Effects of Mycorrhizal and Rhizobial Co-inoculation on the Growth and Mineral Nutrition of Four Varieties of *Bituminaria bituminosa* L

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Citation

Abstract
The present research aims to evaluate the effect of arbuscular mycorrhizal fungi (AMF) and rhizobial inoculations, singly and combined, on the growth and the mineral nutrition of the forage shrub *Bituminaria bituminosa*. Four varieties of *Bituminaria bituminosa* (Mijas, Perdiz, Tenerife and B.bituminosa sp) were used and inoculated with *Rhizobium radiobacter* sole, or with one of the three species of AMF (*Glomus clarum*, *Gigaspora rosea* and *Glomus deserticola*) or with mixture of these symbiotic microorganisms (*Rhizobium + AMF*) at the same time. Plant growth was performed in a mixture of sand and soil poor in phosphorus. The essay was realized at the greenhouse the Faculty of Sciences, Moulay Ismail University. A positive response was recorded in the case of double inoculation and has improved both plant growth and mineral assimilation. The results showed an increase in the growth of the shoot dry matter twice compared with un-inoculated controls. About the relative efficiency of the accumulation of macro-essential elements, we noticed an improvement compared to controls absolute, 3 to 5 times the amount of nitrogen, 5 to 9 times for phosphorus and 3 to 6 times for potassium. The combination that gave the best response was that of Mijas - *Glomus clarum* – *Rhizobium radiobacter*. In summary, the microbial biofertilizers could be recommended to farmers to improve the nitrogen and phosphate nutrition for the deficient soils, which allowed reducing the utilization of the chemical fertilizers and consequently reducing the risk of pollution.

1. Introduction
In Morocco, the arid and semi-arid areas cover 87% of arable land (27% of the territory) and are subject to degradation [1]. The soils of the Mediterranean area are not homogeneous, thin or superficial and are most often calcareous [2]. These soils are often poor in essential minerals. To maintain the fertility of the soil, farmers use high quantities of chemical fertilizers that are expensive and pollutants. To deal with this problem, the use of microorganisms such as the arbuscular mycorrhizal fungi (AMF) and Rhizobacteria as bio-fertilizers will be a good economical, ecological and natural alternative to improve soil fertility.

The arbuscular mycorrhizal fungi (AMF) are obligate biotrophs, establishing a mutualistic relationship with more than 80% of all terrestrial plant species [3, 4] due to
the little selective pressure on either of the mutualistic partners [5-7], yet certain combinations of hosts and fungi are more effective than others for either the fungus or host [8, 9]. These soil inhabitants have shown their effectiveness of colonization on plant growth by the uptake of plant nutrients such as phosphorus [10-12] and micronutrients. These mycorrhizae fungi also enhance the resistance of plants to biotic and abiotic stresses [13] and improve the soil stability [14]. As so their use becomes important for natural and controlled ecosystems [15, 16].

As for the rhizobacteria forming a symbiotic association with legumes, they belong to the Rhizobium genus. These bacteria are of special importance due to their ability to fix atmospheric nitrogen. They produce 50% of 175 million tons of total biological nitrogen fixation, annually providing nearly half of all N$_2$ used in agriculture [17].

Their utilization is of major importance in lessening environmental pollution and the deterioration of nature [18]. Leading thus to friendly environmental and sustainable agricultural practices and organic farming systems [19, 20]. The third part of the combination studied is Bituminaria bituminosa which is perennial specie of legumes and which is widely distributed in the Mediterranean Basin and Macaronesia [21, 22]. This legume is used in Mediterranean region and the Canary Islands to provide hay or forage for livestock [9], although, its nitrogen fixation and drought tolerance make it suitable for sustainable production systems [23].

The present study aims at evaluating the effect of the single inoculation with both AMF or with Rhizobium and their combination on the Bituminaria bituminosa’s growth and mineral nutrition. The parameters evaluated are the fresh and dry weight of the Bituminaria bituminosa’s shoot, the mycorrhizal infection using the staining method of Philip and Hayman (1970) [24] and the observation using the Giovannetti and Mosse (1980) [25] method of intersecting lines [26], the mycorrhizal dependence was determined by the mass ratio of dry matter (DM) [27], the relative efficiency [28], total nitrogen accumulation using Kjeldahl method [29], total phosphorus and potassium accumulation by calcination. The data was statistically analyzed using student test.

