



Original Research Paper

# Plant–Microbe Interactions in Extreme Environments: Functional Adaptation of Bacterial Communities Associated with *Hedysarum perrauderianum* from Algerian Ecosystems

Fatima Zohra. Sebihi<sup>1,2,\*</sup>, Razika. Gharzouli,<sup>1</sup> Mouna. Saoudi<sup>1</sup>, Dounia. Dib<sup>2</sup>, Kanza. Kadi<sup>2</sup>, Katia. Bendjemana<sup>2</sup>

<sup>1</sup> Laboratory of Molecular and Cellular Biology, University Constantine 1, Algeria.

<sup>2</sup> Laboratory of Biotechnology, Water, Environment and Health, Abbes Laghrou University, Khenchela, Algeria

Corresponding author email: [sebihi.Fatima.Zohra@univ-khenchela.dz](mailto:sebihi.Fatima.Zohra@univ-khenchela.dz)

Received: 01/08/2026 ; Accepted: 24/09/2026

## Abstract

Plant-associated microorganisms play a crucial role in ecosystem functioning by enhancing host adaptation to environmental constraints and contributing to soil restoration processes. This study investigates the functional diversity, stress tolerance, and phylogenetic relationships of bacterial communities associated with *Hedysarum perrauderianum*, an endemic Algerian legume growing in environmentally constrained habitats. Ten bacterial isolates recovered from root nodules were characterized using a polyphasic approach integrating morphological, physiological, biochemical, enzymatic, and molecular analyses. The isolates exhibited functional traits associated with plant–microbe interactions, including enzymatic activities involved in root colonization and nitrogen-related metabolism. Phenotypic profiling revealed considerable metabolic variability, particularly in carbohydrate assimilation patterns and amino acid utilization, with distinct nutritional preferences among isolates. All strains demonstrated adaptive capacities to abiotic stresses, including tolerance to elevated salinity (up to 1 M NaCl), acidic conditions (pH 4.5–5.5), and high concentrations of potentially toxic metals, particularly antimony, zinc, copper, and lead (MIC values reaching 1000 µg/ml), whereas sensitivity to mercury and cadmium was observed. Specific interactions with rhizobiophages further revealed ecological differentiation among bacterial isolates. Molecular characterization based on ARDRA and 16S rRNA gene sequencing assigned the isolates to three major phylogenetic groups within Gammaproteobacteria, corresponding to *Enterobacter*, *Pantoea*, and *Pseudomonas*. These findings demonstrate the ecological versatility of *H. perrauderianum*-associated bacteria and highlight their potential contribution to plant resilience in degraded and metal-affected ecosystems. The identified stress-adapted bacterial resources may provide valuable candidates for microbial-based approaches supporting ecosystem rehabilitation and sustainable land management.

**Keywords:** *Hedysarum perrauderianum*; plant-associated bacteria; microbial diversity; environmental stress adaptation; heavy metal tolerance; plant–microbe interactions; ecosystem restoration.

## Introduction:

Algeria represents an important hotspot of Mediterranean plant biodiversity, including numerous wild species adapted to contrasting and stressful environmental conditions. Among these, legumes are particularly valuable due to their ability to establish symbiotic associations with nitrogen-fixing microorganisms, contributing significantly to soil fertility and ecosystem functioning (Sprent, 2021; Laranjo et al., 2023). Wild legumes adapted to Mediterranean and semi-arid environments possess valuable traits related to abiotic stress tolerance, soil

improvement, and reduced dependence on chemical nitrogen inputs, making them promising resources for sustainable agriculture under changing climatic conditions (Liu et al., 2022).

The genus *Hedysarum* represents an important component of Mediterranean legume diversity, comprising annual and perennial species with remarkable ecological and genetic variability (Quezel & Santa, 1962; Abdelguerfi-Berrechia, 1988). In Algeria, *Hedysarum perrauderianum* is of particular interest because of its adaptation to nutrient-poor soils, water limitation, and nitrogen-deficient environments. Beyond

\*Corresponding Author:

Received: 01 st August, 2026 | Revised: 26 th August, 2026 | Accepted: 24 th September, 2026 | Available Online: 28 th September, 2026

(DOI): 10.70102/AEJ.2026.18.2.21

its ecological value, *Hedysarum* species have agronomic importance due to their forage potential, adaptability to marginal habitats, and contribution to soil fertility and ecosystem restoration (Medjebeur et al., 2018; Sebihi et al., 2024).

The ecological success of legumes is largely associated with their capacity to form root nodule symbioses with nitrogen-fixing bacteria. Through complex molecular communication involving flavonoids, nod genes, and nod factors, bacterial partners differentiate into bacteroids capable of biological nitrogen fixation, while receiving carbon resources from the host plant (Andrews & Andrews, 2017; Roy et al., 2021). Recent studies have demonstrated that legumes actively regulate these interactions by selecting bacterial partners according to their symbiotic efficiency, thereby optimizing resource allocation (Porter et al., 2024; Yu & Zhu, 2025).

Although rhizobia are the classical symbiotic partners of legumes, nodulating bacteria associated with *Hedysarum* belong to diverse phylogenetic groups. For example, *Hedysarum coronarium* is mainly associated with *Rhizobium sulae* (Struffi et al., 1998; Squartini et al., 2002), whereas several other *Hedysarum* species, including *H. carnosum*, *H. spinosissimum*, *H. pallidum*, and *H. naudinianum*, harbor nodulating bacteria belonging to Gammaproteobacteria (Benhizia et al., 2004; Torche, 2006). However, the bacterial partners associated with several *Hedysarum* species remain insufficiently characterized.

The establishment and efficiency of legume–bacteria symbioses are strongly influenced by environmental factors, including drought, salinity, temperature, soil pH, and mineral availability, which can affect nodulation, symbiotic gene expression, and nitrogenase activity. Therefore, identifying bacterial strains adapted to Mediterranean environmental constraints represents an important strategy for improving plant resilience and sustainable agricultural systems (Yeremko et al., 2025).

A comprehensive characterization of nodule-associated bacteria requires an integrated approach combining phenotypic, physiological, enzymatic, and molecular analyses. Traits such as carbon and nitrogen source utilization, nutritional requirements, enzyme production, and tolerance to abiotic stresses provide valuable information regarding bacterial adaptation and ecological functionality. Moreover, resistance to heavy metals is an important adaptive trait, as metal contamination can negatively affect bacterial metabolism and biological nitrogen fixation. Similarly, bacteriophage sensitivity patterns provide additional insights into bacterial diversity due to the high specificity of phage–host interactions.

Molecular approaches, including amplified ribosomal DNA restriction analysis (ARDRA) and 16S rRNA gene sequencing, remain essential tools for determining the genetic diversity and taxonomic affiliation of bacterial isolates (Tian et al., 2021; Sinharoy et al., 2024).

