



Evaluation of the Symbiotic Efficiency of *Rhizobium* Strains Applied to *Bituminaria bituminosa* (L.) Stirton

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Authors' contributions

This work was carried out in collaboration among all authors. Author OZ designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author AR participated in the design of the study and managed the analyses. Author MEM contributed in the interpretation of data and managed the literature searches. Authors JI and LN supervised the work, developed discussion and improved the quality of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The study aimed to evaluate the effect of inoculation by different rhizobacteria on *Bituminaria bituminosa* plants grown under greenhouse conditions.

Study Design: An experimental study.

Place and Duration of Study: The study was carried out at the Department of Biology (Environment and valorization of microbial and plant resources Unit), Faculty of Sciences, Moulay Ismail University-Meknes, from November 2019 to February 2020.

Methodology: Eleven species and/or isolates belonging to *Rhizobium* genus are used to inoculate *B. bituminosa* plants; similarly, fresh and dry crushed nodules previously collected from *B. bituminosa* shrubs are tested. The bacterial inoculation effects are evaluated through the estimation of inoculated plants' fresh and dry shoots weight, root dry weight, total nitrogen, nodules number

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and fresh weight in comparison to non-inoculated plants. The infectivity and efficiency of the bacteria and the biological nitrogen fixation are also evaluated.

Results: The results enable us to select the infective strains on the basis of their positive effect on growth and total nitrogen, in order to produce inoculum for *B. bituminosa*. Efficiency and biological nitrogen fixation are also very high compared to the control, especially with the B.b1 strain isolated from *Bituminaria bituminosa* and identified as *Rhizobium tibeticum*. The fresh nodules crushing is also very efficient. as inoculant.

Conclusion: The use of symbiotic complex as *Rhizobium tibeticum* – *Bituminaria bituminosa* or an inoculum produced from fresh nodules are an eco-friendly alternative for the design of sylvo-pastoral systems ensuring increased soil fertility, fodder productivity and sustainable agroforestry.

Keywords: Bituminous clover; Rhizobacteria; inoculation; infectivity; nitrogen fixation; fodder; sustainability.

1. INTRODUCTION

In agriculture, nitrogen is one of the key factors for agricultural productivity and competitiveness [1]. It is a building block of living processes, which is used by animals and plants for nucleic acids and proteins synthesis.

In the plant world, legumes have the particularity of exploiting the molecular nitrogen present in abundance in the atmosphere, unlike the other plants which can use only mineral nitrogen present in limited quantities in the soil [1]. This is possible due to symbiosis with soil bacteria, particularly the *Rhizobium* or *Bradyrhizobium* genera, which fix nitrogen in specialized root organs called nodules. Therefore, legumes are of great agronomic and zoo-technic interest, both for their own nutrition, animal feed [2], and for improving soil fertility, especially in terms of nitrogen [2,3], since part of the fixed nitrogen returns to the soil by rhizodeposition via nodules as well as roots senescence and also via exudation, particularly of ammonium and amino acids [1].

Furthermore, the integration of leguminous species into agricultural systems is very beneficial; firstly, they provide forage from shoots and seeds; secondly, through the set of ecosystem services provided to the crops that succeed them or with which they are associated, they enable better overall productivity with fewer inputs [1].

So, in several tropical African countries, many legume shrubs are integrated in agroforestry and alley-cropping systems; species such as *Cajanus cajan*, *Leucaena leucocephala*, *Gliricidia sepium* and *Albizia lebeckare* associated with food crops as a source of nitrogen [3]. The role of these woody plants is not only fodder through

edible foliage, pods or seeds; they also contribute to improve the soil fertility by fixing carbon and nitrogen, and consequently fight against wind and water erosion by providing permanent plant cover [3,4]. Indeed, the ability of soils to maintain their production potential constitutes the basis for their reproducibility and sustainability [4].

