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# Molecular Identification of Rhizobium Strains Isolated from Cowpea Root Nodules Under Salinity Stress and Assessment of Associated Salt Tolerance Mechanisms

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**ABSTRACT:** This study involved isolating five rhizobial bacterial isolates (AZL-1, AZL-2, AZL-3, AZL-4, and AZL-5) from root nodules of cowpea plants grown in saline environments across five agricultural sites in Basrah Governorate. Following purification, the isolates were molecularly identified via 16S rDNA gene sequencing and compared against reference sequences deposited in the National Center for Biotechnology Information (NCBI) database. Sequences confirmed the identity of all isolates as Brady rhizobium japonicum, except for isolate AZL-3, which was identified as Brady rhizobium sp. A Maximum Likelihood phylogenetic tree was constructed to evaluate genetic relationships among the studied isolates, revealing distinct clustering based on their genetic patterns.

Salt tolerance screening was conducted by culturing the five isolates on Yeast Extract Mannitol Agar (YEMA) media supplemented with varying sodium chloride concentrations (control, 2.5%, 7.5%, and 10%). Isolates AZL-2 and AZL-3 demonstrated growth at 10% NaCl. Consequently, AZL-2 and AZL-3 were selected to evaluate the physiological mechanisms conferring salt stress tolerance. After culturing these resistant isolates in nutrient broth containing 0%, 7%, and 10% NaCl for one-week, intracellular proline accumulation was quantified. Proline accumulation significantly increased with higher salinity levels, peaking at 10% NaCl with values of 0.352 mu mol g-1 and 0.459 mu mol g-1 for AZL-2 and AZL-3, respectively, compared to 0.036 mu mol g-1 and 0.037 mu mol -1 in the non-saline control medium.

Subsequently, these two isolates were evaluated in a pot experiment integrating three irrigation water salinity levels (2, 4, 8 dsm-1), three bio inoculation treatments (uninoculated control, AZL-2 inoculation, and AZL-3 inoculation), and three nitrogen fertilizer application levels (40%, 60%, 80% of the recommended rate of 60 kg N-1 applied as urea, (46%N). Post-harvest evaluations included measuring nitrogen uptake, active root nodule count, and the number of seeds per pod.

## 1. INTRODUCTION

Soil salinization and reduced agricultural productivity represent major challenges contributing to the deterioration of the agricultural sector in Iraq. This issue has drawn significant local and global attention from both theoretical and applied perspectives. Studies indicate that approximately 60–70% of the land in central and southern Iraq is affected by salinity,

rendering it unsuitable for agricultural use (Alhadidi et al., 2024). With the global population projected to reach 9 billion by 2050, the demand for food is escalating rapidly. However, limited arable land, urban expansion, unpredictable climatic conditions, and various biotic and abiotic stresses pose substantial obstacles to meeting these food requirements. Consequently, the application of beneficial microorganisms as eco-friendly biostimulants offers a promising strategy to enhance plant growth and soil fertility. These microbes facilitate nutrient availability, alleviate biotic and abiotic stresses, enhance phytohormone production, and mitigate environmental pollution. In particular, salt-tolerant organisms capable of establishing symbiotic relationships with nitrogen-fixing host plants play a critical role in aiding plant adaptation and enhancing tolerance under saline conditions (Bahuguna et al., 2025; Chaudhary et al., 2022).

Pulse crop cultivation is a key economic activity globally; however, yields remain constrained by low soil nitrogen availability, inadequate chemical fertilizer management, and high soil salinity levels (Cho et al., 2024). Adopting sustainable agricultural technologies to enhance pulse productivity is essential to balance environmental sustainability with growing global food demands (Dhami et al., 2025; Gomoung et al., 2026).

## 2. MATERIALS AND METHODS

### *2.1 Isolation of Rhizobium Bacteria*

*Rhizobia* were isolated from active root nodules of cowpea plants growing in saline soils. Healthy, pink nodules were detached from the roots, washed thoroughly with distilled water, and surface-sterilized with 70% ethanol for 3 minutes. The nodules were then rinsed three times with sterile distilled water and immersed in a sodium hypochlorite (NaClO) solution at a 3:1 (water:NaClO) ratio for 15 minutes, followed by three additional washes with sterile distilled water. The sterilized nodules were transferred to sterile Petri dishes to dry.

Using a sterile glass rod, the nodules were crushed in two drops of sterile distilled water to obtain a uniform suspension. This suspension was streak-inoculated onto Petri dishes containing Yeast Extract Mannitol Agar (YEMA) medium supplemented with 2.5% Congo Red stain (1 mL L-1. The plates were incubated at 30 °C for 5 days. White, unpigmented colonies—indicating non-absorption of Congo Red—were selected, purified, and stored in glycerol stock solutions at -20^ circ C for subsequent use (Beck et al., 1993; Abdullah, 2021).