Therefore, this study aims to isolate and characterize bacterial communities associated with the root nodules of the Algerian endemic legume *Hedysarum perrauderianum*. A polyphasic approach was applied to evaluate their physiological and ecological characteristics, including nutritional profiles, tolerance to abiotic stresses, heavy metal resistance, bacteriophage interactions, and phylogenetic diversity. This work contributes to a better understanding of the adaptive potential and ecological functions of *H. perrauderianum*-associated bacteria and highlights their possible applications in sustainable agriculture and ecosystem restoration.

## Materials and Methods:

### 1. Isolation of bacteria from *H. perrauderianum* nodules

Isolation was performed following the techniques of Vincent (1970) and Somasegaran & Hoben (1994). Nodules were surface-sterilized with ethanol and mercuric chloride, then plated on Yeast-Mannitol-Agar (YMA) supplemented with Congo red (Vincent, 1970). Ten bacterial isolates were obtained from nodules of the legume *Hedysarum perrauderianum* and used in the present study (H.per.1, H.per.2, H.per.3, H.per.4, H.per.5, H.per.6, H.per.7, H.per.8, H.per.9, H.per.10). The isolates were subjected to morphological characterization (Gram staining, growth on YMA) and subsequently preserved in buffered YMA medium (CaCO<sub>3</sub>).

### 2. Determination of rhizobial characteristics

Previous studies on the genus *Hedysarum* have shown that nodulating bacteria belong to different groups: *H. coronarium* is nodulated by *R. sulae* (Squartini, 2002; Struffi, 1998; Benguedouar, 1997), whereas *H. carnosum*, *H. spinosissimum*, *H. pallidum*, and *H. naudinianum* are nodulated by bacteria belonging to the Gamma-proteobacteria (Benhizia, 2004; Torche, 2006).

Based on this, we investigated the main characteristics typical of rhizobia in comparison with reference strains (Table 1) (Vincent, 1970; Vincent, 1982; Jordan, 1984). These characteristics included growth rate (cultivation on YMA medium supplemented with bromothymol blue), dye absorption (growth on YMA + Congo red at 0.0025%), and calcofluor reaction, which highlights the binding of bacterial polysaccharides (EPS, etc.). Distinctive tests differentiating *Rhizobium* from *Agrobacterium* were also performed, since only the former is capable of infecting legume roots. These tests included litmus milk reaction, Ca-glycerophosphate precipitation, and detection of 3-ketolactose (Vincent,

1970; Vincent, 1982; Jordan, 1984).

**Table 1.** Reference strains used

| Strain | Bacterial species            | Host plant                             | Geographic origin    | Source                    |
|--------|------------------------------|----------------------------------------|----------------------|---------------------------|
| A6     | <i>Rhizobium sullae</i>      | <i>H.coronarium</i>                    | Constantine, Algeria | A.Benguedouar-Constantine |
| HS1    | <i>Pseudomonas</i> sp. NZ096 | <i>H.spinossissimum .ssp.capitatum</i> | Constantine, Algeria | Y.Benhizia- Cne           |
| Hp7    | <i>Enterobacter kobei</i>    | <i>H.pallidum</i>                      | Sétif, Algeria       | Y.Benhizia- Cne           |
| Hca1   | <i>Pseudomonas</i> sp. KD    | <i>H.carnosum</i>                      | Biskra, Algeria      | Y.Benhizia- Cne           |
| HnA    | <i>Pantoea agglomerans</i>   | <i>H.naudinianum</i>                   | Sétif, Algeria       | A.Torche- Cne             |

### 3. Screening for metabolic enzymes (related to root infection)

The presence of polygalacturonase and endoglucanase, enzymes associated with nodulation, as well as nitrate reductase and urease, enzymes involved in soil nitrogen metabolism, was investigated.

For the physiological characterization of the isolates and abiotic factors, we used two isolates, Hper 9 and Hper 10, and compared their behavior with that of the reference strains.

### 4. Nutritional assays

Carbon source utilization was tested on B III medium (Dazzo, 1982), in which mannitol was replaced by one of eleven sugars (0.1%): D-arabinose, D-fructose, D-galactose, D-sucrose, D-glucose, D-xylose, D-sorbitol, sodium citrate, lactose, maltose, and raffinose. Nitrogen source assimilation was tested on defined medium 8

(Lindström & Lehtomäski, 1988), where glutamate was replaced by one of seventeen amino acids: valine, tyrosine, leucine, proline, threonine, isoleucine, lysine, glycine, serine, histidine, arginine, phenylalanine, alanine, cysteine, methionine, asparagine, and tryptophan. Vitamin requirements were assessed by growing isolates on B III medium supplemented with 0.1% biotin and/or thiamine.

### 5. Abiotic stress factors (NaCl and pH)

Tolerance to NaCl was tested on **Gloux and Le Rudulier medium (1989)** at different concentrations, while pH tolerance was evaluated on B III medium (Dazzo, 1982).

### 6. Intrinsic resistance to heavy metals

Soil contamination by heavy metals is widespread, originating from industrial activities, power plants, pesticide and fertilizer production, and sewage sludge. Heavy metals such as cadmium (Sofia Cacoilo et al., 2005), lead, mercury, zinc, and copper (Peter M. Kopittke et al., 2005) are known to affect microbial growth, physiology, nitrogen fixation, and legume nodulation. Resistance of isolates, particularly rhizobia, was assessed by determining the minimum inhibitory concentration (MIC). Isolates and reference strains were cultured on TY medium (Behringer, 1974) containing increasing concentrations (20–1500 µg/ml) of heavy metals: CuCl<sub>2</sub>·2H<sub>2</sub>O, HgCl<sub>2</sub>, CdCl<sub>2</sub>·2H<sub>2</sub>O, ZnCl<sub>2</sub>, Pb (CH<sub>3</sub>COO)<sub>2</sub>, and SbO<sub>3</sub>.

### 7. Phage sensitivity assays

Given the high specificity of phage–host interactions, phage sensitivity was used as an ecological tool to differentiate rhizobial populations. Isolates were exposed to a range of rhizobiophages, and lysis profiles were recorded. Phages were isolated from soils where *Hedysarum* plants were cultivated, and bacterial lysis was performed following Vincent (1970).

### 8. Phylogenetic characterization of isolates

Phenotypic analysis was complemented by molecular characterization using:

-ARDRA (Amplified Ribosomal DNA Restriction Analysis),

-16S rRNA sequencing by the Sanger method.

### 9. Statistical analysis

Statistical analyses were performed using R software (version 4.5.0; R Core Team, 2025), developed by the R

Foundation for Statistical Computing (Vienna, Austria), for nutritional and abiotic tests.

## Results

### 1. Morphological and cultural characteristics of isolates

Microscopic observations of the isolates revealed rod-shaped cells that were Gram-negative. Colonies were detectable on YMA after 24 h and were white in color, translucent, featured regular colony margins, and contained abundant PHB. The isolates exhibited rapid growth on YMA supplemented with bromothymol blue. The isolates displayed weak absorption of Congo red and positive reactions to calcofluor (**Fig. 1**).

