

Effect of *Rhizobium* Inoculation on Growth and Nutrient Uptake of Sulla (*Hedysarum coronarium L.*) Grown in Calcareous Soil of Northern Tunisia

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Abstract

Hedysarum coronarium L. cultivated on calcareous soil in Northern Tunisia was the subject of a trial attempted under field condition, to figure out the effect of nitrogen fertilizer and *Rhizobium* inoculation on their growth, nodulation and mineral content. *Rhizobium* inoculation enhanced significantly all growth parameters compared to the nitrogen fertilization especially at the flowering stage. No significance difference was shown between the uninoculated check and the nitrogen fertilized plants concerning the nodule number per plant at all plant stage. At 154 DAS (Day After Sowing) and 174 DAS, *Rhizobium* inoculated plants set a high mean nodule number (86.33 and 77.77 respectively). Nodule weight per plant was high in *Rhizobium* inoculated plants compared to the uninoculated check and the nitrogen fertilized plants especially at 123 DAS, 154 DAS and 174 DAS. Concerning nutrients content in shoot of *sulla* at flowering stage, Nitrogen fertilization significantly enhanced nutrients content. However *rhizobial* inoculation has almost doubled nutrients uptake compared to the nitrogen fertilized control.

Keywords: *Sulla* · *Rhizobium* · inoculation · nitrogen fertilization

1. Introduction

Farmers in arid and semi-arid regions usually mention declining soil fertility as one of their major constraints to crop production. In Tunisia, farmers have to use chemicals in agriculture production. Chemicals create environmental pollution and health hazards. The cost of chemical fertilizers is becoming un-affordable for the farmers of rain fed areas in additions to creating soil and water hazards. Thus, the development and application of environmentally safe biopreparations and most of all, biological agents, are of particular importance in the contemporary methods for plant enhancement. There is a great need to supplement or substitute chemical fertilizers with organic manures or to explore biological means to improve the soil fertility. Legumes have attracting substantial attention because of their potential to fix atmospheric N through symbiosis with N-fixing bacteria, harbored in their root nodules. This phenomenon is often translated in substantial yield grains in a cereal that follows the legume crop. Its fort possible to increase legume plants production by better use of plant rhizobacterium interaction through rhizoidal inoculation. One such technique could safeguard nature from nitrogen pollution. In addition, it increase yield and forage nutritional quality that enhance animal production. In Tunisia *Hedysarum coronarium L.*, known by its Italian name of Sulla, is one of the most popular cultivated legumes. In Tunisia, it covers about 4000 ha and is expected to attain 21000 ha by 2016 (1). Sulla occurs natively in calcareous clay, marnous

slopes and desert soils. Sulla is known as forage crop. It could resist a pronounced drought and alkaline soils as well (2). Sulla is recognized for its high agronomical yield and protein content. It could produce up to 60 tons of green forage per hectare per year, and protein content could be up to 24% of leaf dry weight (3). However, the expansion of this culture is often limited owing to the absence or the inefficiency of its specific *Rhizobium* called *Rhizobiumsullae*. Inoculation with selected strains could be performed to introduce require highly effective strains for nitrogen fixation and adapted to the adverse soil conditions such as salinity (4). The aim of the present work was to compare *Rhizobium* inoculation and nitrogen fertilization effect on growth parameters, nodulation and nutrient uptake of sulla (*Hedysarum coronarium L.*) under field conditions.

2. Materials and Methods

2.1. Experimental location

Field trial was conducted on 1 hectare area during the cropping season of 2009-2010 in Goubellat region situated at the Northern of Tunisia (36°37'N, 9°36'E; 56m), where *Hedysarum coronarium* have not been grown since 2000 considering the absence of indigenous populations of *rhizobia* nodulating Sulla (5). Soil samples were collected from the experimental field before planting to have an idea about the soil fertility status (table 1).