2. Material and Methods

2.1. Substrate

The substrate was a mixture of 20% soil from Khenifra (U35) which is poor soil on phosphorus (P (Olsen-ppm) = 36.02, total P(mgP / Kg) = 1170.00, C/N = 13.10, total N (g / kg) = 2.17, = 2.84 C.org, M.org (%) = 4.90 and total CaCO$_3$ (%) <1.0) and 80% from river sand. The mixture was well washed, dried and sieved to 2 mm, and then sterilized by autoclaving at 120°C for 20 minutes.

2.2. Plants Material

For our study we have tested four varieties of Bituminaria bituminosa which are V1: Mijas, V2: Perdiz, V3: Tenerife and the last one is Bituminaria bituminosa sp (V4).

2.2.1. Seeds Treatment

The seeds were treated in H$_2$SO$_4$ at 95-97% for 30 min and then washed with distilled water to remove the acid excess. The surface sterilization of seeds was performed by successively passing for 5 min in HgCl$_2$ at 3% then in the ethanol at 95% followed by rinsing with sterile water.

2.2.2. Pre-germination and Seeds Sowing

The seed pre-germination was done in Petri dishes containing water agar (7g agar / l water) at 25°C for 2 to 4 days. After pre-germination, the seedlings were transferred into the alveoli, and then in large black plastic bag. The pots were watered three times a week with tap water. The experience was occurred in the greenhouse at the Faculty of Meknes.

2.3. Microorganisms Material

The microorganisms used for this work were 3 species of arbuscular mycorrhizal fungi and one species of rhizobium, isolated from Bituminaria bituminosa’s root nodules.

2.3.1. Arbuscular Mycorrhizal Fungi Inoculums

In the present work we have used three species of arbuscular mycorrhizal fungi: C1: Glomus clarum, C2: Gigaspora rosea and C3: Glomus deserticola.

The inoculation with the AMF was performed simultaneously with the seeding. The roots fragments of the barley were used as arbuscular mycorrhizal fungi inoculum (30 g per pot) mixed with the soil where the seedlings were sowing.

2.3.2. Rhizobial Inoculation

The specie used for this work was of Rhizobium, isolated from Bituminaria bituminosa’s root nodules grown in the experimental field of the faculty of Sciences.

a. Strain isolation and purification

The nodules were detached from the Bituminaria bituminosa roots, their surface was sterilized by soaking in ethanol at 95% for 5 to 10 seconds, then in a solution of HgCl$_2$ at 3% for 5 min and then rinsed thoroughly with sterile distilled water. Finally, the sterilized nodules were crushed in sterile screw cap tubes in the presence of a drop of sterile distilled water with a glass rod soaping alcohol. Then the homogenate was inoculated in a Petri dishes containing YEM (Yeast extract Mannitol) culture medium [30] and then incubated at 28°C for 4 days. A series of planting colonies formed Rhizobium was performed to purify culture.

The strain purity was by their macroscopic and microscopic characteristics (form bacteria and Gram stain).

b. Genotypic characterization

The molecular study involved one selected bacteria and was carried out at functional genomic platform, of the Technical Support Unit for Scientific Research, CNRST in Rabat - Morocco.

(i). Genomic DNA extraction
DNA extraction from bacterial strain on liquid culture using the kit "GenElute Bacterial Genomic DNA kit" from SIGMA, Aldrich according to the protocol provided.

(ii). 16S ribosomal DNA gene amplification

To amplify the 16S rDNA gene two primers were used fD1 (5’ AGAGTTTGATCCTGGCTCAG 3’) and rp2 (5’ ACGGCTACCTTGTACGACTT 3’) [31]. PCR amplification was carried out in a 25 µl reaction volume-containing template DNA (30 ng), Taq buffer (10x), MgCl2 (50 mM), dNTP mixture (10mM), fdl primer (100 µM), rp2 primer (100µM), and (5 U/µl) of Taq DNA polymerase. PCR amplification was performed with a « Verity » thermal cycler model from ABI. The PCR temperature profile used was 96°C for 4 min followed by 35 cycles consisting of 96°C for 10 s, 52°C for 40 s, 72°C for 2 min, with a final extension step at 72°C for 4 min. Reaction efficiency was estimated by horizontal agarose gel electrophoresis (1% w/v) using a molecular weight marker of 100 bp and photographed. The photos were displayed by the "G Box" photo documentation system.

