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# Biodiversity of symbiotic microbes in association with *Sulla aculeata* spp. from semi-arid regions of Morocco

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## Abstract

Twenty-six root nodule bacteria from two native forage legumes namely *Sulla aculeolata* subsp. *aculeolata* and *Sulla aculeolata* subsp. *mauritanica* were isolated and analyzed using a polyphasic approach comprising phenotypic traits, ERIC-PCR, and 16S rRNA gene sequencing. This is the first time a study has been performed to determine the diversity of bacteria associated with *Sulla aculeolata* spp. Phenotypically, all the isolates were identified as fast-growing bacteria and shows high tolerance toward various stressed conditions, particularly those derived from *S. aculeolata* subsp. *mauritanica*. On the other hand, the genotypic characterization revealed high diversity among the isolated bacteria and clustered into 14 clusters at the similarity index of 90% based on ERIC-PCR analysis. Furthermore, the 16S rRNA gene sequencing of representatives strains indicates that all the strains share 99 to 100% identity with bacteria belonging to *Pseudomonas*, *Enterobacter*, *Serratia,* and *Paenibacillus* genera with a clear relation to their host plant. In conclusion, the findings of the present study suggested the inoculation of plants with appropriate bacteria to enhance plant growth and quality of *Sulla aculeolata* under semi-arid conditions of the Mediterranean area.

**Keywords:** Genotypic characterization, Root-nodule bacteria, *Sulla aculeata* spp.



## Introduction

The genus *Hedysarum* spp., tribe Hedysareae, family Fabaceae is one of the most important temperate forage legumes in the Mediterranean region. The genus consists of various annual or perennial herbaceous species, recognizable by their distribution, morphology, genetic diversity (Boussaïd et al., 1995; Ben Fadhel et al., 2006), and ability to adapt to severely marginal and stress-prone environments (Gutierrez-Mas, 1983; Lupi et al., 1988; Abdelguerfi-Berrekia et al., 1991; Moore et al., 2006; Annichiarico et al., 2008) including drought stress (Lefi et al., 2023) and calcaro-saline soil (Jlassi et al., 2013; Tilaki et al., 2016; Tounsi-Hammami et al., 2016). Species such as *Sulla coronaria* L. and *Sulla flexuosa* L. have shown high quantities of green matter (Douglas and Foote, 1985; Chouaki et al., 2006), and good quality fodder (Issolah et al., 2014; Elyemlahi et al., 2019a). Therefore, they have been widely exploited as a forage crop to feed animals both in the pastoral and livestock sectors in several countries (Sulas and Ledda, 2008; Zirmi-Zembri and Kadi, 2021). The genus is also known for its ability to establish nitrogen-fixing symbiosis with soil bacteria (Kishinevsky et al., 2003; Elyemlahi et al., 2019b). However, only a few of them have been identified and characterized. In Morocco, this genus is represented by nine species (Fennane et al., 2007), including the endemic one such as *Sulla aculeolata* syn. *Hedysarum aculeolatum* (Amirahmadi et al., 2014). The plant is a diploide species, displayed an inter-population morphological polymorphism as a function of pedoclimatic variations (Kheffache and Combes, 1992). In addition, the species is known to harbor several genetically distinct bacteria in their root nodules, in relation to the geographical origin of the plants (Bezini et al., 2010). It has been stated that legume nodules in their wild status may be colonized by several endophytic bacteria (Benhizia et al., 2004; Muresu et al., 2019;

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Pang et al., 2021) from *Pseudomonas*, *Enterobacter*, and *Bacillus* genus (Peix et al., 2015), preferentially selected by legumes to cope with stressed environments (Ahemad and Kibret, 2014; Gamalero and Glick Bernard, 2015; Muresu et al., 2019). On other hand, several studies have shown that inoculation of legumes with appropriate bacteria had the advantage of improving crop yield and quality (Zhang et al., 2016; Li et al., 2022; Shome et al., 2022). In this framework, this research aimed to investigate the phenotypic and genotypic characteristics of the root nodules bacteria associated with two forage legumes namely *Sulla aculeolata* subsp. *mauritanica* and *Sulla aculeolata* subsp. *aculeolata* growing spontaneously in natural pastures located in the North of Morocco. Isolation and characterization of native bacteria populations could be a valuable biological resource when searching for biofertilizers that can help to reduce the use of chemical fertilizers while enhancing crop growth and quality.