### *2.2 Molecular Identification of Rhizobium Bacteria*

#### 2.2.1 Genomic DNA Extraction and PCR Amplification

Bacterial genomic DNA was extracted using the Geneaid Mini gDNA Bacteria Extraction Kit according to the manufacturer's protocol. Amplification of the 16S rRNA gene was performed using universal primers: 16S-rRNA-27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 16S-rRNA-1392R (5'-GGTTACCTTGTTACGACTT-3') synthesized by Macrogen (Seoul, South Korea).

The PCR amplification reaction was carried out in a final volume of 50 mu L, containing 100 ng of template DNA, 25 mu L of Master Mix (Promega, USA), 1.25 mu L of each primer 10 pmol mu L^-1, and double-distilled water ddH_2 O to adjust the volume. The thermocycling conditions consisted of an initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 56 °C for 30 s, and extension at 72 °C for 30 s; with a final extension step at 72 °C for 10 min.

**Table 1.** Primers and thermal conditions used for 16S rRNA gene PCR amplification.

| The initiator | Nitrogen base sequence | Correlation temperature ( °C ) | Source |
|---|---|---|---|
| 16S rRNA-27F | AGAGTTTGATCCTGGCTCAG- 3 - F: 5 | 58.4 | |
| 16S rRNA-1392R | GGTTACCTTGTTACGACTT-3 - R: 5 | 53 | |

Table 2 :Polymerase Chain Reaction Program

| Number of courses | the time | Temperature (°C) | Stage |
|---|---|---|---|
| 1 | 5 minutes | 94 | Activation (Initiation) |
| 35 | 30 seconds | 94 | Denaturation |
| | 30 seconds | 56 | Annealing |
| | 30 seconds | 72 | Extension |
| 1 | 10 minutes | 72 | Final Extension |
| | ∞ | 4 | Storage |

The PCR product was qualified and quantified using nanodrop (Thermo Scientific nanodrop 2000, USA) at A260/A280 nm wavelength.Diagnosis of Rhizobium bacteria Based on the sequence of nitrogenous bases (DNA sequencing). After obtaining the PCR amplification product, the amplified samples were sent to the Macrogen (Korea) for sequencing . The sequence results were processed and analyzed by Assis. Prof Dr. Labeed Abdullah Najm of the Department of Intelligent Medical Systems, College of Computer Science and Information Technology, University of Basra. The sequences were initially processed using Chromas software (Technelysium, version 2.6.6), and the samples were identified by comparing the sequences . The isolates under study with the isolates The sequences were stored in the National Center for Biotechnology Information (NCBI) using the BLAST search engine .

**2.2.2 Phylogenetic Tree:** The phylogenetic tree analysis was performed using MEGA11 based on the Maximum Likelihood algorithm.

**2.3 Salt tolerance test of Rhizobium bacteria**

The five bacterial isolates were grown on the special medium . YEMA Sterilizer containing sodium chloride in proportions (control, 2.5 , 7.5 , 10)% They were incubated at 30°C for 3-5 days until growth appeared on the medium (Sharma *et al.* , 2021).

**2.4 Salt tolerance mechanisms experiment**

To understand the mechanism of salt tolerance of rhizobium bacteria used as a bioinoculation to enhance the resistance of legume crops to soil salinity stress, this experiment was conducted based on Bates et al . ( 1973) . This involved the activation and growth of salt-stress-resistant rhizobium isolates AZL-2. AZL -3 in liquid culture medium ( nutrient broth ) at concentrations of 0, 7, and 10% Sodium chloride salt and incubated at 30°C for a week.

**2.5 Agricultural experiment**

A potting experiment was conducted during the spring season to demonstrate the effect of the two efficient isolates. Rhizobia bacteria with nitrogen fertilization levels increased the growth and yield of cowpea plants irrigated with water of varying salinity . After 90 days of cultivation ,the amount of nitrogen uptac ,the number of active root nodules ,and the number of seeds per pod of the cowpea plant were estimated.

**3.RESULTS AND DISCUSSION**

**3.1 Molecular diagnosis of Rhizobium bacteria**

The results of DNA extraction from the isolates showed Nitrogen-fixing rhizobium bacteria in Figure 1 This confirms its purity using the Nano Drop device at a ratio of A280 / A260 The ratio was within the range of 1.8 ,as confirmed by Dauphin et al . )2011 , (Jaafar (2019 , (and Khairallah (2021) as evidence of the best purity and is considered within the recommended ranges for the success of the amplification process .

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The results are shown in Appendix 1 for the molecular diagnosis of the five Rhizobia isolates . Based on the percentage of 16SrRNA gene sequences matching bacterial strains in the global gene bank , the results showed that all isolates belonged to *Bradyrhizobium* . *Japanicum*, except for the third isolate, belongs to *Bradyrhizobium* sp .