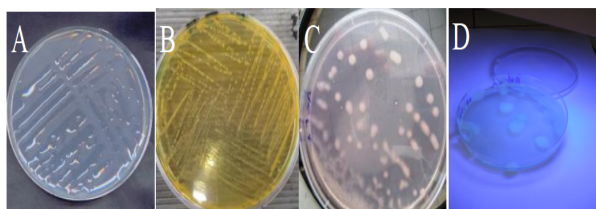


**Figure 1. Morphological aspects of the isolates.** A: Colony morphology on YMA; B: Growth on YMA + BTP; C: Congo red absorption (0.0025%); D: Calcofluor reaction.

The tested isolates, compared with the reference strain A6, exhibited characteristics typical of the genus *Rhizobium*.

The ability of strains to reduce nitrates and nitrites was tested (**Fig. 2**).

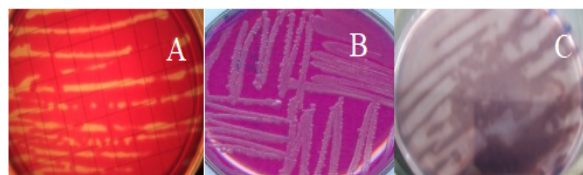


**Figure 2.** Capacity of isolates to produce specific enzymes: A, Polygalacturonase; B, Urease; and C, Endoglucanase.

### 2. Physiological characteristics of isolates

#### 2.1 Utilization of carbon sources

Nutritional tests were performed on BIII medium (Dazzo, 1982), along with the mannitol control. The results indicated that strains were able to grow in the presence of glucose, fructose, sorbitol, and maltose. However, growth was poor in the presence of xylose (**Tab. 2**).

**Table 2:** Phenotypic characteristics of isolates and reference strains

| Characteristic<br>s | Isolates |         | Reference strains |         |         |         |        |
|---------------------|----------|---------|-------------------|---------|---------|---------|--------|
|                     | H<br>pe  | Hp<br>e | A<br>6            | H<br>sl | H<br>ca | H<br>na | H<br>P |

|                     |                | 9              | 10            |               |                   |                   |                   | 7                 |
|---------------------|----------------|----------------|---------------|---------------|-------------------|-------------------|-------------------|-------------------|
| Car<br>sou<br>(O.D) | Sacch<br>arose | 0,<br>31<br>7  | 0,2<br>66     | 0,<br>25<br>5 | 0,<br>1<br>7<br>1 | 0,<br>2<br>1<br>3 | 0,<br>1<br>9<br>1 | 0,<br>2<br>3<br>2 |
|                     | Rafino<br>se   | 0,<br>28<br>5  | 0,1<br>87     | 0,<br>17<br>2 | 0,<br>2<br>1      | 0,<br>3<br>9<br>3 | 0,<br>1<br>9<br>8 | 0,<br>2<br>3<br>1 |
|                     | Sorbit<br>ol   | 0,<br>47       | 0,3<br>43     | 0,<br>31<br>8 | 0,<br>3<br>8<br>5 | 0,<br>5<br>2<br>6 | 0,<br>3<br>1<br>3 | 0,<br>2<br>8<br>2 |
|                     | Arabi<br>nose  | 0,<br>60<br>2  | 0,3<br>44     | 0,<br>29<br>2 | 0,<br>4<br>6<br>5 | 0,<br>5<br>1<br>4 | 0,<br>2<br>7      | 0,<br>3<br>0<br>8 |
|                     | Lactos<br>e    | 0,<br>28<br>8  | 0,0<br>91     | 0,<br>28<br>5 | 0,<br>3<br>4<br>1 | 0,<br>6<br>7      | 0,<br>6<br>9      | 0,<br>3<br>8<br>2 |
|                     | Xylose         | 0,<br>86<br>9  | 0,7           | 0,<br>66<br>5 | 0,<br>3<br>2<br>6 | 0,<br>11<br>7     | 0,<br>1<br>3<br>3 | 0,<br>5<br>5<br>4 |
|                     | Glucos<br>e    | 0,<br>84<br>1  | 0,7           | 0,<br>51<br>8 | 0,<br>5<br>2<br>2 | 0,<br>7<br>1<br>9 | 0,<br>4<br>3<br>7 | 0,<br>7<br>0<br>9 |
|                     | Galact<br>ose  | 0,<br>48<br>6  | 0,5<br>21     | 0,<br>58<br>1 | 0,<br>4<br>4<br>4 | 0,<br>5<br>1<br>5 | 0,<br>4<br>7<br>4 | 0,<br>3<br>2<br>3 |
|                     | Maltos<br>e    | 0,<br>58<br>6  | 0,3<br>68     | 0,<br>36<br>8 | 0,<br>4<br>5<br>5 | 0,<br>5<br>3      | 0,<br>9<br>5<br>9 | 0,<br>3<br>8<br>7 |
|                     | Manit<br>ol    | 0,<br>53<br>6  | 0,5<br>07     | 0,<br>53<br>8 | 0,<br>3<br>7<br>4 | 0,<br>7<br>1<br>8 | 0,<br>2<br>6<br>6 | 0,<br>3<br>4<br>2 |
|                     | Citrat<br>e Na | 0,<br>69<br>7  | 0,4<br>49     | 0,<br>53<br>9 | 0,<br>4<br>8<br>2 | 0,<br>1<br>7<br>2 | 0,<br>2<br>6<br>6 | 0,<br>3<br>7<br>4 |
|                     | Fructo<br>se   | 1,<br>25<br>85 | 0,6<br>69     | 0,<br>62<br>9 | 0,<br>4<br>6      | 0,<br>5<br>3      | 0,<br>5<br>8      | 0,<br>3<br>7<br>7 |
| Vit<br>ami<br>ns    | Biotin         | 0,<br>64<br>4  | 0,4<br>5<br>4 | 0,<br>61<br>9 | 0,<br>6<br>2<br>7 | 0,<br>4<br>8<br>3 | 0,<br>7<br>8<br>1 | 0,<br>6<br>2<br>7 |
|                     | Thiam<br>in    | 0,<br>97<br>9  | 0,4<br>8<br>6 | 0,<br>68<br>3 | 0,<br>7<br>4<br>3 | 0,<br>6<br>1<br>3 | 0,<br>8<br>8<br>1 | 0,<br>7<br>4<br>3 |

