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Enhancing Bio-Fertilization Potential of Introduced *Hedysarum* Species Through Indirect Root Inoculation: Implications for Agro-Ecological Sustainability in Arid Lands

Zwiększanie potencjału bionawozowego introdukowanych gatunków z rodzaju *Hedysarum* poprzez pośrednią inokulację korzeni: implikacje dla rozwoju rolno-środowiskowego w regionach pustynnych

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Abstract: Saharan soils are devoid of *Rhizobium* bacteria specific to the *Sulla* plants which is indispensable for nitrogen fixation and soil fertility. The present study aims to test the possibility of formation nodules on the roots of some species of *Hedysarum* (*Sulla*) genus introduced in Ghardaïa region with indirect inoculation in the reason to optimize the use of nitrogen fertilizers. Three soil samples were chosen from three different districts of Algeria (Tizi-Ouzou, Sétif and El-Tarf) to be used as substrate and source of bacterial strains for three different species of *Sulla* (*Hedysarum coronarium, Hedysarum carnosum, Hedysarum flexuosum*). The results showed the formation of a considerable number of nodules in all treatments. The effect of species and samples of soils on the studied parameter was highly significant ($p<0.000$) at the 5% significance level. All cultivated species in the soil of Sétif could form significant more nodules compared to other soils. The disparity in the average number of nodules formed in the soil of Sétif represents a rate of 78.75% compared to the soil of Tizi-Ouzou. This study could contribute to improve fodder production of *Sulla* species cultivated in Ghardaïa region and also provides a forage rich in protein for local livestock.

Keywords: root inoculation, *Sulla*, *Hedysarum*, leguminous, arid lands, Oases of M'Zab

Abstrakt: Gleby saharyjskie pozbawione są bakterii *Rhizobium*, specyficznych dla roślin bobowatych, niezbędnych do wiązania azotu i utrzymania żyzności gleby. Celem badań było przetestowanie możliwości tworzenia guzków na korzeniach niektórych gatunków z rodzaju

Hedysarum (siekiernica) introdukowanych w regionie Ghardaïa (północna Algieria) metodą pośredniej inokulacji. Próby gleby pobrane z trzech prowincji Algierii (Tizi-Ouzou, Sétif i El-Tarf) zostały wykorzystane jako podłoże oraz źródło szczepów bakterii brodawkowych dla trzech gatunków z rodzaju siekiernica (*Hedysarum carnosum, Hedysarum flexuosum, Hedysarum coronarium*) introdukowanych do tych gleb. Stwierdzono utworzenie się znacznej liczby guzków na korzeniach roślin we wszystkich wariantach eksperymentu. Wpływ gatunku siekiernicy i rodzaju gleby na liczbę guzków był wysoce istotny ($p<0{,}000$) na poziomie istotności 5%. Na korzeniach trzech badanych gatunków siekiernicy w glebie z prowincji Sétif tworzyło się znacznie więcej guzków w porównaniu do pozostałych gleb. Rozbieżność w średniej liczbie guzków utworzonych na korzeniach w glebie z Sétif wynosiła 78,75% w porównaniu z glebą z Tizi-Ouzou. Wyniki badań wskazują, że pośrednia inokulacja korzeni roślin z rodzaju *Hedysarum* przez specyficzne dla nich szczepy bakterii brodawkowych może przyczynić się do zwiększenia produkcji pasz z tych roślin w regionie Ghardaïa, a tym samym zapewnić paszę bogatą w białko dla lokalnych zwierząt gospodarskich.

Słowa kluczowe: inokulacja korzeni, *Sulla*, *Hedysarum*, rośliny motylkowe, rejony pustynne, Oazy M'Zab

Introduction

Leguminous species, belonging to the *Fabaceae* family, exhibit a symbiotic relationship with bacteria, leading to the formation of nodules on their roots. This symbiosis serves the purpose of converting atmospheric nitrogen (N_2) into mineral nitrogen, specifically NH_4^+, which can be readily assimilated by plants (Martinez-Hidalgo and Hirsch 2017). Consequently, legumes play a crucial agronomic role in managing nitrogen flows within cropping systems characterized by low nitrogen inputs and environmentally friendly practices (Voisin et al. 2015). The natural occurrence of the plant-bacteria association takes place in habitats where both biological partners coexist in the rhizosphere, facilitated by specific biological and microbiological mechanisms and guided by a molecular dialogue (Andrews and Andrews 2017). Plants, in this context, excrete different types of flavonoids that induce recognition by compatible bacteria, thereby initiating the induction of nodulation genes (Shamseldin et al. 2016). This biological phenomenon is subject to human monitoring, particularly in the context of re-vegetating degraded areas in the semi-arid regions (Chengyou et al. 2017).