Table 1. Chemical and physical soil analysis of field site

Depth (cm)	Clay (%)	Loam (%)	Sandy (%)	MO (%)	Nt (%)	P ₂ O ₅ (ppm)	Ca (%)	C (%)	pH
0 – 25	54	14	32	3,6	0,22	23	18	2,1	8,7

Goubellat is a semi-arid region receiving a total rainfall of 346 mm during the course of study ranged from 10 mm for April to 82 mm for March, and a mean temperature fluctuated from 11°C in February to 22°C in September (figure 1).

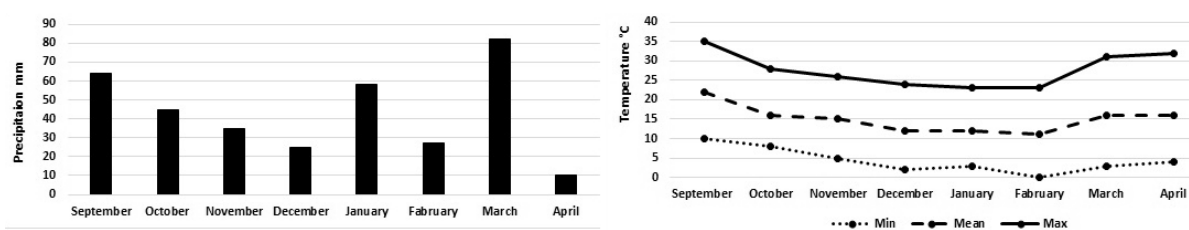


Figure 1. Temperature and precipitation fluctuations during the cropping season 2009 – 2010 in Goubellat region.

2.2. Experimental design and treatments

Bikra 21, the most produced Variety in Tunisia, was used for this survey. Planting was performed after ploughing and harrowing in 23 October 2009 under non-irrigation conditions. Only phosphorous nutrient was applied before few hours of planting to provide P₂O₅ at equivalent rate of 45 Kg ha⁻¹. Plot was arranged in a randomized complete block design with 5 replications. A distance of 2 m was maintained between blocks in order to avoid contamination. Three handlings blocks were effectuated: A) Uninoculated control (T0), B) Inoculated plants (IN) using RSU9 strain (5), and C) Nitrogen fertilizer control (TA). In (TA) block, 90 kg ha⁻¹ of NH₄NO₃, divided into 2 supplies, were added at 60 and 123 days after sowing: (DAS). Each treatment was sown in a 441 m² area (21m × 21 m), in 20 lines (with 21 m long) spaced 1 m apart.

Plant harvest and sample preparation

Thirty plants per treatment were used to assess plant height, leaf area and number of nodules at 4 growing vegetative stages (67, 123, 154 and 174 days after sowing: DAS). Shoot and nodules samples were dried at 70°C for 48h, and thenshoot dry weight for each plant was recorded using a digital balance. At flowering stage (174 DAS), dried shoot was finely ground for nitrogen and micro-nutrients content (P, K, Ca, Mg, Fe) dosage. Effectiveness in nitrogen fixation was evaluated by dosing total N with Kjeldahl method (6). P, K, Ca, Mg, and Fe micro-nutrient were assayed from 1g ground sample ashed in a porcelain crucible at 500°C overnight. Then, the ash was dissolved in 5ml of 1 normal HNO₃ solution and was placed at 50°C for 30min. The residual was dissolved in distilled water and next was filtered. The filtrate was filled to 50 ml with distilled water and was used for the dosage of the five minerals. Phosphorus dosage was set using colorimetric analysis. Potassium and Calcium dosage were determined using flame photometry. The Magnesium and Iron concentration were given using atomic absorption.

2.3. Statistical analysis

Data were subjected to variance analysis of using SAS 8.0 (Statistical Analysis System). Mean significance difference was performed using Least Significant Difference (LSD) at 5% probability level.

3. Results

3.1. Plant growth parameters

Nitrogen fertilization and *Rhizobium* inoculation effect on growth parameters was not significant at 67 DAS, nevertheless, a slight improvement of these parameters with *Rhizobium* inoculation comparing to the nitrogen fertilizer treatment was shown. At this stage, for all treatments, plants showed nitrogen deficiency symptoms with early yellowing leaf, but it disappeared later at 123 DAS for inoculated plants (data not shown). This vegetative stage was characterized by the early development of efficient nodules rose-colored (data not shown). Therefore, it was considered that plants received nitrogen from symbiotic fixation.