In Morocco, several legume shrubs are found in natural rangelands, such as *Argyrocyttisus battandieri* in the Middle Atlas Mountains, *Genista linifolia* in the forest of Maâmora and *Retama sphoeorocarpa* and *Adenocarpus bacquei* in the pre-steppes of the upper Moulouya. Unfortunately, the abusive use of these resources, already weakened by drought, is not without ecological and socio-economic negative impacts [5]. Pastoral resources are becoming scarcer and the vegetation cover is continually shrinking, opening the way to desertification and compromising the natural regeneration of sylvopastoral formations [6,7,8]. Face to this alarming situation and in the context of the national sylvopastoral strategy elaborated in 2016 [9], the implementation of an alternative reconciling fodder needs and the rehabilitation of degraded pastoral land is imperative; that's why multi-purpose plants such as *Bituminaria bituminosa* (L.) Stirton is promising; this leguminous shrub, commonly known as bituminous clover, could provide a significant fodder biomass while improving the fertility of degraded soils due to its hardness, rapid growth and abundant evergreen foliage [10].

In the past, the symbiotic fixation of atmospheric nitrogen has been at the origin of the important place of leguminous plants in cropping systems and pastures; recently, interest in these plants is renewed in an agro-ecological perspective that mobilizes biological processes for sustainable

agricultural production, in respect to the environment [1].

The present study is therefore a part of a serial studies carried out by our research team on the valorization of *Bituminaria bituminosa* (L.) Stirton, with the ultimate objective to introduce it into degraded sylvopastoral sites in the Central Middle Atlas region [11,12,13,14,15,16]. The specific objective of this study is to evaluate the response of *B. bituminosa* to different rhizobia strains inoculation and therefore to assess the efficiency of the symbiosis.

2. MATERIALS AND METHODS

2.1 Seed Pregermination and Experimental Design

To evaluate the effect of rhizobia inoculation on the biometric growth parameters of *B. bituminosa* as well as its ability to fix atmospheric nitrogen, seeds were superficially disinfected under a laminar flow hood to avoid the eventuality of contamination. They were immersed for 3 minutes in a 1% sodium hypochlorite solution and then submitted to a series of rinses with sterile distilled water. After, disinfection, the seeds were sown in plates containing sterile peat and were incubated during two weeks for pre-germination in a culture chamber at a temperature of 28°C.

The seedlings were transplanted into plastic pots containing 750 g of sterile sand; under greenhouse conditions. The experimental design was a completely randomized block with five replicates. The experiment was conducted from November 2019 to February 2020. The plants were watered 3 times a week to keep the pots at the field capacity and during the two first weeks, they were supplied with Hoagland's nutrient solution [17].

2.2 Bacterial Strains and Inoculation Treatments

A total of thirteen types of inoculum were applied to *B. bituminosa* seedlings; there were three strains B.b1, B.b2 and B.b3, previously isolated from *B. bituminosa* nodules by our research team and identified respectively as *Rhizobium tibeticum* (EU256404), *Rhizobium radiobacter* (AJ389904), *Rhizobium nepotum* (FR870231) at the CNRST of Morocco. Three others strains S27, S36 and S39 were isolated from *Adenocarpus boudiyi* nodules and identified respectively as *Rhizobium sp.* (MF767516), *Rhizobium pusense* (MF767516) and *Rhizobium*

radiobacter (MF767518). Also, two strains P11 and P23 isolated from *Vicia faba* and corresponding to *Rhizobium leguminosarum* (USDA 2370) and *Rhizobium nepotum* (39/7) were applied, in addition to three isolates BT1, BT2 and BT3 obtained from nodules of *Bituminaria todghaniensis* and which are not yet identified.

At the same time, fresh nodules collected from *B. bituminosa* roots and others that were kept in a dehydrated state, then soaked, were crushed after surface disinfection with CaCl₂ and used as inoculum (fresh nods, dry nods).

When transplanted into the pots, the seedlings received 5 ml of freshly prepared bacterial suspension (10^9cfu.ml^{-1}) from the above-mentioned strains grown on YMA medium, then on YM broth. Then, the inoculation was repeated three times at regular intervals of one week. Two controls were used; non-inoculated *B. bituminosa* seedlings (inocul control) and seedlings of *Atriplex nummularia*, a non-nitrogen-fixing plant belonging to the Chenopodiaceae family (absolute inocul fix control).

At the end of February 2020, all the pots were sacrificed and the biometric parameters were determined; thus, after the estimation of the fresh aerial biomass, the root and aerial dry biomass were determined by steaming at 65°C for 72 hours.