Nodulation in legume species occurs in association with bacteria from the *Rhizobium* genus, which belongs to the Rhizobiaceae family. Within the realm of intensive agriculture, this symbiosis presents a natural alternative to the utilization of chemical fertilizers, particularly nitrogen fertilizers (Azib et al. 2022; Carpéné 2013). In the *Hedysarum* genus, the symbiotic fixation process is controlled by a distinct genus known as *Hedysari* (Akbarian et al. 2021). The *Sulla rhizobium*, commonly identified as *Rhizobium hedysari*, underwent taxonomic revision in 2002, leading to the recognition of a new name of bacteria, *Rhizobium sullae* (Fitouri et al.

2012a). Nevertheless, these specific strains of *Sulla* are not universally present in all biotopes. However, there exists the potential to transfer these strains to new habitats, facilitating the inoculation of plants and thereby achieving significantly elevated biomass production (Andrews and Andrews 2017).

Presently, there is very high interest in *Sulla* species due to their agronomic and functional traits, exceptional adaptability to marginal environments, versatility as a forage crop (grazing, hay, and silage production), positive impact on soil conservation, and provision of high-quality forage with excellent protein content and moderate levels of condensed tannins (Bouajila and Ben Jeddi 2015). These species possess the ability to combat the gastrointestinal parasitic nematodes (Moussaouali et al. 2023; Mueller-Harvey et al. 2019). They constitute a vital group of temperate forages on pastures in the Mediterranean basin, existing as either annual or perennial herbs.

The specific rhizobium associated with *Sulla* necessitates specific conditions for symbiosis, encompassing abiotic climatic conditions (such as temperature and humidity, etc.), pedological conditions (including salinity, mineral elements, pH, etc.), and biotic conditions (such as genotype, diseases, insects, etc.) (Mansouri 2020). Drought and salinity are generally acknowledged as major factors affecting the nodulation of legume species (Gehlot et al. 2012).

Inoculation (direct or indirect) is the process of introducing certain strains of bacteria into the soil with the goal of establishing symbiosis between the cultivated plants and the bacteria, thereby reducing the need for mineral fertilizers, called bio fertilization. In contrast, in the latter, we only work with a soil sample as part of the agronomical method, as the soil is considered a reservoir of various strains of bacteria. Therefore, there is no need for isolation or purification, etc. as we are confident that the specific *Rhizobium* of *Sulla* (*Rhizobium sullae*) is present in the soil alongside other species and genera of bacteria.

In our research conducted over preceding years, a consistent observation has been the complete absence of nodules in all cultivated plants of introduced *Hedysarum* species. This observation underscores the absolute absence of specific *rhizobia* for these species in Saharan soils. The present study aligns with this context. The initial biological question that prompted this research was: is there a possibility of cultivating *Hedysarum* plants with efficient nodules in the Ghardaïa region (Septentrional Sahara, southern Algeria) to optimize the use of nitrogen fertilizers?

1. Material and Methods

1.1. Site description

Field experiments were conducted in the M’Zab region of Ghardaïa Province, Algeria, situated 600 km south of the capital Algiers (32°29’N, 3°40’W, 450 m above sea level), during 2021/2022 growing season. This region is located in hyper arid bioclimatic stage, and the dry period covers the whole of the year. The average annual precipitation is around 50 mm, and the average annual temperatures are very high, maximums can reach 50°C and minimums are between 2 and 3°C (winter season). The soil of the region is skeletal, predominantly sandy, with 80% sand. It showed a low organic matter amount of 0.52% and a high content of $CaCO_3$. The rest of parameters and their contents are presented in the table 1.