Figure ( 1 ) DNA purity of the five isolates from Rhizobia bacteria Using the Nano Drop device at a ratio of A280 / A260 nanometers

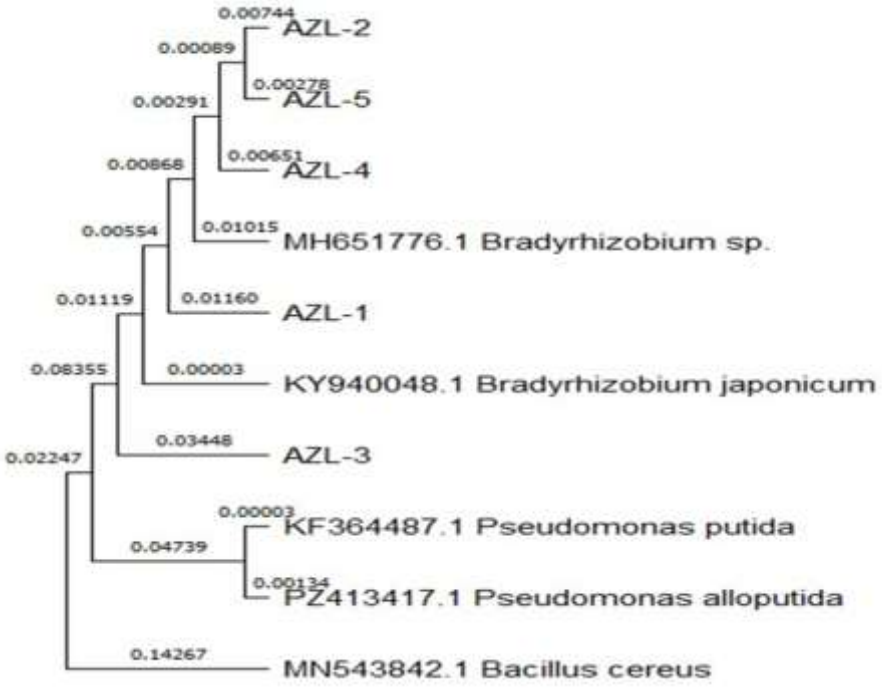


**Figure (2)** Phylogeny and evolutionary tree of Rhizobia bacterial isolates

Evolutionary analysis by Maximum Likelihood method The evolutionary history was inferred by using the Maximum Likelihood method and Tamura- Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (-3322.76) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura- Nei model, and then selecting the topology with superior log likelihood value. This analysis involved 10 nucleotide sequences. There were a total of 1099 positions in the final dataset. Evolutionary analyzes were conducted in MEGA11 (Tamura et al. 2021)

**3.2 Salt tolerance test**

**Table 3** :Salt tolerance test for Rhizobia bacterial isolates

| Salt tolerance of sodium chloride ( NaCl ) % | | | | |
|---|---|---|---|---|
| 10 | 7.5 | 2.5 | Control | Isolates |
| - | - | +++ | +++ | AZL-1 |
| + | ++ | +++ | +++ | AZL-2 |
| + | ++ | +++ | +++ | AZL-3 |
| - | - | +++ | +++ | AZL-4 |
| - | - | +++ | +++ | AZL-5 |

The results in Table 3 illustrate the variation in the growth capacity of Rhizobia isolates in culture media containing different sodium chloride concentrations. Isolates AZL- 2 and AZL- 3 were able to grow at the highest salt concentration of 10 dS /cm , making them the most salt-tolerant isolates. The remaining isolates ceased growth at a concentration of 7.5 dS /cm , indicating the lowest salt tolerance. Saeed (2019) suggested that the tolerance of some bacterial isolates to high osmotic pressure may be due to a mechanism that enables the cell to mitigate the effects of osmotic pressure. This mechanism involves the secretion of various osmotic resistance substances, such as exogenous polysaccharides and amino acids like proline, which raise the intracellular osmotic pressure to counteract the increased extracellular pressure, thus protecting the cell through a process known as osmoprotection . This finding is consistent with Zahir's et al . (2010)

### 3.3 The mechanism of salt tolerance

The results in Figure (3) show a significant increase in the accumulated amount of the amino acid proline with increasing salt concentration. The highest accumulated amount of proline reached 0.459 µmol $g^{-1}$ in the treatment inoculated with AZL-3 bacteria and a salt concentration of 10%, compared to the lowest amount in the control treatment (culture medium only), which was 0.037 µmol $g^{-1}$. This indicates that the higher the salt concentration in the bacterial environment, the greater the stimulation of proline accumulation in its cells, as it is an osmotic compound that helps the organism regulate osmotic pressure, protect proteins and cell membranes, and reduce oxidative damage. The results also showed that the medium inoculated with isolate AZL-3 was superior to the medium inoculated with isolate AZL-2 in increasing the amount of accumulated proline, indicating that AZL-3 is more efficient in resisting salt stress by producing protective compounds such as proline. These results are consistent with Kumar *et al.* (2023) and Egamberdieva *et al* . (2022).