The number and fresh weight of nodules were determined. Therefore, the symbiotic efficiency of the bacterial strains was assessed by evaluating the enhancement of the aerial dry weight (DW) after inoculation [18,19].

$$\text{Efficiency} = (\text{Dry weight of inoculated plant} / \text{Dry weight of control}) \times 100$$

The biological nitrogen fixation (BNF) was evaluated using the classical difference method:

$$\text{BNF} = 100 \times (\text{Total nitrogen of inoculated plant} - \text{Total nitrogen of control plant}) / \text{Total nitrogen of inoculated plant}$$

The total nitrogen (TN) content accumulated by the aerial part was estimated by the Kjeldhal method [20].

2.3 Statistical Analyses

The data related to both the growth parameters, nodulation, total nitrogen (TN) content and symbiotic efficiency were subjected to an

analysis of variance (ANOVA I) using the general linear model procedure via SPSS version 17 software. Means were compared for significance using the least significant difference method (LSD) at $P < 0.05$.

3. RESULTS

3.1 Infectivity of the different strains inoculated to *B. bituminosa*

The results showed that not all of the strains tested in this study were able to induce nodule formation on the roots of *Bituminaria bituminosa* seedlings (Table 1); they can therefore be divided into two groups; the first one contains the infective strains Bb1, Bb2, Bb3, S36, S39 plus the two types of crushed nodules. The second group contains the non-infective strains: S27, P11, P24, BT1, BT2, and BT3. Also, no nodulation was observed on the non-inoculated *B. bituminosa* plants used as controls (inocul).

Furthermore, the degree of infectivity of each strain could be measured by the number of nodules formed on the host plant; also, the nodules fresh weight is another good indicator of infectivity since it provides information on the living cells "bacteroids" inside the nodules. So, the results (Figs. 1-A and 1-B) show that the fresh crushed nodules can be considered as the most infective treatment with more than 78 nodules/plant with an average fresh weight of 1.52 g.plant⁻¹. On the other hand, the lowest value is obtained with the S39 strain, with only 2 nodules/plant and an average fresh weight of 0.02 g.plant⁻¹. Also, the statistical analyses reveal three homogeneous groups for the parameter "number of nodules" where the difference is

significant and within which the values obtained are statistically similar at $p < 0.05$. In descending order, these groups are: fresh nod, dry nod / Bb1, Bb2, Bb3, S36 / S39, non-inoculated control. The parameter "nodules fresh weight" shows the same trend as the one observed with the nodules number, with the addition of the segregation of the first homogenous group into two subgroups, since the value recorded with the crush of fresh nodules is statistically higher than the value obtained with the crush of dry nodules.

3.2 Effect of Bacterial Inoculation on the Shoot Biomass of *B. bituminosa*

In the present study, the objective of the inoculation is the estimation of biological nitrogen fixation by the *B. bituminosa*-*Rhizobium* sp. complex; therefore, non-infective strains were eliminated.

The Fig. 2 shows that rhizobial inoculation has a positive effect on the growth of *B. bituminosa*; indeed, there is a significant increase in fresh and dry shoot weights compared to the non-inoculated control.

The comparison between the strains shows a significant difference in the magnitude of their positive effect on the aerial biomass, both between them and in comparison with the control. S39 is the worst strain for growth stimulation compared to the control. Also, strains B.b1, B.b3 and S36, seem to be the most promising for *Bituminaria bituminosa* growth improvement. Crushed nodules are also effective in stimulating growth, with a slight superiority of the crush of fresh nodules over the crush of the dry ones.

Table 1. Response of *B. bituminosa* to the bacterial inoculation

Qualifier	Strains	Inoculation response
Infectives	B.b1	+
	B.b2	+
	B.b3	+
	S36	+
	S39	+
	Fresh Nod	+
	Dry Nod	+
Non Infectives	S27	-
	P11	-
	P24	-
	BT1	-
	BT2	-
	BT3	-