## Material and Methods

### Plants sampling and bacteria isolation

The whole plant (leaves and stems) of *Sulla aculeolata* subsp. *aculeolata* and *Sulla aculeolata* subsp. *mauritanica* at the early flowering stage was assembled from two distinct sites located in the North of Morocco (Figure 1). Soil chemical and physical characteristics were showing in Table 1. The symbiotic efficiency of native rhizobia was estimated by recording the *in vivo* relative abundance of root nodules (Howieson and Dilworth, 2016), and nitrogen content using the Kjeldahl method.

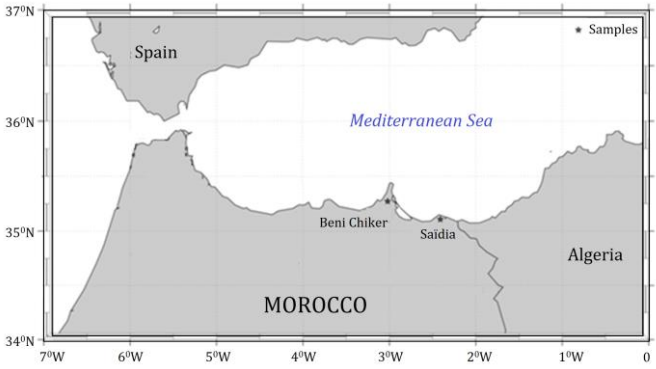


Figure 1. Location of sampling sites of *Sulla aculeolata* spp.

Table 1. Soil characteristics of the sampling site

| | Beni Chiker | Saïdia |
|---|---|---|
| Slope | 30°(NE) | 0° |
| pH (water) | 8.75 | 8.82 |
| N, % | 0.11 | 0.08 |
| $P_2O_5$, ppm | 5.13 | 6.21 |
| $K_2O$, ppm | 111.46 | 250.04 |
| Organic Matter, % | 2.00 | 0.60 |
| $CaCO_3$, % | 33.6 | 21.03 |
| Clay, % | 10.20 | 5.13 |
| Fine silt, % | 10.20 | 20.51 |
| Coarse silt, % | 4.14 | 17.33 |
| Fine sand, % | 27.45 | 35.18 |
| Coarse sand, % | 14.39 | 0.82 |

### Bacteria isolation and purification

Bacteria were isolated from naturally *Sulla aculeolata* spp. root nodules as described by Ezzakkioui et al. (2015). The isolates were verified for purity by repeated streaking on YEM (Howieson and Dilworth, 2016) agar medium and Gram staining. Pure isolates were stored at -20°C using 50% glycerol until analysis.

### Phenotypic characterization

### Response to environmental stress conditions

The tolerance ability of different bacteria isolates to grow under stress conditions was inspected using YEM agar as a basic medium. The pH tolerance was examined by growing isolates on YEM agar medium adjusted to a pH range from 4 to 10. Heat tolerance was assessed by incubating strains at 28, 37, 40, and 42°C. Salt tolerance was tested on YEM agar medium containing different levels of NaCl (0.5%, 1% and 2%w/v). Finally, drought resistance was conducted in YEM broth using polyethylene-glycol 6000 (PEG 6000) as indicated by Busse and Bottomley (1989).

### Utilization of carbon and nitrogen sources

All isolates were tested for their ability to utilize different carbon (Glucose, Sucrose, Maltose Fructose, Raffinose, and Lactose) and nitrogen (Asparagine, Histidine, $NH_4NO_3$, and $KNO_3$) sources, using modified YEM agar medium as described by Kishinevsky et al. (2003).

### Intrinsic heavy metal and antibiotic resistance

Determination of intrinsic and heavy metals and antibiotic resistance were examined on a solid YEM medium containing the filter-sterilized in µl.mL[1]: Spectinomycine (100), Chloramphenicol (150), Streptomycin (10), Kanamycin (10), Tetracyclin (50), Erythromycin (50), Ampicillin (100), $ZnCl_2$ (200), $CdCl_2$ (20), $CoCl_2.6H_2O$ (100), $HgCl_2$ (20), $AlCl_3.6H_2O$ (400), $CuCl_2.6H_20$ (100), and $MnCl_2.4H_2O$ (400).