Table 1. Soil physico-chemical properties of the experimental field

Depth (cm)	pH	Conductivity (mS/m)	Clay (%)	Silt (%)	Sand (%)	Organic matter (%)	C/N ratio	Nitrogen (%)	Phosphorus (ppm)
0-30	8.27	153.30	4	16	80	0.52	1.79	0.17	38.40

1.2. Experimental design and treatment description

Three soil samples for testing nodulation coming from three different regions of Algeria were brought back: Sétif and El-Tarf (S19 and S36), representing the natural habitats of *H. coronarium* (H3) and *H. carnosum* (H1) respectively, collected in June 2021. The 3rd sample, collected in September 2021 from Tizi-Ouzou (S15), corresponds to the dispersal zone of *H. flexuosum* (H2) and was provided by the LABAB (Analytical Biochemistry and Biotechnologies Laboratory) at UMMTO (Mouloud MAMMERI University of Tizi-Ouzou). The experiment was conducted using perforated seedling bags with dimensions of 20 cm in upper diameter, 15 cm in lower diameter, and 30 cm in height. This setup was employed under controlled conditions to mitigate climatic hazards, especially prevalent sand winds during the period from February to May.

The bags were filled with three different substrates, in layers (1/3 of the volume each): the top layer of soil was wadi flood sand rich in minerals, the middle layer was commercial seedling soil (chosen for its water retention) and the bottom layer was soil from Sulla’s natural habitats i.e. S19, S36 or S15 (in a mixture of 1/2 topsoil, 1/4 commercial seedling soil and 1/4 test soil).

The experimental design followed a factorial test with two controlled factors and three repetitions (Table 2):

- Factor 1: 3 species of *Sulla*: *H. carnosum* (**H1**), *H. flexuosum* (**H2**), and *H. coronarium* (**H3**).
- Factor 2: 3 types of soil: Soil of Sétif (**S19**), Soil of Tizi-Ouzou (**S15**), and soil of El-Tarf (**S36**).

For the second factor, the 4th variant, which corresponds to the local soil of Ghardaïa (S47), was excluded due to previous findings its lack of strains.

Table 2. Experimental design adopted

H1 – S36	H2 – S15	H3 – S19	H1 – S15	H2 – S36	H2 – S19	H3 – S19	H1 – S19	H1 – S15
H1 – S19	H2 – S36	H3 – S15	H2 – S15	H1 – S19	H3 – S19	H2 – S15	H3 – S15	H2 – S19
H1 – S15	H2 – S19	H3 – S36	H3 – S36	H3 – S15	H1 – S36	H1 – S36	H2 – S36	H3 – S36

The manual sowing took place on January 2022, following the established protocol of placing five seeds of each species in a bag. All sown bags were consistently maintained at 90% relative humidity through regular irrigation with tap water from the time of sowing until the initiation of flowering (marked as the period of uprooting the plants and observing the roots). Upon emergence, plant thinning was conducted, with the decision to retain 3 plants per bag for the continuation of the trial. At the early flowering stage, the seedling bag was carefully cut to release the root ball, and the roots were manually unearthed to prevent damage to the nodules. Subsequently, the roots were separated from the aboveground part at the base of the stem, briefly soaked in water, underwent a meticulous yet gentle rinse with tap water to eliminate residual soil on the roots, and were then dried on filter paper. The nodules were counted by carefully examining each root, with the option of using a binocular if necessary.

1.3. Statistical analysis

All obtained data underwent appropriate statistical analyzes. To assess whether the three types of soils significantly affected the roots of plants, a two-way ANOVA (F-test value) was employed. The Shapiro-Wilk normality test was used at the 5% significance level to confirm the normality of the data, and the Bartlett test was employed to check the homogeneity of variances. The significance of differences between means was evaluated using the Tukey post-hoc test, with a probability value of $p \leq 0.05$, using R software (version 4.0.0).

2. Results

2.1. Evaluation of the number of nodules according to species of *Sulla* and types of soil

The assessment of number of nodules formed on the roots of cultivated *Sulla* plants was conducted at a 100-day culture period. A distinct discrepancy in nodular activity was observed among the 3 species, evident in the number of nodules on the root system (Figure 1A).