|                        |                                |       |       |       |       |       |       |       |
|------------------------|--------------------------------|-------|-------|-------|-------|-------|-------|-------|
| Amino acids (O.D λ600) | Biotin +Thiamin                | 0,641 | 0,492 | 0,651 | 0,937 | 0,504 | 0,597 | 0,937 |
|                        | BIII medium                    | 0,475 | 0,509 | 0,39  | 0,54  | 0,517 | 0,526 | 0,544 |
|                        | “BIII medium without vitamins” | 0,311 | 0,25  | 0,505 | 0,662 | 0,811 | 0,306 | 0,622 |
|                        | Isoleucine                     | 0,615 | 0,797 | 0,547 | 0,790 | 0,670 | 0,660 | 0,660 |
|                        | Serine                         | 0,222 | 0,249 | 0,248 | 0,297 | 0,311 | 0,323 | 0,293 |
|                        | Proline                        | 0,437 | 0,669 | 0,374 | 0,764 | 0,633 | 0,732 | 0,544 |
|                        | Méthionine                     | 0,246 | 0,247 | 0,177 | 0,221 | 0,256 | 0,235 | 0,229 |
|                        | Tyrosine                       | 0,189 | 0,778 | 0,206 | 0,721 | 0,409 | 0,677 | 0,471 |
|                        | Arginine                       | 0,723 | 0,835 | 0,305 | 0,479 | 0,613 | 0,499 | 0,413 |
|                        | phenylalanine                  | 0,305 | 0,382 | 0,202 | 0,419 | 0,358 | 0,310 | 0,322 |
|                        | Glutamine                      | 0,102 | 0,559 | 0,263 | 0,580 | 0,604 | 0,697 | 0,476 |
|                        | Alanine                        | 0,968 | 0,956 | 0,234 | 0,588 | 0,580 | 0,611 | 0,513 |
|                        | Valine                         | 0,324 | 0,800 | 0,133 | 0,343 | 0,543 | 0,546 | 0,361 |
|                        | Histidine                      | 0,724 | 0,598 | 0,576 | 1,117 | 1,119 | 0,969 | 1,009 |

|           |                  |       |       |       |       |       |       |       |
|-----------|------------------|-------|-------|-------|-------|-------|-------|-------|
| NaCl (mM) | tryptophane      | 0,306 | 0,275 | 0,537 | 0,471 | 0,326 | 0,312 | 0,902 |
|           | Lysine           | 0,667 | 0,141 | 0,275 | 0,209 | 0,103 | 0,148 | 0,115 |
|           | Leucine          | 1,409 | 0,195 | 0,326 | 0,511 | 0,225 | 0,227 | 0,722 |
|           | cystéine         | 1,910 | 0,506 | 0,442 | 1,498 | 0,413 | 0,592 | 0,622 |
|           | Thréonine        | -     | 0,307 | 0,125 | 0,183 | 0,204 | 0,216 | -     |
|           | Asparagine       | 0,855 | 0,452 | 0,674 | 0,771 | 0,620 | 0,642 | 0,436 |
|           | Sans acide amine | 0,179 | 0,544 | 0,328 | 0,270 | 0,264 | 0,206 | 0,167 |
|           | Glutamate Na     | 1,860 | 1,927 | 0,616 | 0,839 | 0,632 | 0,693 | 0,333 |
|           | Glycine          | 0,858 | 0,140 | 0,661 | 0,451 | 0,159 | 0,222 | 0,200 |
|           | 0                | 0,208 | 0,231 | 0,284 | 0,212 | 0,247 | 0,255 | 0,209 |
|           | 20               | 0,223 | 0,21  | 0,217 | 0,218 | 0,244 | 0,212 | 0,226 |
|           | 40               | 0,257 | 0,239 | 0,244 | 0,244 | 0,273 | 0,211 | 0,223 |
|           | 100              | 0,137 | 0,269 | 0,185 | 0,244 | 0,238 | 0,231 | 0,217 |
|           | 200              | 0,127 | 0,254 | 0,169 | 0,291 | 0,218 | 0,222 | 0,248 |
|           | 400              | 0,181 | 0,174 | 0,123 | 0,299 | 0,210 | 0,213 | 0,217 |

|    |        |       |       |       |       |       |       |       |
|----|--------|-------|-------|-------|-------|-------|-------|-------|
|    |        |       |       |       | 2     | 5     |       | 2     |
|    |        | 0,026 | 0,057 | 0,023 | 0,064 | 0,044 | 0,028 | 0,023 |
|    | 1000   |       |       |       |       |       |       |       |
|    |        | 0,017 | 0,001 | 0,003 | 0,002 | 0,008 | 0,001 | 0,005 |
|    | 1500   |       |       |       |       |       |       |       |
|    | 2000   | 0     | 0     | 0     | 0     | 0     | 0     | 0     |
| pH | pH=5,3 | 0,443 | 0,647 | 0,493 | 0,639 | 0,526 | 0,033 | 0,454 |
|    | pH=5,6 | 0,388 | 0,89  | 0,211 | 0,544 | 0,677 | 0,227 | 0,337 |
|    | pH=5,8 | 0,215 | 0,268 | 0,287 | 0,433 | 0,588 | 0,687 | 0,937 |
|    | pH=6,8 | 0,34  | 0,347 | 0,346 | 0,499 | 0,447 | 0,379 | 0,899 |
|    | pH=7,8 | 0,329 | 0,592 | 0,543 | 0,511 | 0,633 | 0,911 | 0,764 |
|    | pH=8,8 | 0,294 | 0,357 | 0,269 | 0,311 | 0,288 | 0,644 | 0,933 |
|    | pH=9,8 | 0,504 | 0,41  | 0,926 | 0,399 | 0,399 | 0,399 | 0,577 |

A principal component analysis of the isolates (Hpe9 and Hpe10) together with reference strains (Hna, Hca, Hp7, Hs1, and A6) revealed that the first two dimensions accounted for 77.1% of the total variance represented by the samples (Dim 1 accounted for 52.3% of the variance and Dim 2 accounted for 24.8%). The first dimension mainly represented the isolates (Hpe9 and Hpe10) together with the reference strains A6 and Hp7, indicating the similarity of these strains. The second dimension contained the majority of the reference strains Hna and Hca, which exhibited a different pattern of metabolic activity than the isolates studied (Fig. 3). The isolates that were studied were able to utilize a wide range of carbohydrates as carbon sources.