### Genotypic characterization

### DNA extraction and ERIC-PCR fingerprinting

Total genomic DNA was extracted using the phenol/chloroform procedure as outlined by Chen and Kuo (1993). The quantity of DNA was ascertained by using a NanoDrop spectrophotometer (NanoDrop ND2000/2000c, Thermo Fisher Scientific). To assess the genotypic diversity among the isolates and to avoid any duplicates or clonality, ERIC-PCR (Enterobacterial repetitive intergenic consensus polymerase chain reaction) was performed using primers ERIC1R and ERIC2 according to Versalovic et al. (1991). Amplification was verified by horizontal gels electrophoresis (2%w/v agarose (Bioline) in Tris-acetic-EDTA buffer) at 70 V for 3 h, and finally photographed under UV light using the ENDUROTM GDS Gel Documentation System (Labnet International, Inc., US). Analyses of the ERIC-PCR profiles were carried out with GelCompar II software (version 2.5 Applied Maths, Belgium) using Dice similarity coefficient and UPGMA (Unweighted Pair Group Method with Arithmetic Averages) clustering method.

### 16S rDNA gene sequence and phylogenetic analysis

PCR amplification of 16S rRNA was performed using two universal primers fD1 and rD1 (Weisburg et al., 1991). Amplification products were firstly cheek by horizontal electrophoresis in 1% (w/v) agarose (Bioline) gels at 70V for 1 h, then purified using the purification system of Qiagen and finally subjected to cycle sequencing using the same primers as for PCR amplification. The sequences obtained were compared with those from the GenBank database, then corrected manually using MEGA 7 software (Kumar et al., 2016).

## Results

Twenty-four strains were recovered from root nodules of *Sulla aculeolata* spp. and characterized as phenotypic and genotypic features. All strains were fast-growers and failed to absorb Congo red (RC) when incubated in YEM-RC agar plates. Some phenotypic properties are presented in Table 2 and 3.

Table 2. Nodulation and efficiency of *Sulla aculeata* spp. evaluated at different sites in Morocco.

| | Infectivity[1] | Nodule color | Nodule size | Aerial dry matter (%) | Nitrogen content (%) |
|---|---|---|---|---|---|
| *Sulla aculeolata* subsp. a*culeolata* | Ample | Brown | Small | 18.25±0.48 | 2.23±0.23 |
| *Sulla aculeolata* subsp. *mauritanica* | Extremely abundant | Pink to brown | Big | 36.28±1.13 | 2.20±0.25 |

[1] using the chart proposed by (Howieson and Dilworth, 2016).

Physiologically, all the isolates were capable to grow at 37°C, or when incubated in presence of 0.5% of NaCl (Table 3). Most of them grow at a pH range from 4.00 to 9.00 independently of their origin-soil pH (Table 1). By contrast, most of them were sensitive to high temperatures, where three isolates from *Sulla aculeolata* subsp. *mauritanica* were able to grow at 42°C, and only those from the same species were capable to tolerate high salinity level (up to 2%w/v). Furthermore, the evaluation of intrinsic resistance to heavy metals showed that all of the isolates were more endurable and exhibited the highest tolerance to manganese (up to 400 µl.mL$^{-1}$), and most of them tolerate cadmium and zinc (Table 3). However, only those from *Sulla aculeolata* subsp. *mauritanica* resisted copper and five isolates from the same plant grown in presence of cobalt. Finally, all the isolates appear sensitive toward aluminum and mercury, except one isolate from *Sulla aculeolata* subsp. *aculeolata* (Table 3). In compliance with these results, the evaluation of antibiotic sensibility showed that the majority of the isolates from *Sulla aculeolata* subsp. *mauritanica* were resistant to the tested antibiotic, while those from *Sulla aculeolata* subsp. *aculeolata* were highly sensitive to most antibiotics except for Streptomycin and Ampicillin (Table 3). By the same token, drought resistance shows that 27% of the isolates from *Sulla aculeolata* subsp. *aculeolata* were highly tolerant and able to grow at a water potential of – 0.25 MPa, while, more than 78% of the isolates from *Sulla aculeolata* subsp. *mauritanica* survived at the same water potential. However, only 14% of isolates from *Sulla aculeolata* subsp. *mauritanica* were capable to grow at – 0.5 MPa and none of the isolates tolerate PEG-induced drought stress set to -1 MPa (Figure 2).