The *Hedysarum flexuosum* species from the Tizi-Ouzou region exhibited the highest average number of nodules (94.77 nodules), followed by *Hedysarum coronarium* species with a value of 88.66 nodules, and finally, *Hedysarum carnosum* had the lowest average value of 52.55 nodules. These recorded values are particularly noteworthy when compared to the control (local soil, devoid of bacteria specific to *Sulla* species), which consistently showed a value of zero. The lower count recorded for *H. carnosum* species is primarily attributed to the absence of a soil sample from its natural habitat. Importantly, it is observed that all the three *Sulla* species can establish a symbiotic relationship in all treatments.

A)

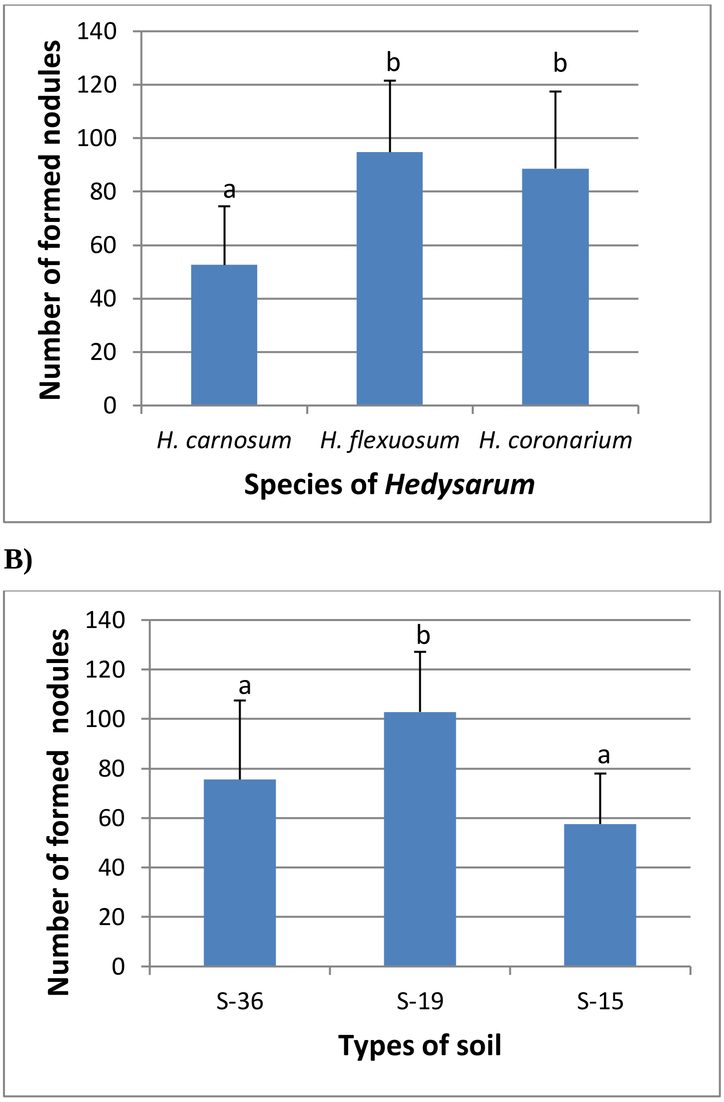

Figure 1. Number of formed nodules on the root system of cultivated plants according to species (A) and types of soil (B); (S-15: soil of Tizi-Ouzou; S-19: soil of Sétif; S-36: soil of El-Tarf). Mean values + SD are given. Different lowercase letters indicate significant differences among the species (A) or soil types (B).

For the same parameter, another evaluation was realized from a different perspective, focusing on the types of soil employed. The soils are gathered from three different regions (El-Tarf, Sétif, Tizi-Ouzou), primarily chosen based on the natural habitats of the cultivated species. The examination revealed the presence of specific *rhizobium* for all three cultivated species in the selected soil. Consequently, varying values were recorded for all treatments, signifying a notable difference in terms of nodule formation on the roots of the selected plants (Figure 1B).

A detailed analysis of the combined impact of the two factors (*Sulla* species, soil origins) on nodule production yielded several observations (Figure 2):

- The soil of Sétif (S19) yielded very interesting values regarding the number of nodules for all cultivated species (116; 115.3; 77.33 nodules), especially on *H. coronarium* and *H. flexuosum*, affirming the presence of the three specific *rhizobia* for the three *Sulla* species in this soil.
- The soil of Tizi-Ouzou (S15) caused the formation of a lowest number of nodules on cultivated species, including that originating from this soil (*H. flexuosum*).
- The soil of El-Tarf (S36) exhibited notably distinct values among the species (high number of nodules for *H. flexuosum* and low number for *H. carnosum*).