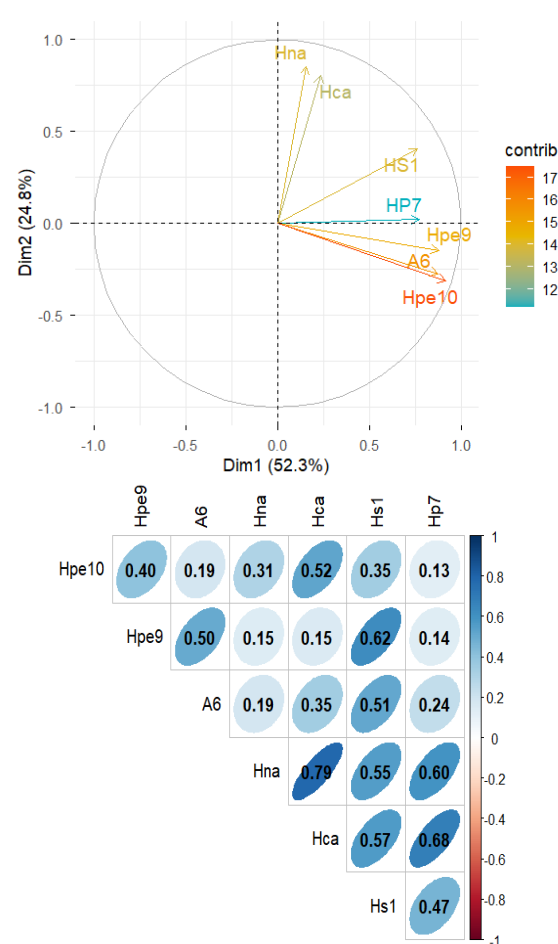


**Figure 3.** Principal Component Analysis (PCA) and correlation matrix of isolates based on sugar utilization. The correlation circle (left) represents the projection of the variables Hpe10, Hpe9, A6, Hna, Hca, Hs1 and Hp7 on the first two dimensions (Dim1, Dim2) of the PCA, indicating the contribution of each variable to those dimensions with colors. The graph on the right represents the Pearson correlation coefficients between each of these variables, represented with ellipses of various shades and shapes.

## 2.2 Utilization of nitrogen sources

On defined medium 8, growth depended on the source of nitrogen. All strains exhibited good growth on all amino acids, with a preference for histidine, sodium glutamate, asparagine, proline and isoleucine (Tab. 2). Growth was slightly reduced on threonine, methionine, serine, lysine and glycine.

The PCA of the responses to the different nitrogen sources in the media explained 70% of the total variance (Dim1: 45.6%; Dim2: 24.8%). The clustering of the reference strains (Hs1, Hp7, Hca, A6) indicates a homogeneous utilization of standard amino acids such as glutamine and asparagine. In contrast, isolates Hpe9 and Hpe10 are more separated along Dim1, indicating a particular affinity for certain amino acids (proline, histidine) (Fig. 4).

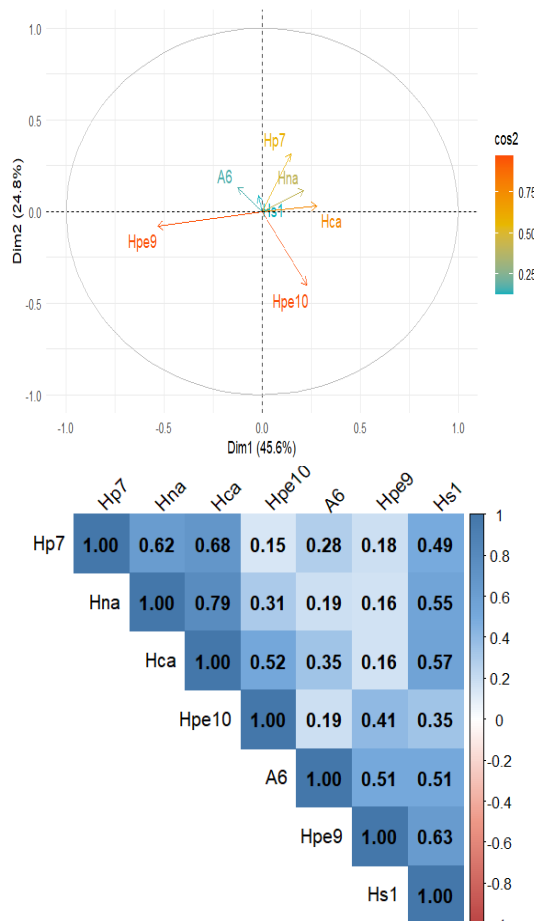


**Figure 4.** Principal Component Analysis (PCA) and correlation matrix of isolates based on amino acid utilization. The correlation circle on the left represents the projection of the seven variables on the first two dimensions of the correlation circle, with the color scale indicating the contribution of each variable. The graph on the right represents the correlation coefficients of the variables on the left, with the ellipse indicating the correlation between each of the variables.

### 2.3 Vitamin requirements

Growth on BIII medium (Dazzo, 1982) supplemented with thiamine and/or biotin led to good development of the isolates (Tab. 2). Growth occurred with thiamine alone.

The PCA on the effect of vitamins indicated that the first two axes accounted for 85.8% of the variance (Dim1: 68.8%; Dim2: 17%). Dim1 grouped isolates Hpe9, Hpe10 and the majority of the reference strains (Hca, Hna, Hp7, A6), while the majority of the strains lay along Dim2 with the Hs1 strain (Fig. 5).

The correlation matrix indicated that most isolates and strains correlated with one another, with correlations greater than 0.6, and that the majority of correlations within the highest percentage of variance (Dim1) correlated with one another with correlation strengths of  $r > 0.7$  (Fig. 5).

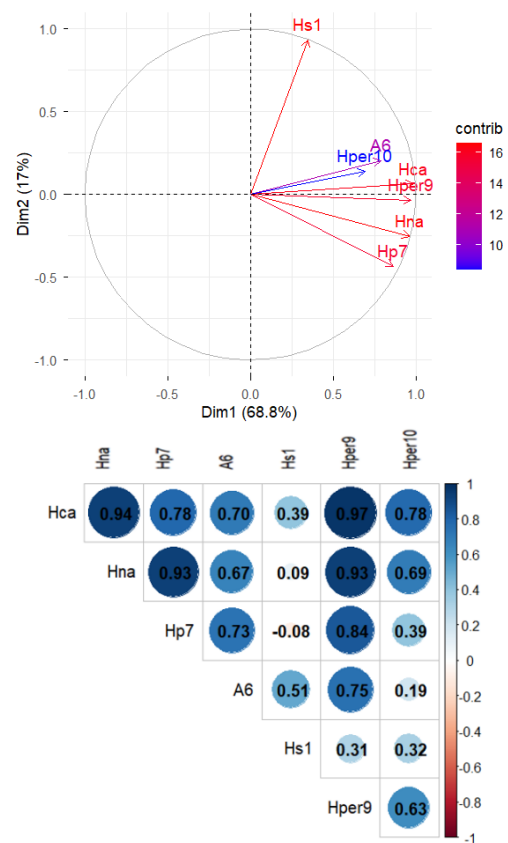


**Figure 5.** Principal Component Analysis (PCA) and correlation matrix of isolates based on vitamin utilization. The correlation circle (left) represents the projection of the variables on the first two dimensions of the HCA analysis (Dim1 and Dim2), indicating the contribution of each variable to the construction of the graph on the right. The graph on the right indicates the Pearson correlation coefficients among the variables represented; the darker the blue, the stronger the positive correlation between the strains according to the amino acids they utilized for growth.