Table 3. Phenotypic characterization of root-nodule bacteria of Sulla aculeata spp.

| | *Sulla aculeolata* subsp. *aculeolata* | *Sulla aculeolata* subsp. *mauritanica* |
|---|---|---|
| *Strain* | (n=12) | (n=14) |
| *Growth at temperature* | | |
| 37°C | + | + |
| 40°C | 1 | + |
| 42°C | - | 3 |
| *Growth at pH* | | |
| 4 | + | + |
| 5 | + | + |
| 8 | + | + |
| 9 | 10 | + |
| *NaCl tolerance* | | |
| 0,5 % | + | + |
| 1 % | 1 | + |
| 2 % | - | + |
| *Carbohydrate assimilation, 1%* | | |
| Sucrose | + | 3 |
| Fructose | 5 | 3 |
| Maltose | 7 | 13 |
| Glucose | 7 | - |
| Lactose | - | 12 |
| Raffinose | + | 4 |
| *Utilization of nitrogen sources, 0.1%* | | |
| Histidine | 2 | 4 |
| Asparagine | - | 1 |
| $KNO_3$ | 1 | + |
| $NH_4Cl$ | 2 | + |
| *Antibiotic sensibility, µl.mL$^{-1}$* | | |
| Chloramphenicol (150) | - | 11 |
| Spectinomycin (100) | - | 9 |
| Streptomycin (10) | + | 9 |
| Tetracyclin (50) | - | 9 |
| Erythromycin (50) | - | + |
| Ampicillin (100) | 8 | 13 |
| Kanamycin (10) | | |
| *Heavy metal resistance, µl.mL$^{-1}$* | | |
| Zinc (200) | 11 | 12 |
| Cadmium (20) | 8 | 12 |
| Cobalt (100) | - | 5 |
| Mercury (20) | 1 | - |
| Aluminum (400) | - | - |
| Copper (100) | - | 12 |
| Manganese (400) | + | + |

(+): positive growth; (-): no growth and numbers indicate the number of positive strains

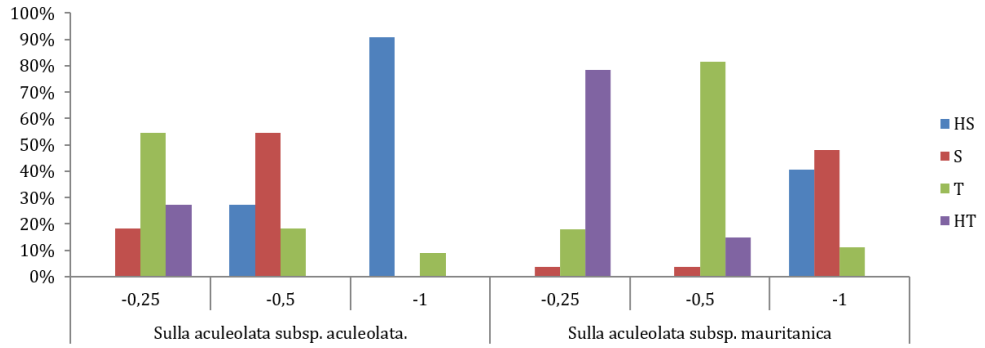


Figure 2. Effect of different water potential levels (-0.25; -0.5 and -1MPa) on growth percent of root nodule bacteria of *Sulla aculeolata* spp. HS: highly sensitive (DO<0.5); S: sensitive (DO=0.3-0.4); T: tolerant (DO=0.4-0.5); TT: highly tolerant (DO>0.5).

Finally, the metabolic properties (Table 3) of *Sulla aculeolata* spp. root-nodule isolates disclose great variations among the strains. In this regard, the majority of the strains from *Sulla aculeolata* subsp. *aculeolata* appear able to use a large range of carbohydrates as the sole carbon source for growth, except lactose, preferentially Sucrose and Raffinose. While the isolates from *Sulla aculeolata* subsp. *mauritanica* were unable to grow in a Glucose-based medium, and only 3 and 4 (out of 14) isolates used Sucrose and Raffinose respectively. In addition, all the isolates were greatly inhibited in presence of Asparagine and Histidine as nitrogen sources, and only isolates from *Sulla aculeolata* subsp. *mauritanica,* except for 2 and 1 isolates from *Sulla aculeolata* subsp. *aculeolata,* were able to use respectively $NH_4Cl$ and $KNO_3$ for growth.