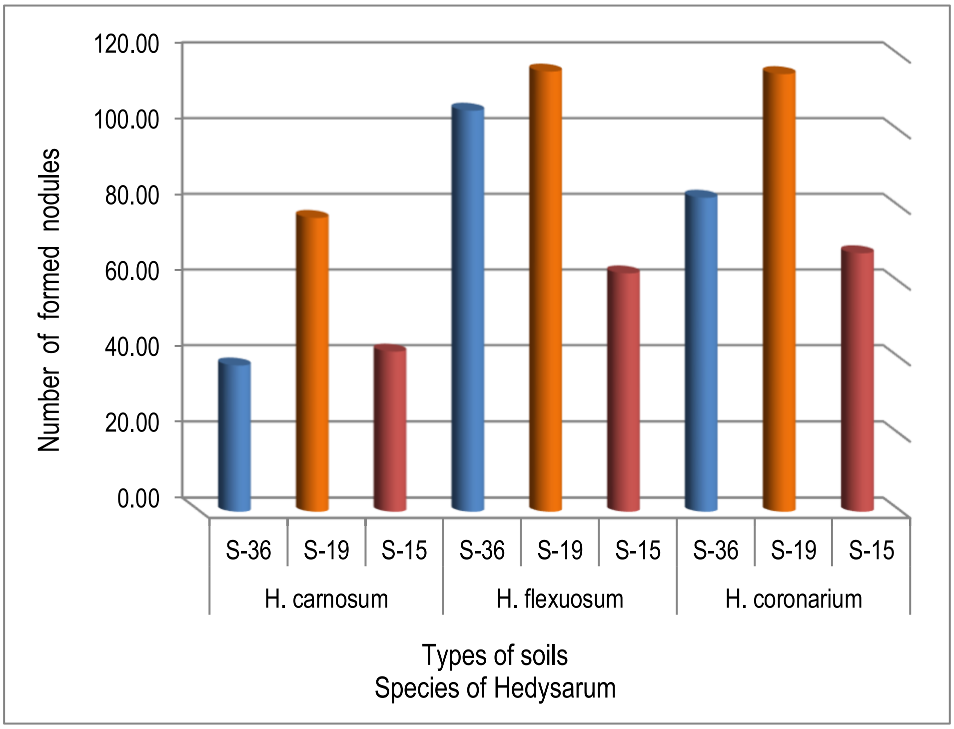

Figure 2. Combined outcomes of the number of formed nodules on the root system of cultivated plants of *Sulla* according to species and types of soil (S-15: soil of Tizi-Ouzou; S-19: soil of Sétif ; S-36: soil of El-Tarf)

The analysis of variance revealed a highly significant difference between the cultivated species of *Sulla* (*Hedysarum*) and the selected soil types (p = 8.439e-05; p = 8.405e-05 < 0.001 respectively). Both *Sulla* species and soil types exerted a discernible effect on the process of nodule formation on plant roots and the subsequent increase in their number. However, no significant difference was observed in the interaction concerning the parameter in question (p > 5%).

The soil sample from Sétif notably enhanced the activity and efficiency of the bacterial strains present in the soil, significantly influencing the formation of nodules in all cultivated species. The disparity in the average number of nodules formed in the soil from Sétif represents a rate of 78.75% compared to the soil from Tizi-Ouzou and 47.48% compared to that of El-Tarf (Figure 1).

The preceding findings highlighted the impact of the two factors under investigation on the number of nodules per plant (*p-values* < 0.001). The application of the Tukey test aims to categorize the means of the response variable into distinct groups. A highly significant difference is observed between the species *H. coronarium – H. carnosum* on one side, and between *H. flexuosum – H. carnosum* on the other side (p<0.001, Fig. 1). However, there is no difference between the two species *H. flexuosum – H. coronarium* concerning the parameter of the number of nodules per plant, indicating a similar effect. A remarkably significant difference is revealed in the number of nodules per plant between the effects of soils S19 and S15 (p<0.00, Fig. 1). Consequently, a highly significant effect is observed between the two treatments S36 and S19 (p<0.01). Finally, there is no discernible effect between the two soils S36 and S15 concerning the parameter of the number of nodules per plant suggesting a similar effect.

