:

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2023
DEFINITION  Rhizobium sp. strain ZAZ 16S ribosomal RNA gene, partial
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ACCESSION   OQ254797
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KEYWORDS    .
SOURCE      Rhizobium sp.
  ORGANISM  Rhizobium sp.
            Bacteria; Proteobacteria; Alphaproteobacteria;
Hyphomicrobiales;
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REFERENCE   1  (bases 1 to 946)
  AUTHORS   Qaddawi,Z.T., Mohammed,A.A. and Hassan,Z.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (15-JAN-2023) Sciences, University of Mosul, Al
Majmoaa,
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      241 ggaggcagca gtggggaata ttggacaatg ggcgcaagcc tgatccagcc atgccgcgtg
      301 agtgatgaag gccttagggt tgtaaagctc tttcaccgat gaagataatg acggtagtcg
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      421 tgttcggaat tactgggcgt aaagcgcacg taggcggata tttaagtcag gggtgaaatc
      481 ccgcagctca actgcggaac tgcctttgat actgggtatc ttgagtatgg aagaggtaag
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      661 ataccctggt agtccacgcc gtaaacgatg aatgttagcc gtcgggcagt atactgttcg
      721 gtggcgcagc tnacgcatta aacattccgc ctggggagta cggtcgcaag attaaaactc
      781 aaaggaattg acgggggccc gcacaagcgg tggagcatgt ggtttaattc gaagcaacgc
      841 gcanaacctt accagctctt gacattcggg gtatgggcat tgganacgat gtccttcagt
      901 tagnctggcc cagaacangt gctgcatggc tgtcgtcagc tcgngt
```

Fig. 7: Registration of novel *Rhizopium* sp. ZAZ in NCBI.

Production of root nodules on the *Lupinus albus* seedlings roots grown on NF medium which inoculated with *Rhizobium* sp. ZAZ for15, 30 minutes

The results indicated the ability of *Rhizobium* sp. ZAZ isolate to re-infect the roots of *Lupinus albus* seedlings when treated with them and the success of the root nodule formation process, with different ratio according to the time of inoculation (Table 3).

Table (3): Production of *Lupinus albus root* nodules after inoculation with *Rhizobium* sp. ZAZ for 15 and 30 minutes.

Medium	Inoculation time (min.)	Number of seedlings inoculated / infected	Percentage of infected seedling (%)	Number of nodules	Rate of nodes number /seedling
NF	15	40/16	40	32	2
	30	40/26	65	62	2.3

The treatment with 30 minute was the most prominent for the root nodules formation, and superiority on the other treatment in the root nodules formation at 65% with 2.3 of nodules/seedling. The hairs of their seedling's roots appeared various changes after 15 days of inoculated with bacteria represented by their transformed from a straight shape to a curved shape Fig. (8-A), then continued to grow and form a spherical shape represented by a primary nodule Fig. (8-B) after 28 days, and elongated to oval shape, it developed into a complete root nodule Fig. (8-C) after 35 days.

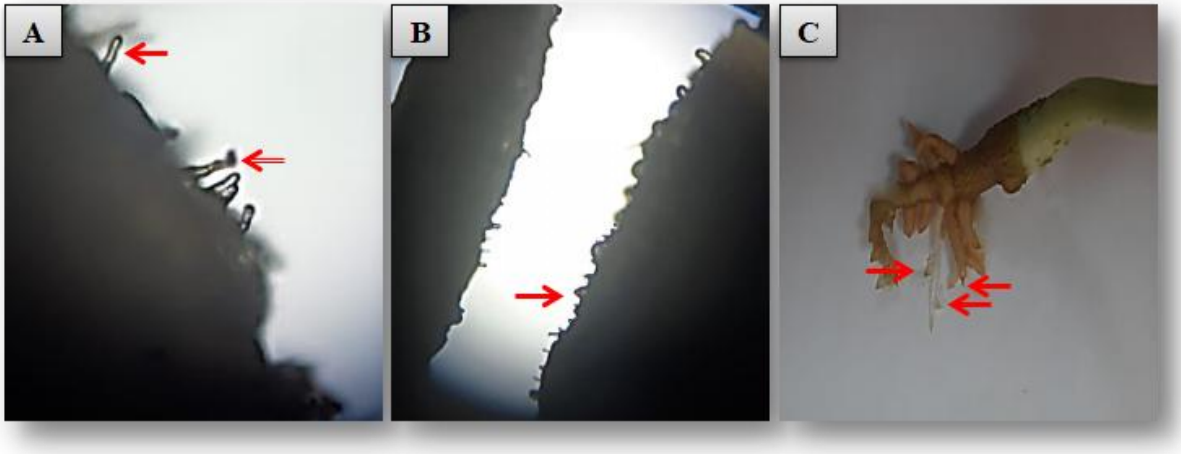

Fig. 8: Production of nodules on the *lupinus alb*us seedling roots inoculated with *Rhizobium* sp. ZAZ for 30 minutes.
A: Root hairs malformed after 15 days of inoculation (arrows).
B: Production of the primary root nodule after 28 days (arrows).
C: Mature root nodule formation after 35 days (arrows).

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الخصائص الجزيئية للبكتريا المعزولة من العقد الجذرية لنبات الترمس وتحديد تخصصها العائلي

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الملخص

البكتيريا الداخلية المعزولة من العقد الجذرية للترمس الأبيض (*Lupinus albus*) والمزروعة على وسط خلاصة الخميرة Mannitol (YEM)، وبعد مرور 48 ساعة من التحضين كانت بيضاء اللون، ذات مظهر لزج، وأظهرت إنتاج مادة مخاطية. وكانت موجبة لفحص الاوكسديز والكتاليز وسالبة لصبغة جرام. وكانت البكتيريا مقاومة للمضاد الحيوي 30 ميكروجرام/مل ميترونيدازول. بناءً على تشخيص العزلة على المستوى الجزيئي باستخدام تقنية تسلسل الحمض النووي لتحليل تسلسل القواعد النيتروجينية لجين 16SrRNA مع قاعدة البيانات العالمية المركز الوطني لمعلومات التكنولوجيا الحيوية (NCBI)، فقد اظهرت النتائج تشابهاً بنسبة 99.26% مع العزلة على المستوى الجزيئي، ولذلك تم تسجيلها لأول مرة باسم *Rhizopium* sp. سلالة ZAZ في NCBI وأظهرت النتائج استجابة شتلات الترمس الأبيض المزروعة على وسط خالي من النيتروجين (NF) للتطعيم بالعزلة *Rhizobium* sp. ZAZ بالتحضين لمدة 30 دقيقة. ونجحت في تكوين عقيدات على الجذور بنسبة 65% وبنسبة عدد العقد/الشتلة 2.3.

الكلمات الدالة: الترمس الابيض، بكتريا الرايزوبيوم، جين 16SrRNA، البكتريا الداخلية.