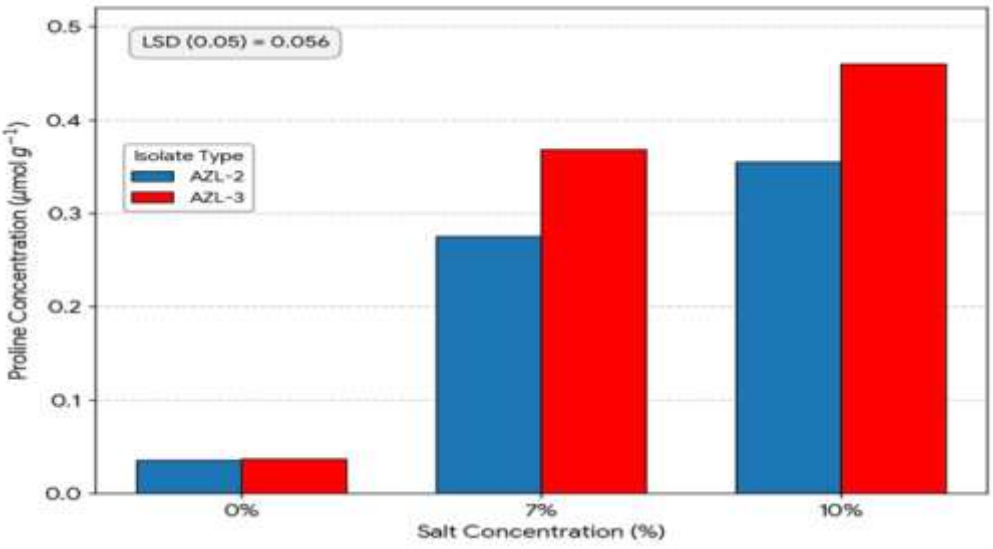


**Figure (3)** Effect of different salinity levels on the accumulated amount of proline in Bradyrhizobium bacteria strains Japanicum and Bradyrhizobium sp.

### 3.4 Agricultural experiment

#### 3.4.1 The amount of nitrogen Uptake (mg $Pot^{-1}$)

The results in Table (4) indicate that the increase in the salinity level of irrigation water significantly affected the amount of nitrogen absorbed by the cowpea plant , as the average decreased to 1661.710, 1238.106, and 642.621 mg $pot^{-1}$ at salinity levels S2 , S4 , and S8 respectively. The decrease in the amount of nitrogen absorbed with increasing salinity of irrigation water is attributed to the negative effects of salt stress on the plant. Salinity leads to a decrease in the osmotic potential of the soil solution, which limits the absorption of water and nutrients by the plant. In addition, there is competition between sodium and chloride ions with nitrate and ammonium ions for absorption sites in the roots. Salinity also inhibits root growth and reduces the activity of enzymes responsible for nitrogen metabolism, which leads to a decrease in the efficiency of nitrogen use and a reduction in the amount absorbed. These results are consistent with Aslam *et al.* (2023). The results showed a significant effect