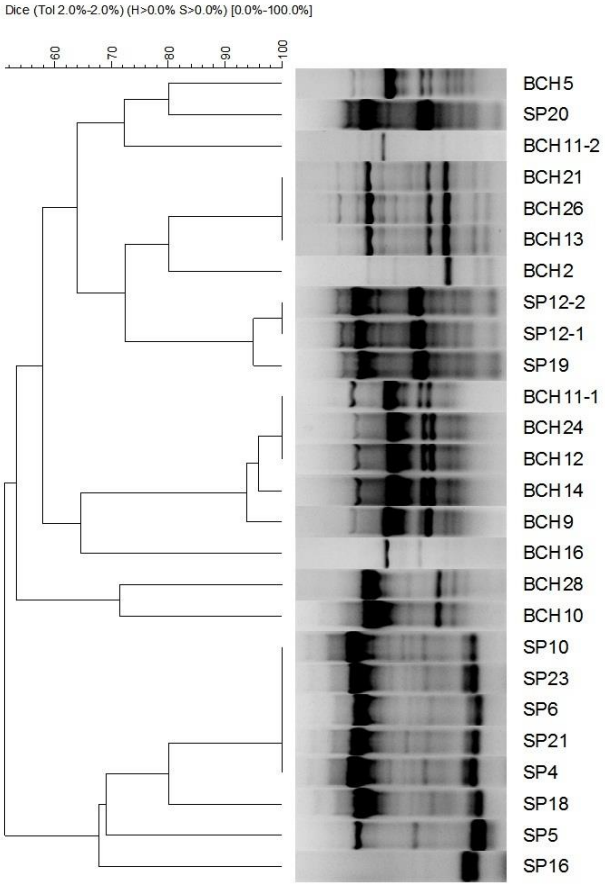


Figure 3. Dendrogram generated by UPGMA clustering from ERIC-PCR fingerprinting of 26 bacterial strains isolated from *Sulla aculeolata* spp. BCH: *Sulla aculeolata* subsp. *mauritanica* and SP: *Sulla aculeolata* subsp. *aculeolata*.

From a genetic perspective, the 26 indigenous bacteria isolated from root nodules of *Sulla aculeata* spp. were first examined for their genetic diversity using ERIC-PCR analysis. Results revealed a high level of genetic divergence among studied strains and form 14 distinct groups at 90% of similarity according to their host plant (Figure 3). Furthermore, analysis of the 16S rRNA gene sequence from the representative isolates originated from each ERIC-PCR pattern and their comparison with the sequences retained in the GenBank database showed that the majority of strains belonged to *Pseudomonas, Enterobacter,* and *Paenibacillus* genera, depending on their host plant (Table 4).

Table 4. Molecular identification of representative root nodules bacteria associated with *Sulla aculeata* spp. based on 16S rDNA sequence analysis.

| Host plant | Strain | Closest related bacteria[1] | Sequence Similarity (%) |
|---|---|---|---|
| *Sulla aculeolata* subsp. *mauritanica* | BCH16 | *Pseudomonas* sp. BT1 | 100 |
| | BCH10 | *Serratia plymuthica* strain IHB B 12183 | 99.80 |
| | BCH5 | *Pseudomonas* sp. BSP24 | 100 |
| | BCH24 | *Pseudomonas moraviensis* strain SP9 | 99.80 |
| | BCH2 | *Enterobacter* sp. BSP12 | 99.93 |
| | BCH13 | *Enterobacter hormaechei* strain AUH-ENM30 | 99.87 |
| *Sulla aculeolata* subsp. *aculeolata* | SP6 | *Pseudomonas frederiksbergensis* strain DSM 13022 | 99.61 |
| | SP4 | *Pseudomonas frederiksbergensis* strain DSM 13022 | 99.74 |
| | SP12-1 | *Pseudomonas thivervalensis* strain SBK26 | 99.58 |
| | SP5 | *Paenibacillus polymyxa* strain DSM 36 | 99.87 |

[1] determined using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi/).

## Discussion

In the Mediterranean basin, *Hedysarum* spp., widely Known as Sulla (Choi and Ohashi, 2003), are an important component in many ruminant diets. Those groups of forage legumes establish symbiosis with indigenous soil microorganisms such as mycorrhizal fungi (M'Saouar et al., 2020), reputable for their mineral absorption enhancement under several environmentally stressed conditions (Labidi et al., 2012; 2015), and rhizobial bacteria with high Plant Growth Promoting Rhizobacteria (PGPR) activity (Achkouk et al., 2018; Hamane et al., 2020). Within this framework, two Sulla species, namely *Sulla aculeolata* subsp. *aculeolata* and *Sulla aculeolata* subsp. *mauritanica* (native to Morocco), located in northern Morocco, were harvested and analyzed for symbiotic diversity of native soil bacteria that colonized their root nodules. Primary results (Table 1) show the occurrence of *Sulla aculeolata* spp. on calcareous silty soil, rich in potassium and total limestone and poor in nitrogen and phosphoros. Similar findings were reported by Ionesco and Stéfanesco (1967); Abdelguerfi-Berrekia et al. (1991); Hannachi-Salhi et al. (2002) who indicated the occurrence of *Sulla aculeolata* spp. in a very limited distribution area in scrub and pasture zones at low altitudes, on sloping sandy-clayey to clayey soils moderately watered, under the sub-humid and semi-arid climates of Morocco and Algeria.