2.2. Qualitative analysis of formed nodules

Based on the findings of this study, a distinct variance is noted in the form and structure of the nodules formed on the roots of cultivated plants (Figure 3). These shapes exhibit clear dissimilarities when compared to nodules from their original regions. This discrepancy is likely attributed to environmental conditions, particularly edaphic factors, namely the texture and structure of the soil (grain size fraction). Alternatively, it may be result of phenotypic deformation, given that these nodules were formed for the first time in this soil.

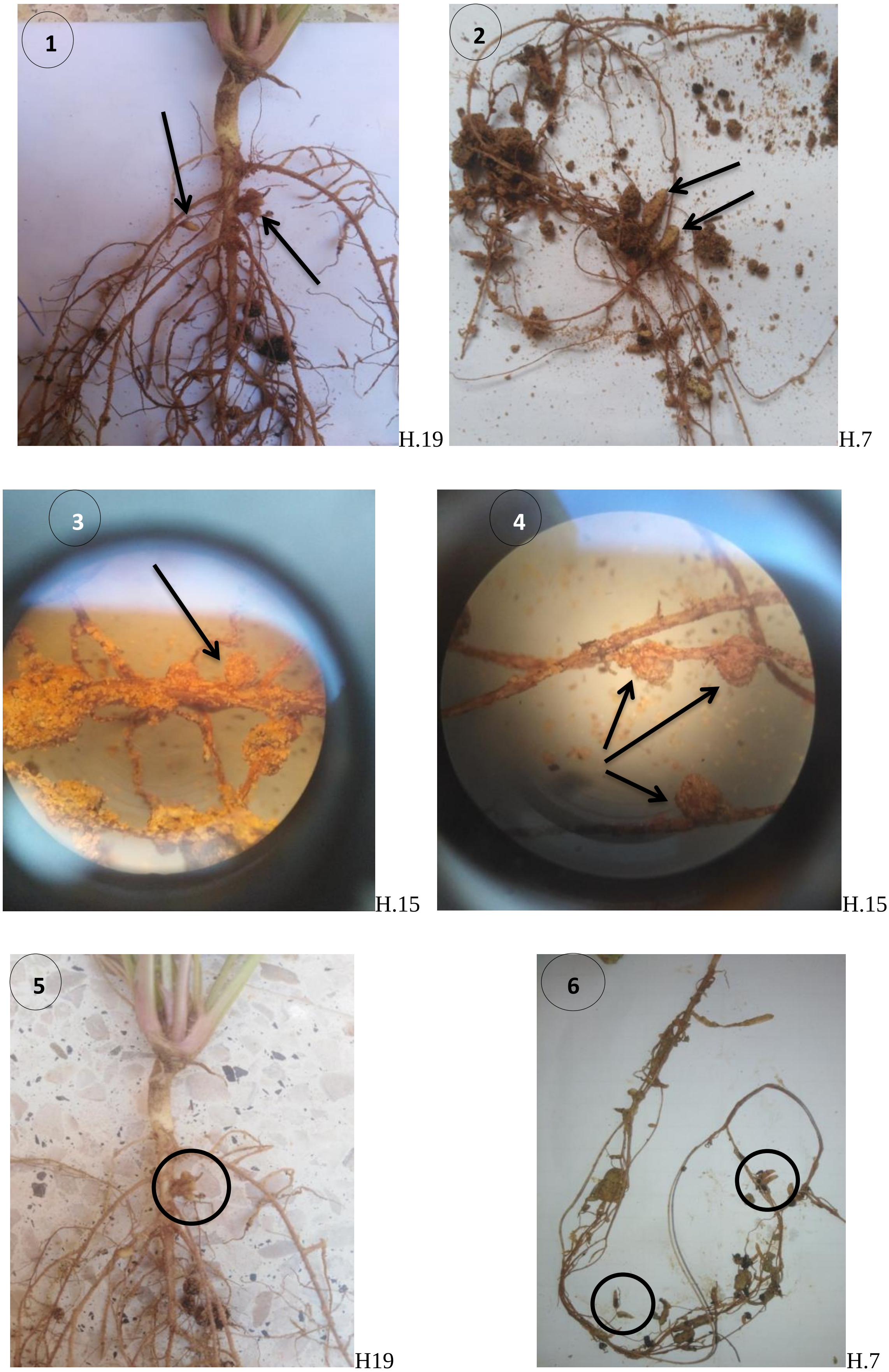

Figure 3. Various categories of formed nodules observed on the roots of cultivated *Sulla* plants (Moussaouali 2022). H19: *H. coronarium* / H7: *H. carnosum* / H15: *H. flexuosum*.

3. Discussion

3.1. Evaluation of the number of nodules according to species of *Sulla* and types of Soil

Our study aimed to assess the potential formation of nodules on the roots of *Sulla* plants cultivated in an environment lacking the specific *Rhizobium*. The obtained results illustrate the capability of *Rhizobia* present in the soil to establish symbiosis under regional conditions (116; 115.3; 105.67 nodules per species). This outcome is deemed noteworthy. In a Tunisian study on inoculated *Sulla*, the number of nodules per plant ranged from 3 nodules per plant in a water deficit (25% useful reserve) to 88 nodules per plant in the case of 75% useful reserve (Fitouri et al. 2012b). Azib (2020) working on the lucerne in the region of Ouargla and Ghardaïa, found a very low number of nodules ranging from 4.3 to 12.9 nodules per plant (without inoculation). In another experiment conducted in Tunisia on *H. coronarium* plants cultivated in pots with adequate irrigation and the presence of bacterial strains, the number of nodules increased to 230 nodules per plant (Fitouri 2011). As previously reported in Moussaouali and Hamdi-Aïssa (2017), *H. coronarium*, an indigenous population of El-Tarf, exhibited abundant nodality ranging between 51 and 167 nodules per plant, with an average of 82.26 nodules.