of inoculation with Rhizobia bacteria on the amount of nitrogen absorbed by cowpea plants . An increase in the average was observed from 561.163 mg $pot^{-1}$ in the uninoculated control treatment ( R0 ) to 1330.103 and 1651.171 mg $pot^{-1}$ when inoculated with Rhizobia bacteria species AZL-2 and AZL-3 respectively. The significant increase in the amount of nitrogen absorbed by cowpea plants when inoculated with Rhizobia bacteria is explained by the increased efficiency of atmospheric nitrogen fixation through the formation of efficient root nodules, which provided a continuous source of nitrogen available for absorption by the plant. Rhizobia also contributed to improving root system growth and increasing the efficiency of water and nutrient absorption, in addition to improving the efficiency of nitrogen use within the plant through increased protein and chlorophyll formation. This was reflected in increased vegetative growth and biomass, and thus an increase in the amount of nitrogen absorbed, as concluded by Mupambwa *et al.* (2024) , and the results showed a significant effect of adding levels of nitrogen fertilizer on the amount of nitrogen absorbed by the cowpea plant , as an increase in the rate was observed from 952.311 mg $pot^{-1}$ at level N40 to 1174.427 and 1415.698 mg $pot^{-1}$ at nitrogen levels N60 and N80 respectively. This significant increase in the amount of nitrogen absorbed is attributed to the increased concentration of available mineral nitrogen in the soil solution , which enhanced its absorption by the roots . Nitrogen fertilization also contributed to improving the growth of the root and shoot systems, and increasing the formation of chlorophyll and proteins, which improved the efficiency of the photosynthesis process and thus led to an increase in the amount of nitrogen absorbed. These results are consistent with many recent studies that indicated that balanced management of nitrogen fertilization increases nitrogen uptake efficiency and improves the growth and productivity of cowpeas, including Danso *et al* . (2025) and Kim *et al* . (2025) demonstrated a significant interaction effect between irrigation water salinity levels and bioinoculation on the amount of nitrogen absorbed by cowpea plants . The highest rate 2355.688 mg $Pot^{-1}$ was recorded at salinity level S2 and the AZL-3 inoculation treatment, compared to the lowest rate at salinity level S8 . The treatment without vaccination R0 reached 329.557 mg $Pot^{-1}$. The significant increase in the amount of nitrogen absorbed is attributed to the ability of salt-resistant rhizobium bacteria strains to mitigate the negative effects of salt stress on the plant , as they improved root nodule formation and nitrogen biofixation efficiency, as well as promoting root system growth, maintaining osmotic balance , and reducing ion toxicity resulting from the accumulation of sodium and chloride . This led to an increase in the amount of nitrogen absorbed compared to unpollinated plants irrigated with saline irrigation water. This result is consistent with recent studies, including Wang *et al* . (2025) and Kaya *et al.* (2026) The study confirmed that using salt-tolerant bio-inoculations is an effective way to improve nitrogen nutrition in legumes and increase its uptake. The results showed a significant interaction between irrigation water salinity levels and nitrogen fertilizer levels on the amount of nitrogen uptake in cowpea plants , with the highest rate 1954.849 mg $Pot^{-1}$ recorded at salinity level S2 and nitrogen fertilizer level N80, compared to the lowest rate at salinity level S8 . The nitrogen level N40 reached 519.069 mg $Pot^{-1}$ because the addition of nitrogen improved the plant's ability to cope with salt stress, as it provided an additional source of mineral nitrogen , which helped the plant maintain its growth and vital activity, thus leading to increased nitrogen absorption and accumulation compared to plants irrigated with saline water without the addition of nitrogen fertilizer (Gonzalez *et al.*, 2026). showed a significant interaction effect between rhizobium bacteria bioinoculation and nitrogen fertilizer application on the amount of nitrogen absorbed by cowpea plants , with the highest rate reaching 1872.261 mg $Pot^{-1}$ when inoculated with AZL-3 bacteria and a nitrogen fertilizer level of N80, measured at the lowest rate of 371.783 mg $Pot^{-1}$ when using the control treatment without R0 inoculation and a nitrogen fertilizer level of N40 , This is due to the integration of nitrogen biofixation by efficient strains of rhizobium bacteria and the addition of mineral nitrogen . This integration contributed to improved root and shoot growth, increased nitrogen utilization efficiency, enhanced photosynthetic activity, and improved dry matter production, which was reflected in an increased amount of nitrogen absorbed by cowpea plants. These results are consistent with Danso *et al* . (2025) . The results showed a significant effect of the three-way interaction between irrigation water salinity levels, bioinoculation, and nitrogen fertilizer levels on the amount of nitrogen absorbed by cowpea plants . The highest rate was 2642.430 mg $Pot^{-1}$ at irrigation water salinity level N2, inoculation with bacteria AZL-3, and nitrogen fertilizer level N80 , compared to the lowest rate of 235.597 mg $Pot^{-1}$ at irrigation water salinity level S8, control treatment without inoculation R0, and nitrogen fertilizer level N40 . This indicates that the complementary effect between chemical and biological management factors plays a role in mitigating the negative effects of salinity on the plant , as each factor compensated for part of the negative effects of salinity, leading to improved nitrogen uptake and increased accumulation in plant tissues . Studies have confirmed that combining bacterial inoculation with efficient strains of Rhizobia bacteria with moderate nitrogen fertilization is one of the most efficient strategies for improving nitrogen nutrition in cowpea plants under salt stress conditions . (Nelwamondo *et al* ., 2026).

**Table (4)** Effect of inoculation with Rhizobia bacteria, levels of nitrogen fertilization, and salinity of irrigation water on the amount of nitrogen absorbed by cowpea (mg Pot$^{-1}$)

| Inoculation × Nitrogen level | salinity level | | | nitrogen level | Inoculation |
|---|---|---|---|---|---|
| | 8 S | 4 S | 2 S | | |
| 371.783 | 235.597 | 364.063 | 515.690 | 40 N | R0 |
| 551.644 | 294,323 | 523.430 | 837,180 | 60 N | |
| 760.060 | 458,750 | 725.510 | 1095.920 | 80 N | |
| 1057.110 | 610.183 | 1095.117 | 1466.030 | 40 N | AZL-2 |
| 1318.427 | 727.253 | 1380.717 | 1847.310 | 60 N | |
| 1614.773 | 906.457 | 1811.667 | 2126.197 | 80 N | |
| 1428.041 | 711.427 | 1435.427 | 2137.270 | 40 N | AZL-3 |
| 1653.211 | 887,240 | 1785.030 | 2287.363 | 60 N | |
| 1872.261 | 952.360 | 2021.993 | 2642.430 | 80 N | |
| | 642.621 | 1238.106 | 1661.710 | S rate | |
| SRN = 129.0, RN = 74.5, S = 43.0 | | | | LSD (0.05) | |
| N rate | 8 S | 4 S | 2 S | | |
| 952.311 | 519.069 | 964.869 | 1372.997 | 40 N | Salinity × Nitrogen |
| 1174.427 | 636.272 | 1229.726 | 1657.284 | 60 N | |
| 1415.698 | 772.522 | 1519.723 | 1954.849 | 80 N | |
| 43.0 | 74.5 | | | LSD (0.05) | |
| R rate | 8 S | 4 S | 2 S | | |
| 561.163 | 329.557 | 537.668 | 816.263 | R0 | Salinity × Inoculation |
| 1330.103 | 747.964 | 1429.167 | 1813.179 | AZL-2 | |
| 1651.171 | 850.342 | 1747.483 | 2355.688 | AZL-3 | |
| 43.0 | 74.5 | | | LSD (0.05) | |