(iii). 16S rDNA gene sequencing

Sequencing was performed on the 515 bp to 907 bp region of the 16S rRNA gene using the 3130XL Dye Terminator Cycle Sequencing (DTCs) Quick Start kit (Applied Biosystems) according to manufacturer's instructions with 25–100fmol template DNA and 0.2 µM 515F and 907R primers (515F: GTGCCAGCMGCCGCGGTAA, 907R: CCGTCAATTCCTTTRAGTTT) [32; 33; 34] For the purposes of this study, both strands of the 16S rDNA gene were sequenced. For one sample only the forwards strand was sequenced. The optimal thermocycling conditions for the cycle sequencing reaction were as follows: 25 cycles of 96°C for 1 min 96°C for 10s, 50°C for 5s, and 60°C for 4 min, followed by a 4°C infinite hold. The Sephadex G50 superfine (SIGMA ALDRICH) was used to remove unincorporated dye terminators from the cycle sequencing reaction, according to manufacturer’s instructions with an additional 300 µL wash of the column with distilled H2O and centrifugation at 15000×g for 3 min prior to applying the sample to the column. The sequences of the labeled and purified DNA samples were separated by capillary electrophoresis and determined with the ABI 3130xl Genetic Analyzer (Applied Biosystems).

c. Inoculum production and inoculation

The rhizobial inoculum was produced by sowing the pure and identified colony into an Erlenmeyer flask containing a broth yeast mannitol (YMB) and stirred at 28°C for 7 days.

The inoculum produced was applied three times; the first application was at the seeding time; the two other applications were separated from each other by a week, with the aim to ensure good root infection.

3. Results and Discussion

3.1. Genotypic Characterization

According to the expected size of the 16S rRNA gene [14] and for the rhizobial strain nodulating B. bituminosa, the 16S rDNA were amplified and a band of about 1500 was obtained.

The sequence analysis of 16S rDNA showed that the strain isolated corresponds to Rhizobium radiobacter with 100% whose accession number was AJ389904ALR.

3.2. The Shoot Dry Matter

The single inoculation by one of the three types of mycorrhizae showed a net increase in the dry weight of the shoot for the four varieties of Bituminaria bituminosa. The Glomus clarum was the better fungal inoculum applied for both varieties Mijas and B. bituminosa sp (620 mg / plant and 612 mg / plant respectively). Also the response to the single inoculation with Rhizobium radiobacter has shown a net increase on their shoot dry matter compared to the absolute control plants. And the best response was recorded by Mijas which has a weight of 319mg/plant compared to the absolute control which its weight was 191mg/plant. Whereas in the case of the double inoculation (Mycorrhizae + Rhizobium), the improvement of the dry matter weight of the aerial part is more important in comparison to the single inoculation, especially for the variety Mijas, inoculated with Glomus clarum and Rhizobium radiobacter, whose dry matter weight was 980 mg/plant (Figure 1).

![Figure 1](image_url)
confirms that reported by Ibijbijen (1996) [40] tested for the bean, which was testified Fikri benbrahim (2002) [1] in the case of Acacia Saliga and confirmed even for Chamaecytisus proliferus [37]. This improvement can be attributed to the better exploitation of soil phosphorus [41], to the enhancement of the biological fixation of atmospheric nitrogen and can also be a good acquisition of other mineral elements including Zn, Mn and even nitrates [42, 12].

### 3.3. The Mycorrhizal Dependency

The Mycorrhizal dependency differs according to different mycorrhizal species used and also in terms of the four varieties of *Bituminaria bituminosa* studied. Thus this parameter showed identical means 329% as better value as a result of mycorrhizae treatment particularly the genus Glomus. A wide variety, the variety *B. bituminaria* sp leads with a dependency that varied around 419.19% under *Glomus clarum* or *Glomus deserticola* treatments.

The dual inoculation increased this parameter that was more important than the single inoculation, and its increase was of 62.51% and 103.29% for *Glomus clarum* + *Rhizobium* and *Gegaspora rosea* + *Rhizobium* respectively. However, the *Rhizobium* effect was negligible when combined with the *Glomus deserticola* treatment.

In conclusion, the best combination which gave the best relative efficiency was better in the case of *Glomus clarum* and *Rhizobium* - Tenerife (659.84%) (Figure 2).

These results show that mycorrhizal dependency is closely related to the shoot dry weight and the mineral nutrition. In general they increased when the dependence increases with either single or double inoculation. And this may be due to the extra root hyphae that absorb more minerals when they are abundant, which can improve the shoot dry weight production.

Mycorrhizal dependency observed in plants inoculated with *Rhizobium* and AMF was higher than in plants inoculated with mycorrhizae alone. The same result was found by Kucey and Bonetti (1988) [5] in the fields of beans, El-Kherbawy et al. (1989) [43] for alfalfa and Xie et al. (1995) [44] for soybean. Because our plant material used is a forage shrub, so our results confirm those recorded by Siequira J.O and Franco A.A (1988) [45].