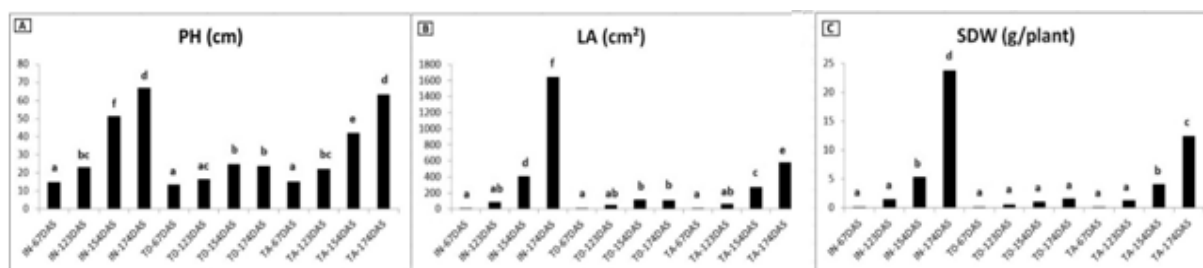


Figure 2. Nitrogen and *Rhizobium* fertilization effect on plant growth parameters. PH= Plant Height, LA= Leaf Area, DSW= Dry Shoot Weight. Mean values marked by the same letter are not significantly different at 5% level.

At 154 and 174 DAS, Nitrogen fertilization and *Rhizobium* inoculation significantly increased plant height compared to the uninoculated plants. Only at 154 DAS, plant height was significantly enhanced under *Rhizobium* inoculation in comparison with nitrogen fertilization (Figure 2A). Plant leaf area was significantly enlarged under nitrogen fertilization and *Rizobium* inoculation compared to the check trial. Mean leaf area for nitrogen fertilized plants at 154 and 174 DAS (274.95 cm² and 576.25 cm² respectively) were significantly lower than the *Rhizobium* inoculated plants at 154 and 174 DAS (408.96 cm² and 1641.5 cm², respectively) (Figure 2B). As plant height and leaf area, shoot dry weight (SDW) for nitrogen

fertilized and *Rhizobium* inoculated plants at 154 DAS and 174 DAS was significantly higher than the uninoculated check. Between nitrogen fertilized plants and *Rhizobium* inoculated plants, only at 174 DAS, a significant difference for SDW was registered. At this stage, mean SDW was 23.68 g/plant for *Rhizobium* inoculated plants and 12.35 g/plant for nitrogen fertilized plants (Figure 2C).

3.2. Nodulation parameters

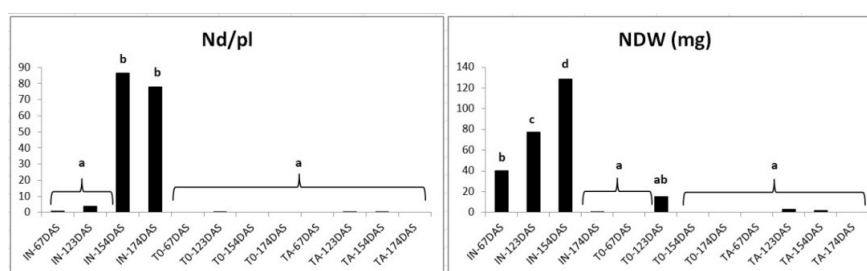


Figure 3. Nitrogen and *Rhizobium* fertilization effect on nodule weight and number per plant. Mean values marked by the same letter are not significantly different at 5% level.

No significance difference was shown between the uninoculated check and the nitrogen fertilized plants concerning the nodule number per plant at all plant stages. *Rhizobium* inoculated plants showed a restricted mean nodule number per plant at 67 DAS and 123 DAS (0.8 and 3.93 respectively), but at 154 DAS and 174 DAS, *Rhizobium* inoculated plants set a high mean nodule number (86.33 and 77.77 respectively) (Figure 3A). Nodule weight per plant was high in *Rhizobium* inoculated plants compared to the uninoculated check and the nitrogen fertilized plants especially at 123 DAS, 154 DAS and 174 DAS (Figure 3B).