### 3.4.2 Number of active root nodes (plant node$^{-1}$)

The results in Table (5) indicate that the increase in the salinity level of irrigation water significantly affected the number of effective root nodules of cowpea plants, as the average decreased to 41.674, 30.527, and 15.937 plant node$^{-1}$ at salinity levels S2 , S4 , and S8 respectively. The significant decrease in the number of active root nodules is attributed to the fact that salinity leads to a decrease in the proportion of active nodules capable of fixing atmospheric nitrogen due to the high concentrations of sodium ions. Chloride, which causes osmotic and ionic stress, limits the growth of rhizobia bacteria and reduces their efficiency in root colonization and the formation of effective nodules containing active bacteria and the nitrogenase enzyme responsible for nitrogen fixation. Recent reviews have shown that salt stress weakens the symbiotic relationship between the plant and the rhizobium and leads to a decrease in the efficiency of effective nodules . Salinity also leads to a reduction in

leghemoglobin content . Within the root nodules , it is considered an essential protein for regulating oxygen access to the bacteria while maintaining the activity of the nitrogenase enzyme . With a decrease in this protein, the nitrogen fixation process is inhibited, and many nodules become inactive , leading to their premature aging and consequently a decrease in their numbers Gupta *et al* . (2026). The results showed a significant effect of inoculation with types of Rhizobia bacteria on the number of active root nodules of cowpea plants, as an increase in the average was observed from 10,031 plant node$^{-1}$ in the uninoculated control treatment ( R0 ) to 35,679 and 42,428 plant node$^{-1}$ when inoculated with the Rhizobia bacteria types AZL-2 and AZL-3 respectively. The increased number of active nodules is attributed to the fact that the bioinoculation supplies the root zone with selected and highly efficient strains of *Bradyrhizobium* bacteria capable of forming a successful symbiotic relationship with cowpea plants. This increases the formation of active root nodules containing active bacterioids and the nitrogenase enzyme responsible for fixing atmospheric nitrogen . In addition, the rhizobia bacteria produce plant growth regulators such as auxins, cytokinins, gibberellins , and the ACC deaminase enzyme . This stimulates root system growth and increases the number of root hairs, providing more sites for bacterial infection and nodule formation . Rhizobia bacteria also contribute to increased availability of nutrients, particularly phosphorus, iron, and molybdenum, which are essential for nodule formation and nitrogenase enzyme activity. This leads to an increase in the number of effective root nodules and improves their efficiency in nitrogen fixation. Compared to unpollinated plants (Kim *et al* ., 2025). The results showed a significant effect of adding levels of nitrogen fertilizer on the number of effective root nodules of cowpea plants, as an increase in the average was observed from 24,999 plant node$^{-1}$ at level N40 to 29,400 and 33,740 plant node$^{-1}$ at nitrogen levels N60 and N80 respectively. The significant increase in the number of active root nodules in cowpea plants with increased nitrogen fertilizer application is attributed to the moderate addition of nitrogen. The plant's needs were met during the early stages of growth, leading to improved root system development and an increased number of root hairs, thus enhancing root colonization efficiency by Rhizobium bacteria. Furthermore, the addition of nitrogen fertilizer improved photosynthetic efficiency and increased carbohydrate translocation to the root nodules, maintaining nitrogenase enzyme activity and increasing leghemoglobin content within the nodules, resulting in a higher number of functional root nodules. (Kundu *et al*., 2025). The results showed a significant interaction effect between irrigation water salinity levels and bioinoculation on the number of effective root nodules in cowpea plants, with the highest average of 58,253 plant node$^{-1}$ at salinity level S2 and the AZL-3 inoculation treatment compared to the lowest average at salinity level S8. The treatment without vaccination had an R0 of 6.888 plant node$^{-1}$ , this increase is attributed to inoculation with vigorous and salt-tolerant strains of *Bradyrhizobium* bacteria This mitigates the harmful effects of salt stress on the symbiotic relationship, as these strains possess a higher ability to survive and reproduce in the root zone under saline conditions, thus increasing the success of the infection process and the formation of effective root nodules capable of fixing atmospheric nitrogen. (Kaur *et al* ., 2025) . The results showed a significant interaction effect between irrigation water salinity levels and nitrogen fertilizer levels on the number of active nodules, with the highest rate reaching 47,302. plant node$^{-1}$ at salinity level S2 and nitrogen fertilizer level N80 compared to the lowest rate at salinity level S8 The nitrogen level ( N40) , which reached 13,117 plant node$^{-1}$ , is attributed to balanced nitrogen fertilization . It enhances the ionic balance within the plant by promoting the absorption of essential nutrients, particularly potassium, phosphorus, and calcium, and reducing the toxic effects of sodium and chloride ion accumulation. It also increases the efficiency of antioxidant systems, thus reducing oxidative damage to root nodule tissues and maintaining their activity for a longer period. Therefore, the interaction between salinity and moderate nitrogen fertilization may lead to an increase in the number of active root nodules Xu *et al*., (2025). The results showed a significant interaction effect between bioinoculation with rhizobia bacteria and the addition of nitrogen fertilizer levels on the number of active root nodules in cowpea plants. The highest average of 46,396 plant node$^{-1}$ was reached with inoculation using AZL-3 bacteria and a nitrogen fertilizer level of N80, compared to the lowest average. 7.178 plant node$^{-1}$ when using the control treatment without R0 pollination and a nitrogen fertilizer level of N40 , Attributed This effect moral in more number The contract radical to presence relationship Integration between Rhizobia Nitrogen fertilization Metallic, so shares vaccination bio in to provide numbers Effective from Rhizobia bacteria The capable on colonialism root plant and motivation formation The contract radical in when save fertilization nitrogen amount suitable from nitrogen Necessary To support Growth early and formation total radical good, as that to improve the condition Nutrition For the plant a result overlap between staff may Increase from efficiency formation The contract And continuation Her activity And from then to rise Their number comparison In transactions The solitary one, And agreed This is amazing Results with what He pointed To him Tran and Hoang (2026) and Budiastuti *et al.* (2025) .