### 3.4. The Relative Efficiency

According to Figure 3, the single inoculation was the most efficient especially that applied with *Glomus deserticola* with an efficiencies average of 64.27% for the 4 *Bituminaria bituminosa* varieties.

Generally, the treatments with *Glomus clarum + Rhizobium* and *Rhizobium + Gegaspora rosea* were the most effective because they showed an average of 78.78% and 76.50% respectively of relative efficiencies. Whereas a negligible effect was observed when applying the treatment *Glomus clarum + Rhizobium* on *B. bituminosa sp* and the treatment *Glomus deserticola + Rhizobium* on Tenerife and *B.bituminosa sp*.

Between the four varieties tested, Mijas and Tenerife were the leading in response to the *Glomus clarum + Rhizobium* treatment (80.44% and 83.80% respectively).

The Mycorrhizal dependency differs according to different mycorrhizal species used and also in terms of the four varieties of *Bituminaria bituminosa* studied. Thus this parameter showed identical means 329% as better value as a result of mycorrhizae treatment particularly the genus Glomus. A wide variety, the variety *B. bituminaria* sp leads with a dependency that varied around 419.19% under *Glomus clarum* or *Glomus deserticola* treatments.

![Figure 2. The mycorrhizal dependency of the shoot of the four varieties of Bituminaria bituminosa under different treatments (No. C- No Rh = absolute control, No C-With Rh = Rhizobium alone, C1 = Glomus clarum, C1 + Rh = Glomus clarum and Rhizobium, C2 = Gegaspora rosea, C2 + Rh = Gegaspora rosea and Rhizobium, Glomus deserticola = C3, C3 + Rh = Glomus deserticola and Rhizobium). (P=5%).](image)

The dual inoculation increased this parameter that was more important than the single inoculation, and its increase was of 62.51% and 103.29% for *Glomus clarum + Rhizobium* and *Gegaspora rosea + Rhizobium* respectively. However, the *Rhizobium* effect was negligible when combined with the *Glomus deserticola* treatment.

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### 3.5. The Shoot Content on the Total Phosphorus

The results have shown that the accumulation of the...
absolute control plants on the total phosphorus was low in all results knowing that we have used a substrate poor in phosphorus.

However, an improvement of this accumulation was observed even in the case of single inoculation as in the double. The accumulation rate on the total phosphorus was highly dependent on the applied treatment. When applying single inoculation with the AMF we have observed that in response to the Glomus clarum, Gegaspora rosea and Glomus deserticola treatments the total phosphorus accumulated was of 550.43mg/plant, 364.34mg/plant and 473.04mg/plant respectively.

![Figure 4](image)

**Figure 4.** The phosphorus content on the shoot plant of the four Bituminaria bituminosa varieties in response to the different treatments (No. C- No Rh = absolute control, No C- With Rh = Rhizobium alone, C1 = Glomus claram, C1 + Rh = Glomus clarum and Rhizobium, C2 = Gegaspora rosea, C2 + Rh = Gegaspora rosea and Rhizobium, Glomus deserticola = C3, C3 + Rh = Glomus deserticola and Rhizobium) (P=5%).

Plants under single inoculation with Rhizobium have accumulated more than the double amount of phosphorus (0.251mg/plant) comparing to control plants (0.115mg/plant). Indeed the best result was recorded in the case of the dual inoculation concerning all Bituminaria bituminosa’s varieties except of the tripartite interaction Rhizobium – Glomus deserticola – Mijas.

This result has shown the strong relationship between mycorrhizal colonization and plants phosphorus content. Our result is in well concordance with the results found by Ibijibjen 1996 [40] and Liu 2002 [46]. The shoot phosphorus content was more important under mychorrizal treatment and especially in the case of double inoculation. This last result confirms that reported by Fikri Benbrahim (2002) for Acacia saligna [1].

Generally, the efficiency of mycorrhizae to improve the plants phosphorus nutrition is mainly due to the ability of mycelial hyphae to better exploit the pool of available soil phosphorus [4]. Thus, the phosphate accumulated at the ends of hyphae mycelium is translocated into the system that transfers to the various organs of the host plant. These attributes allow an adequate improvement of the biomass of inoculated plants.