In other study involving *Phaseolus vulgaris*, the recorded number of nodules per plant was 25.22 (Mansouri 2020). These values appear considerably low when compared with those obtained in our trial. Viçosi et al. (2020) found wide range of nodules on the same species, ranging from 8.87 to 63.62 nodules per plant in both cases of inoculation and non-inoculation. The total absence or low presence of nodules in the roots of leguminous species may be explained by the low population of *Rhizobium* in the soil or the inhibition of the nodulation due to unfavorable environmental conditions, such as salinity (Mansouri 2020). Additionally, nitrogen fertilization is another cause of the inefficiency of nodulation.

The number of nodules per plant is evidently influenced by the soil's origin, or in other words, by the presence of specific *Rhizobium* in the soil. So, the three specific *Rhizobia* of the three *Sulla* species are present in the three sampled soils. This confirms the existence of these *Hedysarum* species in most soils of northern Algeria. According to Abdelguerfi-Berrekia et al. (1991), several species of *Hedysarum* were encountered in floristic surveys of natural grasslands unaffected by agriculture or development. They added that *H. flexuosum* was found mixed with *H. coronarium*. *Hedysarum flexuosum* can establish efficient symbiosis and forms active nodules with only *Rhizobium sullae*; no other strain can achieve this (Ezzakkioui et al. 2015).

The inoculation of *Sulla* species by rhizobial strains appears more advantageous than nitrogen fertilization in terms of fodder production and the number of formed nodules per plant, even in drought conditions (Fitouri et al. 2012b). Pollution of agricultural soils (e.g. heavy metals) acts as a barrier to the *Rhizobium*-legume symbiotic relation, inhibiting the root infection process and affecting nodule formation, ultimately reducing the number of nodules (Rahal and Chekireb 2021).

Hedysarum flexuosum demonstrated the highest average number of nodules (94.78 nodules) compared to the other species. This number represents a difference of 6.89% and 80.33% compared to the medium and low values, respectively (*H. coronarium, H. carnosum*). These numbers are likely to have a significant and direct impact on fodder production, nutritional quality, nitrogen content in leaves, the use of chemical inputs, export of mineral nitrogen in the soil, and microbial activity in the living soil compartment.