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The results showed a significant effect of the three-way interaction between irrigation water salinity levels, bioinoculation, and nitrogen fertilizer levels on the number of active root nodules in cowpea plants. The highest average of 61,507 plant node$^{-1}$ was recorded at irrigation water salinity level N2, bioinoculation with AZL-3 bacteria, and nitrogen fertilizer level N80, compared to the lowest average of 5,220. plant node$^{-1}$ at irrigation water salinity level S8 and control treatment without inoculation R0 and nitrogen fertilizer level N40 , inoculation led Vital to effective strains of rhizobium bacteria with Level Suitable from fertilization nitrogen under conditions salinity to to improve growth Roots and efficiency relationship symbiosis between plant rhizobia Which mirror in more number The contract radical Effective comparison In transactions that Exposed salinity Don vaccination or levels not balanced from Nitrogen, And it is possible that Reflect more number The contract Effective to improve capacity plant on Governorate on Solidarity Installation nitrogen under stress Salty and agree This is amazing Result with Danial and Basset (2024) .

**Table (5)** Effect of inoculation with Rhizobia bacteria, levels of nitrogen fertilization and salinity of irrigation water on the number of effective root nodules of cowpea plants (plant node$^{-1}$ )

| Inoculation × Nitrogen level | salinity level | | | nitrogen level | Inoculation |
|---|---|---|---|---|---|
| | 8 S | 4 S | 2 S | | |
| 7.178 | 5.220 | 6.667 | 9.647 | 40 N | R0 |
| 9.964 | 6.557 | 9.107 | 14,230 | 60 N | |
| 12.952 | 8.887 | 9.443 | 20.527 | 80 N | |
| 29.942 | 12.603 | 31.987 | 45.237 | 40 N | AZL-2 |
| 35.221 | 18.633 | 36.237 | 50.793 | 60 N | |
| 41.873 | 20,230 | 45.517 | 59.873 | 80 N | |
| 37.876 | 21.527 | 39.940 | 52.160 | 40 N | AZL-3 |
| 43.013 | 23.723 | 44.223 | 61.093 | 60 N | |
| 46.396 | 26.057 | 51.623 | 61.507 | 80 N | |
| | 15.937 | 30.527 | 41.674 | S rate | |
| SRN = 3.07, RN = 1.77, S = 1.02 | | | | LSD $_{(0.05)}$ | |
| N rate | 8 S | 4 S | 2 S | | |
| 24.999 | 13.117 | 26.198 | 35.681 | 40 N | Salinity × Nitrogen |
| 29,400 | 16.304 | 29.856 | 42.039 | 60 N | |
| 33,740 | 18.391 | 35.528 | 47.302 | 80 N | |
| 1.02 | 1.77 | | | LSD $_{(0.05)}$ | |
| R rate | 8 S | 4 S | 2 S | | |
| 10.031 | 6.888 | 8.406 | 14.801 | R0 | Salinity × Inoculation |
| 35.679 | 17.156 | 37.913 | 51.968 | AZL-2 | |
| 42.428 | 23.769 | 45.262 | 58.253 | AZL-3 | |
| 1.02 | 1.77 | | | LSD $_{(0.05)}$ | |

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**3.4.3 Number of seeds per pod ( seed pod$^{-1}$ )**