### 3.6. The Shoot Content on the Total Nitrogen

Belonging to the result found, the most important accumulation of total nitrogen was reported for B. bituminosa sp under the Glomus deserticola treatment (10.297mg/plant), then Mijas inoculated with Glomus claram and then Mijas treated by Gegaspora rosea (8.921mg/plant and 10.210mg/plant respectively). The single inoculation with Rhizobium has improved this parameter in comparison with the absolute control. The Rhizobium is known for its main role on fixing biologically the atmospheric nitrogen. In the present study, the plants under single inoculation by the Rhizobium have shown a beneficial effect on its shoot total nitrogen comparing to the control plants. This result corroborates that of Alves (2003) [47]. This improvement was greater in the case of the double inoculation with mycorrhiza and Rhizobium. For this parameter the best tripartite interaction was shown for Mijas which was under Glomus claram + Rhizobium treatment (Figure 5).

![Figure 5](image)

**Figure 5.** The total nitrogen content on the shoot plant of the four Bituminaria bituminosa varieties in response to the different treatments (No. C- No Rh = absolute control, No C- With Rh = Rhizobium alone, C1 = Glomus claram, C1 + Rh = Glomus claram and Rhizobium, C2 = Gegaspora rosea, C2 + Rh = Gegaspora rosea and Rhizobium, Glomus deserticola = C3, C3 + Rh = Glomus deserticola and Rhizobium).

In light of these results the importance of mycorrhiza on improving nodulation and nitrogen fixation and hence the response to double inoculation was better than that to the single inoculation with Rhizobium or mycorrhiza separately. This effect is similar to results found testing the soybean [14, 48], the Medicago [36] and the Stylosanthes [10]. The significant improvement of nitrogen in the case of single inoculation by different species of AMF can be explained by the proper exploitation of soil nitrogen by these microorganisms.

### 3.7. The Shoot Content on the Potassium

Generally, mycorrhizal inoculation induced increases in the amount of potassium absorbed by all varieties of Bituminaria bituminosa particularly under Glomus claram treatment which its content on the potassium was of an average of 10.417mg/plant. The single inoculation with Rhizobium has improved slightly the macronutrient accumulation (4.783mg/plant for Mijas comparing to the control 2.655mg/plant). The combination of mycorrhizae and Rhizobium effects increased significantly the accumulation
of potassium in the shoot plant and especially the association *Glomus clarum + Rhizobium* which revealed a good accumulation for Mijas, Perdiz, Tenerife and *B. bituminosa* sp (21.514mg/plant, 16.440mg/plant 14.592mg/plant, 13.495mg/plant and respectively). However and exceptionally the combination of *Glomus deserticola + Rhizobium* had shown a negative effect for Mijas and *B. bituminosa* sp. (Figure 6).

![Figure 6](image-url)

*Figure 6. The potassium content on the shoot plant of the four Bituminaria bituminosa varieties in response to the different treatments (No. C: No Rh = absolute control, No C-With Rh = Rhizobium alone, C1 = *Glomus clarum*, C1 + Rh = *Glomus clarum* and *Rhizobium*, C2 = *Gegaspora rosea*, C2 + Rh = *Gegaspora rosea* and *Rhizobium*, *Glomus deserticola* = C3, C3 + Rh = *Glomus deserticola* and *Rhizobium*) (p=5%).*

The mycorrhizal inoculation single or combined with Rhizobium increased the potassium concentration in the shoot of *Bituminaria bituminosa*, this result confirmed that reported by Ibijbien (1996) for beans [40]. We noticed that the best response was that of Mijas co-inoculated by *Glomus clarum* and the *Rhizobium radiobacter*. The main effect of the inoculation was noteworthy comparing to the absolute control and also compared to the result showed by Ventura [49].

Since the potassium is a very mobile in soil, and its accumulation in plant may be explained by the role of the mycorrhizal inoculation and / or rhizobia alone, as shown by our results, which improve greatly the absorption of many mineral element.

4. Conclusion

In light of the results, we can conclude that the single inoculation with AM fungi or with rhizobium improves the biomass production and the mineral nutrition of all varieties of *Bituminaria bituminosa* studied, and the best result was shown in the case of the double inoculation. This leguminous studied is known as a useful perennial shrub. This plant is very used as good fodder due to its high nutritional value on one hand, and also for its contribution to restore and enhance soil fertility on ensuring the biological fixation of the atmospheric nitrogen. All these advantages and benefits may be provided once we identify the most promoting tripartite symbiosis: Plant – Rhizobium - AMF.

These microbial biofertilizers could be recommended to farmers to improve the nitrogen and phosphate nutrition for the deficient soils, which allowed reducing the utilization of the chemical fertilizers and consequently reducing the risk of pollution.

References