The soil sample from Sétif notably enhanced the activity and the efficiency of bacterial strains existing in the soil. They effectively influenced the formation of nodules in all cultivated species. The disparity in the average number of formed nodules in the soil from Sétif represents a rate of 78.75% compared to the soil from Tizi-Ouzou and 47.48% compared to that of El-Tarf.

3.2. Qualitative analysis of formed nodules

Nodules typically exhibit a rounded shape and can vary in size depending on the plant species and bacteria involved. The shape of the nodule is a specific genetic trait. Both determinate and indeterminate shapes can be observed (Azib 2022). It is undoubtedly influenced by external factors and conditions, resulting in multiple forms (Figure 3).

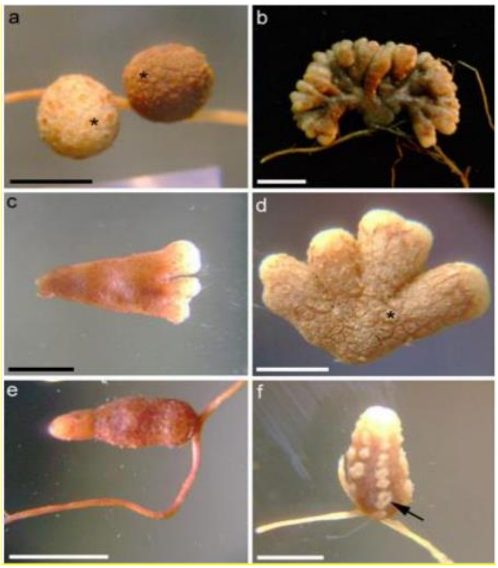

Figure 4. Various shapes of nodules identified on the root system of leguminous species (Gehlot et al. 2012)

The shape or form of nodules is a specific and genetic characteristic; determinate and indeterminate forms were observed (Azib 2020). Nodality is undoubtedly influenced by external factors and conditions, such as edaphic and climatic.

In our discussion, we will refer to images illustrated in figure 4. The pictures in figure 3 depict various nodalities observed in the roots of cultivated species of *Sulla*, whether these photos are taken directly or under binocular magnifying glass. Morphologically, these nodules appear highly differentiated. According to Fitouri et al. (2012b), the morphology of nodules is closely related to the adaptation of *Sulla* to environmental stress, especially water stress.

From the preceding photos, plants have formed nodules in different shapes, even within the same plant (e.g. *Hedysarum carnosum*). Comparing photos (1 to 6) with figure 3, photos 1 and 2 are closely resemble to the photo “c” and “d” in figure 3, while the photo 6 exhibits a flattened appearance and a low volume, resembling photo “e” in the figure 3. The bulky shape of the nodules likely reflects their adaptation to severe water stress in the rhizosphere, as small nodules quickly lose their water reserves (Fitouri 2011). Bacterial strains highly tolerant to water stress can be found in marginal areas, subsequently, the use of selected bacteria tolerant to drought conditions is very useful for the best crop production in drylands (Ashry et al. 2022).

Conclusion

Root nodulation in leguminous species represents a genuine biological mechanism facilitating mutualism between host plants and bacteria, making agriculture more sustainable, healthy and organic.

The study reveals that *Sulla* plants were capable of forming nodules with *Rhizobia* already present in the soil. This is a significant finding as it suggests that *Sulla* plants can be cultivated in areas where the specific *Rhizobium* is not naturally present. The results obtained in this experimental trial constitute a crucial element of the success of implementing this project on the ground. To date, no experimental study has been conducted in the region to explore the diversity of symbiotic bacteria in the soil of Ghardaïa, let alone its application for inoculation purposes.

This work concludes by proposing several perspectives that must be pursued in the near future:

- Implementation of direct inoculation by introducing the specific rhizobial strain of *Sulla* from its regions of origin, amplifying it, and subsequently inoculating plants directly at the roots;
- Evaluation of the inoculation effect (both direct and indirect) on green fodder production, forage yield, and nutritive value;
- Characterization of bacterial strains found on the roots of *Sulla* cultivated in the region in response to biotic and abiotic constraints, namely salinity, drought, and high temperatures, etc.

The study's results hold promise for the cultivation of *Sulla* plants in new areas. However, further research is necessary to validate the findings of this study and determine the best practices for cultivating *Sulla* plants in the absence of the specific *Rhizobium*.

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