### 3. Effect of abiotic factors

#### 3.1 Effect of NaCl

All strains grew in the presence of NaCl from 0.04 M to 1 M, demonstrating the halotolerant character of the strains. However, no growth was observed at 1.5 M NaCl, with the exception of strains isolated from *H. coronarium*, *H. pallidum*, and *H. carnosum*. The optimal pH for growth for the strains was around 7.0 in liquid medium (BIII), although some strains were able to grow at pH 5.3 (Tab. 2).

The first two axes of the PCA explained 95.9% of the total variance of the strains (Dim1: 90.7%; Dim2: 5.3%). The first dimension grouped most of the strains along a gradient of NaCl tolerance, while the second dimension, which explained a smaller portion of the variance, grouped certain strains apart from the others, most notably Hpe10 (Fig. 6).

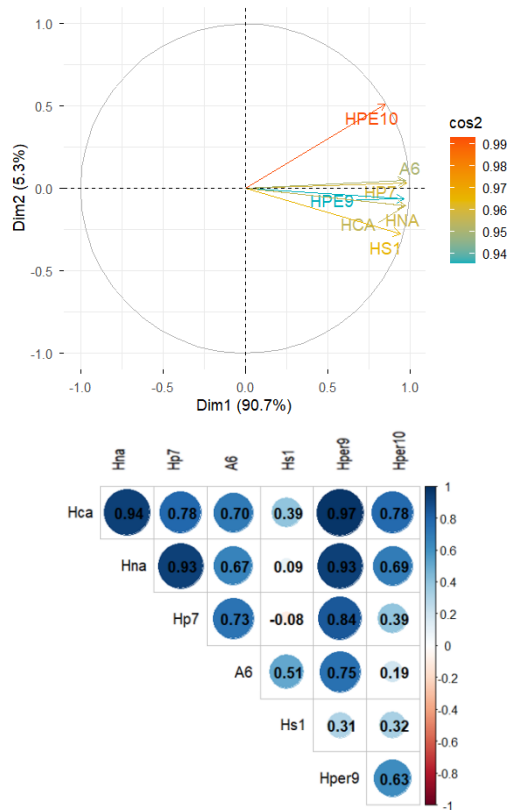


**Figure 6.** Principal Component Analysis (PCA) and correlation matrix of isolates as a function of NaCl concentration. The correlation circle (left) represents the projection of the variables Hca, Hna, Hp7, A6, Hs1, Hpe9, and Hpe10 onto the first two dimensions (Dim1 and Dim2) of the PCA, with the color scale indicating the contribution of each variable. The graph on the right depicts the Pearson correlation coefficients between these same variables, where correlations close to 1 are represented by dark blue ellipses and correlations close to 0 are represented by light blue ellipses.

### 3.2 Effect of pH

Strains grown in liquid BIII medium exhibited optimal growth around pH 7.0. However, growth was also observed at pH 5.3, indicating the ability of strains to maintain internal cellular pH (Tab. 2).

The first two axes of PCA encompassed 77% of the variance of the data set (Dim1: 52%; Dim2: 25%). Isolates Hpe7 and Hpe10 were represented on the right side of the graphic along Dim1, indicating their ability to tolerate acidic pH values between 4.5 and 5.5 ( $\cos^2$ : 0.91-0.95). Isolates Hca, Hna, Hp7, and Hs1 were represented along the left side of the graphic along Dim1, indicating their preference for neutral pH between 6.5 and 7.5 ( $\cos^2$ : 0.6-0.8). Isolate Hs1 was represented with the lowest contribution along Dim1, indicating its susceptibility to acidic pH levels (Fig. 7).

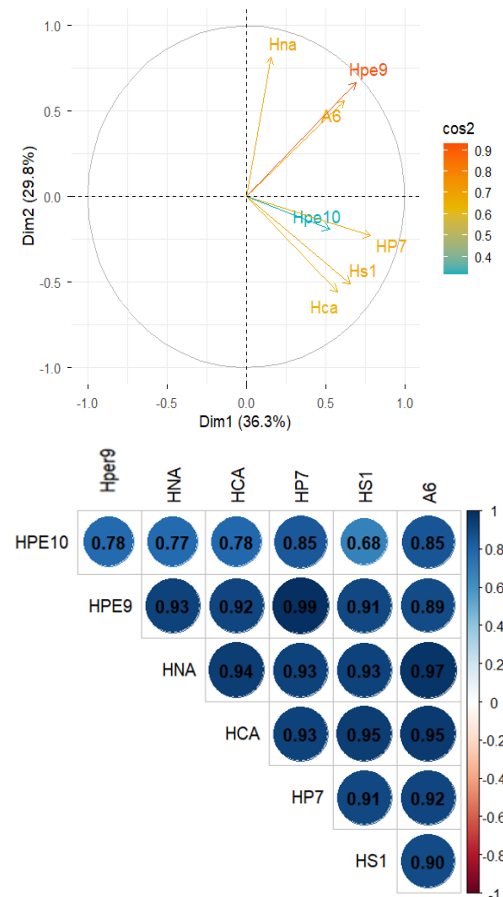


**Figure 7.** Principal Component Analysis (PCA) and correlation matrix of isolates as a function of pH. The correlation circle (left) illustrates the projection of the variables Hca, Hna, Hp7, A6, Hs1, Hpe9, and Hpe10 onto the first two dimensions (Dim1 and Dim2) of the PCA, indicating the contribution of each variable to the model via its color. The graph on the right plots the Pearson correlation coefficient for each of these variables, indicating the strength of the relationship between the growth profiles of these bacterial strains under the various pH conditions (represented by the dark and light blue ellipses, respectively).

### 4. Intrinsic resistance to heavy metals

The presence of heavy metals revealed the presence of MICs from various bacterial strains (both isolates and reference strains). Some strains were found to be tolerant to antimony, zinc ( $\text{ZnCl}_2$ ), copper ( $\text{CuCl}_2$ ), and lead [ $\text{Pb}(\text{CH}_3\text{COO})_2$ ], with MIC values reaching up to 1000  $\mu\text{g/ml}$ . In contrast, the isolates revealed sensitivity to mercury ( $\text{HgCl}_2$ ) and cadmium, with MIC values reaching only 200  $\mu\text{g/ml}$ .

### 5. Phage sensitivity characterization

Using soil extracts obtained from the sampling site and nodules, the tested isolates exhibited lysis zones. These lysis zones, appearing as large clear circles, correspond to areas where bacteria were lysed (Fig. 8).