The results in Table (6) indicate that the increase in the salinity level of irrigation water significantly affected the number of seeds per pod for cowpea plants, as the average decreased from 8.970, 8.111, and 6.756 seed pod$^{-1}$ at salinity levels S2 , S4 , and S8 respectively. This is attributed to the fact that to rise salinity He causes stress Osmotic and ionic Reflected In picture clear on Stages Reproductive For the plant, no Sima flowering and vaccination and fertilization And it developed pod and the fetus And with more concentration salts It decreases absorption Water And it happens accumulation for ions Like $Na^+$ and $Cl^-$ , Which Limits from Growth And representation photo and supply Members Reproductive With materials These carbohydrate results were consistent with those of Khalil *et al* . (2022). The results showed a significant effect of inoculation with Rhizobia bacteria on the number of seeds per pod in cowpea plants. An increase in the average was observed from 7.292 seed pod$^{-1}$ in the uninoculated control treatment (R0) to 8.139 and 8.406 seed pod$^{-1}$ when inoculated with the Rhizobia bacteria species AZL-2 and AZL-3, respectively. This is attributed to the fact that Inoculation with viable strains of rhizobium bacteria Hassan formation The contract radical Effective And increased from efficiency stabilizing nitrogen Air Which Hassan the condition Nitrogen For the plant and raised His ability on formation Save flowers and the horns and seeds (Dhami *et al* ., 2025). The results showed a significant effect of adding nitrogen fertilizer levels on the number of seeds per pod in cowpea plants, as an increase in the average was observed from 7.554 seed pod$^{-1}$ at the N40 level to 7.919 and 8.363. The seed pod$^{-1}$ at nitrogen levels N60 and N80 respectively, indicating that nitrogen component essential in formation proteins and enzymes and chlorophyll Therefore for Providing it at the level Suitable improves Growth and representation photo and it increases amount Materials carbohydrates Materials Nitrogen that It moves to flowers and the horns developing Therefore more number seeds that It continues in Growth (Danso *et al* ., 2025). The results showed a significant interaction effect between irrigation water salinity levels and bioinoculation on the number of seeds per pod in cowpea plants, with the highest average of 9.318 seeds seed pod$^{-1}$ occurring at salinity level S2 and the AZL-3 inoculation treatment . Based on a minimum rate of 5.770 seed pod$^{-1}$ at a salinity level of S8 Treatment without vaccination R0 , This indicates that vaccination bio shares in mitigation part from Effects Negativity salinity on Growth and reproduction from during to improve formation The contract radical Effective and increase stabilizing nitrogen Therefore to improve the condition food For the plant His ability on Governorate on flowers And the horns and formation The seeds (Dhami *et al* ., 2025) . The results showed a significant interaction effect between irrigation water salinity levels and nitrogen fertilizer levels on the number of seeds per pod in cowpea plants, with the highest average reaching 9.453. seed pod$^{-1}$ at salinity level S2 and nitrogen fertilizer level N80 compared to the lowest rate of 6.388 seed pod$^{-1}$ at salinity level S8 And the nitrogen level (N40 ), indicating that addition nitrogen At the level Suitable Helped plant on compensation part from Deficiency in Growth and jobs physiology Output on stress salty no Sima during My stage flowering and formation The results showed that there was no significant effect of the two-way interaction between biopollination and nitrogen fertilizer addition , and the three-way interaction between irrigation water salinity levels , biopollin addition , and nitrogen fertilizer addition, on the number of seeds per pod for cowpea plants.

**Table (6)** Effect of inoculation with Rhizobia bacteria, levels of nitrogen fertilization and salinity of irrigation water on the number of seeds per pod of cowpea plant (seed pod$^{-1}$ )

| Inoculation × Nitrogen level | salinity level | | | nitrogen level | Inoculation |
|---|---|---|---|---|---|
| | 8 S | 4 S | 2 S | | |
| 6,900 | 5.467 | 7.217 | 8.017 | 40 N | R0 |
| 7.288 | 5.690 | 7.433 | 8.740 | 60 N | |
| 7.688 | 6.153 | 7.860 | 9.050 | 80 N | |
| 7.768 | 6.783 | 8.093 | 8.427 | 40 N | AZL-2 |
| 8.088 | 7.093 | 8.190 | 8.980 | 60 N | |
| 8.562 | 7.623 | 8,500 | 9.563 | 80 N | |

| | | | | | |
|---|---|---|---|---|---|
| 7.994 | 6.913 | 8.217 | 8.853 | 40 N | AZL-3 |
| 8.382 | 7.283 | 8.510 | 9.353 | 60 N | |
| 8,840 | 7.793 | 8.980 | 9.747 | 80 N | |
| | 6.756 | 8.111 | 8.970 | S rate | |
| SRN = NS, RN = NS, S = 0.081 | | | | LSD (0.05) | |
| N rate | 8 S | 4 S | 2 S | | |
| 7.554 | 6.388 | 7.842 | 8.432 | 40 N | Salinity × Nitrogen |
| 7.919 | 6.689 | 8.044 | 9.024 | 60 N | |
| 8.363 | 7.190 | 8.447 | 9.453 | 80 N | |
| 0.081 | 0.140 | | | LSD (0.05) | |
| R rate | 8 S | 4 S | 2 S | | |
| 7.292 | 5.770 | 7.503 | 8.602 | R0 | Salinity × Inoculation |
| 8.139 | 7.167 | 8.261 | 8.990 | AZL-2 | |
| 8.406 | 7.330 | 8.569 | 9.318 | AZL-3 | |
| 0.081 | 0.140 | | | LSD (0.05) | |

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