3.3. Mineral contents parameters

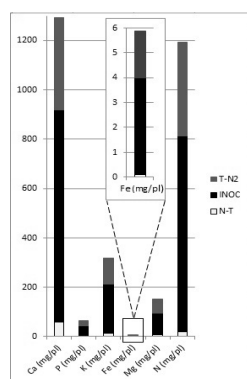


Figure 4. Nitrogen fertilization and *Rhizobium* inoculation effect on mineral content of sulla. LSD test showed a highly significant difference between treatments at $\alpha \leq 0.5$.

LSD test at $\alpha \leq 0.5$ showed a highly significance difference between treatments. For all minerals, *Rhizobium* inoculated plants showed the highest mean mineral content per plant, then the nitrogen fertilized plants. Restricted mineral contents was shown in the uninoculated check (Figure 4).

4. Discussion

Increasing and spreading the role of biofertilizers such as *Rhizobium* would decrease the necessity for chemical composts and decline adverse environmental effects. Consequently, in the improvement and application of ecological agricultural techniques, biofertilization is of

great importance in relieving environmental pollution and the corrosion of nature (7). This survey, aimed to compare the effect of *Rhizobium* inoculation and synthetic nitrogen fertilizer on the growth parameters, nodulation parameters and mineral contents parameter of *Hydysarum coronarium* grown in field. Despite the edaphic conditions of Goubellat area which were characterized with high pH (8.7) and active calcium carbonate (18%) (table 1), nitrogen fertilization and *Rhizobium* inoculation showed a significant increase of growth parameters compared to the control check (figure 2). Inoculation with diazotrophic bacteria like *Rhizobium* enhanced the plant growth as a result of their ability to fix atmospheric nitrogen (8). Basu et al. (9) reported in 2011, that *Rhizobium* inoculation was more effective for increasing the growth traits, yield and oil content of *Arachis hypogaea*. Deshwal et al. (10) concluded that the use of rhizobia inoculant enhanced plant growth in *Mucuna* plant. Not only Growth parameters were significantly influenced by biological and chemical fertilization but also it depended on growth stages. The most important finding is that *Rhizobium* inoculation significantly improve growth parameters compared to nitrogen fertilization only at Advanced growth stage (154 DAS for “plant height”; 154 and 174 DAS for “Plant leaf area” and 174 DAS for “Shoot dry weight”) (figure 2). Sulla at 154 DAS was characterized by the beginning of the button floral development, and at 174 DAS, it was the flowering stage. Notable variation was found for biological nitrogen fixation at different growth stages of *Pisum sativum* (11). Studies in activity of nodules in fixing nitrogen have suggested that flowering is a critical period for the symbiotic system in Soybean (12). This confirms the study conducted by Pedersen (13), which notes that the demand for nitrogen is extremely high during seed formation immediately after flowering stage. Nitrogen fertilization enhance Sulla growth parameters compared to the uninoculated check, but not as much as the bio-fertilized plants. This result could be explained by the fact that *Rhizobium* bacteria possibly come into coaction with Plant Growth Promoting Rhizobacteria (PGPR). The coinoculation with bradyrhizobia and PGPR leads to an augmented number of the furthestmost vigorous nodules and plant yield as well as a better nitrogen fixation. PGPR could provide some nitrogen to plants via nitrogen fixation and might rise the nodule number, nodule dry weight, and plant dry weight of soybean (14). In fact, the greatest usually apprehensive modes of action of PGPR were phytohormone production, which improves plant development and growth (15). In Tunisia, it has been revealed that rhizobial inoculation of *Phaseolus vulgaris* increased abundance of the bacterial and Rhizobiaceae communities especially at the flowering and the harvesting stage more than nitrogen fertilized controls (16). Uninoculated plants were not able to form efficient nodules as those that had been inoculated at all plant stages. Nitrogen fertilization has no significant effect on nodulation (number and weight) at different growth stages (figure 3A, 2B). Conversely, *Rhizobium* inoculated plants showed a significant high mean nodule number per plant especially at 154 DAS and 174 Das (figure 3A), and a significant high mean nodule weight per plant mainly at 123 DAS, 154 DAS and 174 DAS (figure 2B). These results suggested that Goubellat soil is free of indigenous efficient rhizobia specific to *Sulla coronarium* L. Several authors have reported the beneficial effects of N₂-fixing bacteria and phosphate solubilizing microorganism on nodulation and legume yield (17). Possible coaction of *Rhizobium* bacteria inoculum and PGPR hosted in Goubellat soil could increase nodule number and nodule dry weight (14). Selecting efficient *Rhizobium* strains under greenhouse and field conditions for lentil, pigeon pea, clover and Chickpea increase nodule dry weight and shoot dry weight (18). Pii et al. (19) reported that *Rhizobium* inoculation increased nodule number on *Medicago* spp. Yadav and Verma (8) stated that the dual combinations of *Rhizobium leguminosarum* either with *Pseudomonas aeruginosa*,