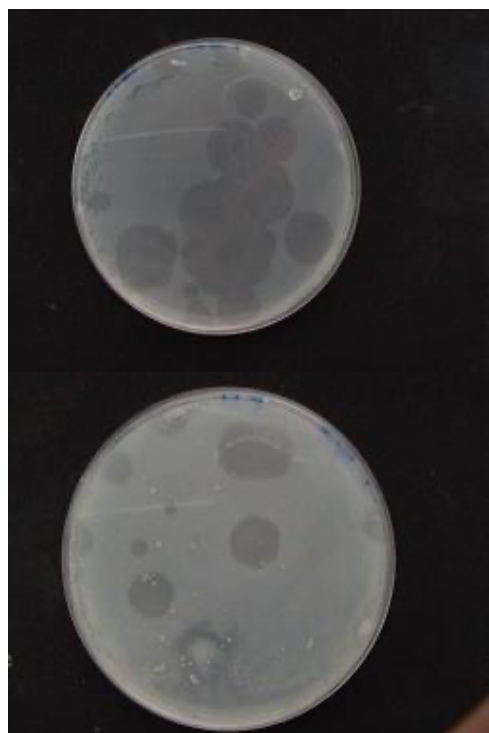


**Figure 8.** Lysis zones produced by soil bacteriophages.

#### 6. Strain characterization by molecular analysis

The amplified rDNA profiles obtained after enzymatic digestion and agarose gel electrophoresis revealed three distinct clusters with different patterns (**Fig. 9**):

- Group 1: strains Hpe1, Hpe2, Hpe5, and Hpe10 shared the same profile.
- Group 2: strains Hpe3 and Hpe8.
- Group 3: strain Hpe9.

The restriction profiles obtained by RFLP (ARDRA) and sequencing of the isolates, analyzed through the NCBI Sequence database, classified the isolates as Gamma-proteobacteria. Genotypic techniques (PCR-RFLP and 16S rRNA sequencing) determined the genus (*Enterobacter* sp.) and the species (*Pantoea agglomerans*).

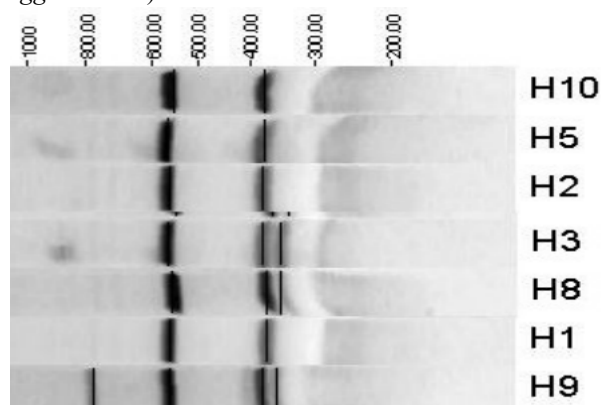


**Figure 9:** ARDRA (Amplified Ribosomal DNA Restriction Analysis) profiles of the isolates.

#### Discussion

These morphological traits are consistent with rhizobial characteristics (**Vincent, 1970; Jordan, 1984; Ge Hong Wei et al., 2006**). The tested isolates exhibited characteristics typical of the genus *Rhizobium* as described by **Vincent (1982)**, who is well known for defining the morphological, cultural, and biochemical traits of *Rhizobium*, notably their ability to form nodules on legume roots and their nitrogenase enzyme, which fixes atmospheric nitrogen. These bacteria are also characterized by enzymatic traits such as the presence of polygalacturonase (involved in root infection) and enzymes of nitrogen metabolism (urease, nitrate reductase).

The ability of strains to reduce nitrates and nitrites is an important taxonomic criterion for differentiating *Rhizobium* from *Agrobacterium*. Nitrates are reported to be the preferred nitrogen source for most microorganisms, including their plant hosts, in agreement with the studies of **El-Hilali (2006)** and **Joffin et al. (2006)**. This metabolic capacity represents a key aspect in the taxonomy and physiology of *Rhizobium*.

The ability of *Rhizobium leguminosarum* to enzymatically break down the cellulose and pectin components of the plant's cell wall is thought to play a pivotal role in allowing the bacteria to penetrate the legume's root. According to **Christine F. et al. (1998)**, *R. leguminosarum* is capable of secreting two different enzymes known as PlyA and PlyB, which can hydrolyze the polysaccharide carboxymethylcellulose (CMC). However, the authors were able to observe only the degradation of CMC occurring within the bacterial colonies. Other studies, however, such as those by **Mateos et al. (1992)**, indicate that *R. leguminosarum* is also able to secrete a cellulase enzyme that can break the glycosidic bonds of the cell wall. The enzymatic activity of this cellulase is thought to allow the bacteria to penetrate the plant's root.

The cellulolytic and pectinolytic enzymes that are produced by *Rhizobium* play a crucial role during the early stages of the infection of the root of legume plants. These enzymes play a role in the hydrolysis of the wall polysaccharides (cellulose and pectins), which enables the bacteria to cross the cell wall microfibrils and initiate the nodulation process (**Roberts & Keen, 1992**). Additionally, observations of these hydrolase enzymes indicate that they remain anchored to the bacterial cell wall, limiting their diffusion within the environment. As a result, the cellulase and pectinase enzymes limit the degradation of non-specific substrates (carboxymethylcellulose, exopolysaccharides), indicating that the enzymes have a specific enzymatic activity that facilitates the crossing of the plant cell wall while preserving its structural integrity.

The ability of the isolates to utilize various types of carbohydrates as a carbon source was present, as indicated by **Somasegaran and Hoben (1994)**, **Struffi et al. (1998)**, and **Dekak et al. (2020)**. The ability of the bacteria to utilize the various nitrogen sources was found to be in agreement with the findings of **Lindström and Lehtomäki (1988)**. The stimulatory effect of the vitamins upon the growth of the bacteria was in agreement with the findings of **Vincent (1970)**, **Dazzo (1982)**, and **Jordan (1984)**, and the ability of thiamine alone to enable the bacteria to exhibit robust growth is in agreement with **Struffi et al. (1998)**. Furthermore, the strong correlations between the isolates indicate the similarity in the metabolic capabilities of those bacteria to utilize those vitamins, which is one of the main performance criteria of rhizobia in general (**Vincent, 1970; Dazzo, 1982; Jordan, 1984; Ben Salem & Ben Abdelaziz, 2021; Deghim & Dahoum, 2024**).

Regarding the effect of NaCl, the results showed that the level of tolerance to high salt concentrations varied between isolates, which is in agreement with previous studies on the topic (**Gloux & Le Rudulier, 1989; Csonka & Hanson, 1991; Graham et al., 1991; Fujihara, 1993**). The adaptation of rhizobia to saline conditions requires the accumulation of osmo-protectants within the cell. In contrast, adaptation to acidity is related to the ability of cells to maintain a stable intracellular pH despite exposure to high external acidity, along with membrane adaptations to maintain integrity under acidic conditions. Generally, rhizobia are sensitive to both salt and acidic conditions. However, some isolates exhibit higher levels of adaptation to these stressors (**Vargas & Graham, 1998; Hungria & Vargas, 2000**). The PCA results indicate that all strains of bacteria studied have a similar response to salinity. The predominance of strain Dim1 accounts for 90.7% of all strains, demonstrating a principal gradient in the tolerance of these strains to NaCl. The remaining contribution of Dim2 indicates the different responses to salt stress of specific strains, most notably strain Hpe10. However, overall, all strains exhibit similar levels of tolerance to NaCl, with some utilizing different mechanisms to ensure their adaptation to high levels of salt in their environment.