From a symbiotic point of view, the on-field survey (Table 2) of examined leguminous species showed great productivity (up to 36.28% of dry matter in the case of *S. aculeolata subsp. mauritanica*) and high nitrogen content (in mean: 2.21%), comparable with others species evaluated under field conditions within the genus *Hedysarum* (Fitouri et al., 2012; Elyemlahi et al., 2017). A particular result was linked to the capacity of the species to establish a specific nitrogen-fixing symbiosis with soil bacteria known as rhizobia.

In this study, the phenotypic analysis of the 24 sampled isolates of both *Sulla aculeolata* spp. subspecies revealed a high degree of variation toward a large set of stressful treatments such as salinity, drought, and heavy metals, in concordance with previously reported root nodules isolates of *Sulla spinosissuma* L. (Oubohssaine et al., 2022), and *Sulla pallida* Desf. (Hamane et al., 2020) from the mining sites of the northeast region of Morocco and *Sulla aculeolata* spp. growing wild in Algerian soils (Bezini et al., 2010). Such results indicate a possible adaptation of those isolates to prevailing growth conditions and allow the selection of strains with ecological important traits. Indeed, molecular characterization of representative isolates, shows they belong to different bacterial genera (Table 4), however, no clue of the occurrence of rhizobia species. Similar findings were advanced by Benhizia et al. (2004), who reported no evidence of any rhizobial-like strains isolated from root-nodules of wild *Hedysarum* species, which could be related to the putative oxidative stress caused during bacteria isolation (Muresu et al., 2013).

Some of those strains such as *Enterobacter hormaechei*, *Serratia plymuthica*, *Pseudomonas frederiksbergensis,* and *Paenibacillus polymyxa* have been previously isolated from the root-nodules of different legume species such as *Hedysarum carnosum* Desf. (Muresu et al., 2008), *Sphaerophysa salsula* Pall. (Deng et al., 2011), *Glycine max* L. (Annapurna et al., 2013), *Phaseolus vulgaris* L. (Kawaka et al., 2018), and *Lupinus* (Ferchichi et al., 2019), usually regarded as plant growth promoter rhizobacteria (PGPR) (Hamane et al., 2020). Therefore, they were preferentially selected by the host plants as they can promote not only plant growth and health but also nodulation and N availability in sustainable agriculture systems under stress conditions including drought stress (Benhizia et al., 2004; Muresu et al., 2019; Hanaka et al., 2021).

On the other hand, it was reported that inoculation by some of those root nodule non-rhizobial endophytic bacteria such as *Pseudomonas frederiksbergensis* isolated from root nodules of *Sulla aculeolata* subsp. *aculeolata*, has been proven to be an effective inoculant for enhancing plant abiotic stress tolerance (Subramanian et al., 2015; Chatterjee et al., 2017). Bacteria like *Pseudomonas* sp. BT1 isolated from root nodules of *Sulla aculeolata* subsp. *mauritanica*, was identified as a barophilic bacterium (up to 60MPa), can be used for plant inoculation under drought-stress environments (Kaneko et al., 2000). While, other bacteria such as *Paenibacillus polymyxa* are demonstrated in their ability to increase organic dry matter digestibility and forage quality (Zayed et al., 2020).

## Conclusion

The present study provides the first characterization of root nodules bacteria associated with two forage legumes i.e. *Sulla aculeolata* subsp. *aculeolata* and *Sulla aculeolata* subsp. *mauritanica* growing wild in natural pastures located North of Morocco. From an applied point of view, the leguminous species chosen for this survey are appropriate for revegetation and soil-restoration of degraded pasturelands. Furthermore, the inoculation of *Sulla aculeolata* spp. with appropriate bacteria resistant to water stress would ensure root nodulation and improve plant performance under semi-arid conditions of the Mediterranean area. However, further study should be conducted, under a controlled environment, to determine the main microsymbiot of *Sulla aculeolata* spp.

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