Bacillus megaterium and *Azobacterchroococcum*, significantly enhanced nodules number and dry weight of nodules.

Calcareous soil often contains excess of nutrients, however high pH and active calcium carbonate reduces its availability. Then minerals deficiencies are often observed in these soils (20, 21). This study has demonstrated that *Rhizobium* has an additional advantage apart from the basic function of fixing atmospheric nitrogen; it significantly enhanced the uptake of Ca, P, K, Fe and N in *Sulla coronarium L.* shoots. Our results confirm the study conducted by Ndakidemi (22) which revealed that *Rhizobium* inoculation of *Phaseolus vulgaris L.* significantly improved uptake of Mn, Fe, Cu, Zn, B and Mo in all organs (roots, shoots, pods and whole plants except the Mo uptake in roots). In addition, Heggio and Barakah (23) confirmed that the enhancement of mineral uptake depended on *Rhizobium* density in the soil. In contrast, Togay et al. (24); Purbey and Sen (25) and Guo et al. (26) reported that rhizobial inoculation has not a significant effect in P and K shoot content. The capacity of bacteria to solubilize mineral phosphates and outturn siderophores has been of great interest to agricultural microbiologists, as it can improve the availability of phosphorus and iron for plant growth (8). Although, it has been suggested that different nitrogen fixing organisms may produce which may facilitate the solubility of nutrients such as Fe (27), P (28) and Zn (29) from different sources. Alikhani et al. (30) reported that many rhizobia isolated from Iran soil are able to mobilize P from organic to inorganic sources which can improve P plant nutrition. Similarly, Chabot et al. (31); Antoun et al. (32) confirm that most strains nodulating *Phaseolus* have been reported as P solubilizers bacteria. Plant growth improvement of rice plants under field condition was attributed to the ability of rhizobacteria to solubilize precipitated phosphates and increase phosphate attainability (33). Obtainability of P in soil and its uptake by plants could be increased due to solubilizing effect of acidic exudates produced by effective *Rhizobium* isolates and other microbes conspicuously existing in the rhizosphere⁸. Increased nutrient uptake with plant age has been reported in *Sesbania sesban* inoculated with *Mesorhizobium*³⁴. The better concentration and uptake of N and P in plants treated with microbial inoculations suggest that an affirmative interaction occurs among root colonization, N and P uptake, and growth raise (35).

5. Conclusion

Rhizobium inoculation was more effective for increased plant height, leaf area, shoot dry weight and nodulation in term of number and dry than supplying nitrogen fertilizers especially at 154 DAS and 174 DAS. Regarding nutrient content in shoot, *Rhizobium* inoculation had also better performance in enhancing nutrients uptake of this crop than nitrogen fertilization. It is important to state that the use of *Rhizobium* bacteria as a source of N, P, K and other micronutrients can also minimize dependence on chemical fertilizers.

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