Concerning the pH of the environment, the majority of bacteria can tolerate conditions close to neutrality, but a few strains are adapted to acidity. The pH optimum for the growth of these strains is around 7.0, the pH characteristic of the native soil from which these strains were isolated. The growth of these bacteria at pH 5.3 indicates the presence of specific physiological mechanisms that allow them to grow in acidic environments. These results are in agreement with **Fujihara (1993)** and **Graham et al. (1994)**, who also noted that specific physiological adaptations allow these bacteria to grow at acidic pH values. Additionally, these results are in agreement with **Vargas and Graham**

**(1998)** and **Hungria and Vargas (2000)**, who have noted that the microsymbiont is the most sensitive to pH, but the rhizobial strains have some degree of adaptation to acidic soils. The position of strains Hpe7 and Hpe10 relative to the other strains indicates the expression of specific mechanisms that allow adaptation to acidic soils (proton pumps, exopolysaccharides) that are either not expressed or less effective in the reference strains. The first dimension (Dim1) indicates a clear opposing view of acid tolerance (Hpe7 and Hpe10) versus the neutrophilic behavior (reference strains) of these strains, as indicated by the angles between the pH vectors and the positions of the strains. Additionally, the second dimension (Dim2) indicates some degree of alkaline tolerance in strains Hca and Hna; however, only to a moderate extent. These findings establish Hpe7 and Hpe10 as promising candidates for inoculation into acidified soils, especially those with high concentrations of toxic aluminum ions and in arid regions. Thus, these strains' acid tolerance gives them a competitive advantage over the reference strains, making them promising for agricultural use.

Regarding their heavy metal resistance, these results are consistent with those reported by **Bourameni et al. (2024)**, who found that rhizospheric bacteria demonstrated variable levels of tolerance to heavy metals, with increased sensitivity to cadmium and lead ions. However, other studies have found that the tolerance of rhizobia to heavy metals depends upon both the species of bacteria and the metal species itself. For instance, **Balakrishnan et al. (2017)** found that even rhizobial bacteria of the same genus exhibited variable levels of resistance to heavy metals. Cadmium and mercury are the most toxic metals for these rhizobia species, while zinc, copper, and lead are less toxic and can be present in higher concentrations without adversely affecting rhizobial growth. Thus, these high MICs for these metals suggest some level of adaptation or resistance within the rhizobia strains, which opens perspectives for their use in the bioremediation of soils contaminated with these metals. Rhizobia and other microorganisms are generally more sensitive to heavy metals in polluted soils than animals or plants. Their essential processes are adversely impacted by heavy metals, such as nitrogen fixation and legume nodulation. Various studies into the sensitivity of heavy metals, such as those conducted by **Biro et al. (1995)** on *Rhizobium leguminosarum* bv. viciae, demonstrate the importance of utilizing rhizobial isolates adapted to the soils in which they are to be utilized. The various mechanisms of resistance that rhizobia may exhibit against heavy metals include the mobilization or transformation of those heavy metals in soils (**Gremion, 2003**). The sensitivity of rhizobia to heavy metals and the varying resistance rates among different isolates of rhizobia to these metals present an opportunity for further study into the isolation of rhizobial strains that can be utilized in polluted soils to enhance phytoremediation processes.

In discussing the sensitivity of these bacteria to bacteriophages, Kleczkowska was the first to establish the presence of bacteriophages within soil samples, as well as in relation to the specific interactions between those bacteriophages and their bacterial hosts. Somasegaran and Hoben utilized phage typing to identify strains of the soil bacterium *Bradyrhizobium*, while Struffi et al. discovered strains of bacteria that were resistant to those identified phage viruses. These bacteria have played a crucial role in the diversity within bacterial strains through both lytic cycles (in which the bacteriophage leads to the destruction of the bacterial host) and lysogenic cycles (in which the bacteriophage integrates with the bacterial genome), leading to the development of strains with selective pressures that enable both the bacteria and bacteriophages to coexist and create new strains of those bacteria (Hatfull, 2015). Due to the specific interactions between rhizobia and their bacteriophage counterparts, these rhizobiophages have acted as tools in ecological studies of those rhizobia bacteria (Somasegaran & Hoben, 1994). Our results are consistent with those of Saoudi (2008) on legume-nodulating bacteria of the genus *A. armatus*. Furthermore, the work of De Lajudie et al. (1998) on *R. lupini*, *R. trifolii*, and *R. leguminosarum* demonstrated that bacteriophages have been isolated from most rhizobial species. The primary role of rhizobiophages is to regulate rhizobial populations in different soil types, and these bacteriophages can also be used to identify and differentiate bacterial strains (Mehdipour-Moghaddam et al., 2008).

Finally, regarding the molecular characterization of strains, the classification of the isolates as Gammaproteobacteria is consistent with previous findings on bacteria isolated from nodules of *H. naudinianum* (*Pantoea agglomerans*), *H. spinosissimum* ssp. *capitatum* (*Pseudomonas* sp. NZ096), *H. carnosum* (*Pseudomonas* sp. KD), and *H. pallidum* (*Enterobacter kobei*) (Benhizia et al., 2004; Torche, 2006). According to the polyphasic taxonomy approach (Vandamme, 1996; Zakhia & de Lajudie, 2006; Balachandar et al., 2007), only ARDRA and 16S rRNA sequencing provide discriminatory resolution for identifying the isolates. At the same time, phenotypic methods suggested a behavior similar to the genus *Rhizobium*; genotypic techniques, PCR-RFLP and 16S rRNA sequencing (Weisburg et al., 1991), clearly determined the genus (*Enterobacter* sp.) and the species (*Pantoea agglomerans*).

## Conclusion

The integrated morphological, physiological, and molecular characterization of bacterial isolates associated with the root nodules of *Hedysarum perrauderianum* revealed a high degree of functional diversity and environmental adaptability. The isolates exhibited broad metabolic capacities and remarkable tolerance to abiotic constraints, including salinity, acidic

conditions, and multiple heavy metals, highlighting their potential role in supporting plant survival under stressful Mediterranean environments. Although phenotypic characteristics suggested affiliation with classical rhizobial groups, ARDRA profiling and 16S rRNA gene sequencing revealed that the isolates were mainly related to Gammaproteobacteria, including *Enterobacter* and *Pantoea agglomerans*, emphasizing the complexity of bacterial partnerships associated with wild legumes. These findings demonstrate the importance of combining polyphasic approaches for accurately assessing the diversity and ecological functions of plant-associated bacteria. The stress-adapted isolates identified in this study represent valuable microbial resources for future applications in sustainable agriculture, soil rehabilitation, and ecosystem restoration; however, their effectiveness as bioinoculants requires further validation through plant-based inoculation experiments under controlled and field conditions.

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