(+): Presence of root nodules; (-): Absence of root nodules

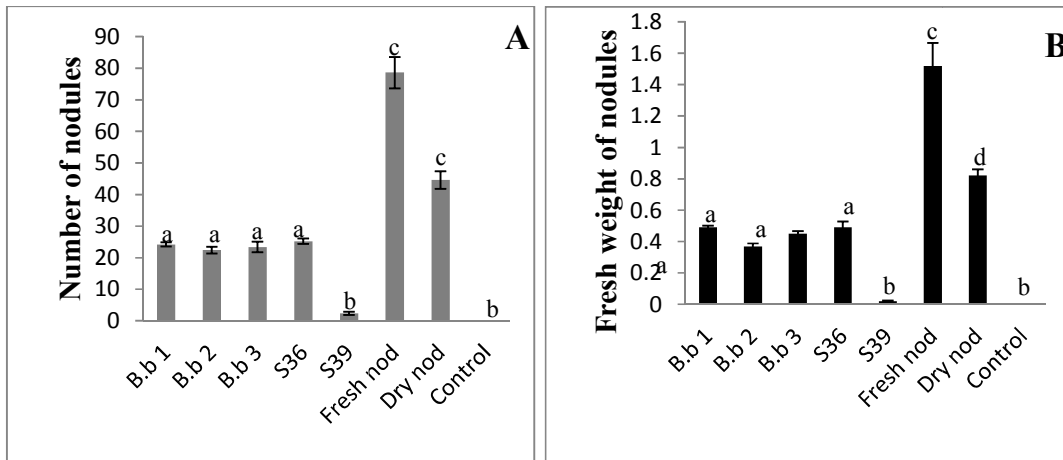


Fig. 1. A- Number of nodules and B- Fresh weight of nodules formed on *B. bituminosa*
(Means with the same letter do not differ significantly at $P < 0.05$)

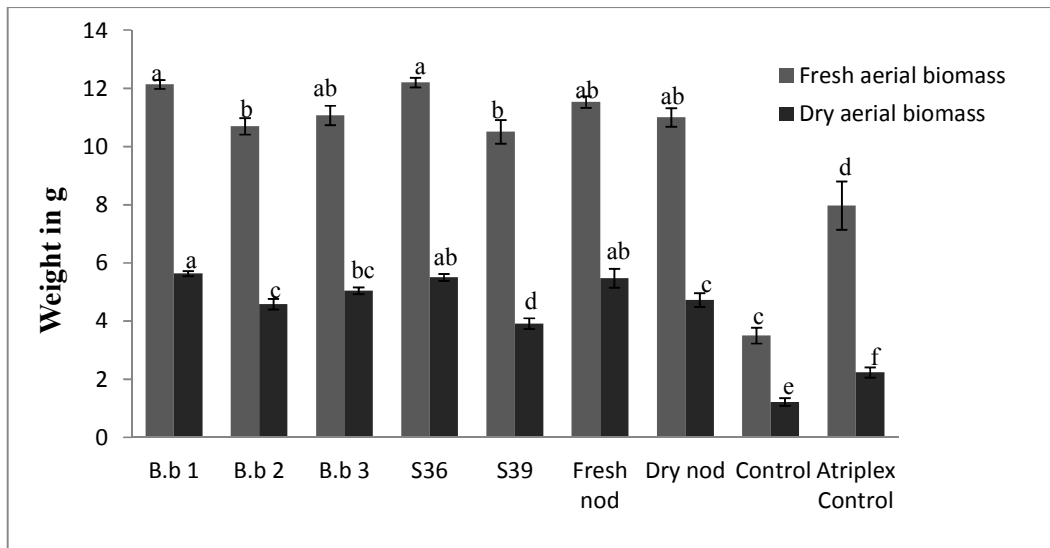


Fig. 2. Effect of the bacterial inoculation on fresh and dry above-ground biomass of *B. bituminosa* plants
(Means with the same letter do not differ significantly; LSD Test at $P < 0.05$)

Expressed as a rate of improvement over the non-inoculated control, the amplitude of fresh weight stimulation is 2.49; 2.49; 2.47; 2.16; 2.14 2.06; 2, respectively, for treatments S36, fresh nod, Bb1, Bb3, dry nod, Bb2, S39. For the dry weight, these rates are 3.58; 3.48; 3.45; 3.10; 2.85; 2.73; 2.19, respectively for B.b1, S36, fresh nod, B.b3, dry nod, Bb2 and S39.

3.3 Effect of Bacterial Inoculation on the Root Biomass of *B. bituminosa*

The root dry weight is shown in Fig. 3; the difference between inoculated plants and the

non-inoculated control is clearly significant, and the lowest root dry weight value is obtained with the S39 strain.

The strains value obtained with B.b1, B.b3 and S36 are statistically similar, and are higher than the dry weight of the control as well as than the dry weight of the others inoculation treatments at $P < 0.05$ (Fig. 3). However, contrary to what has been observed with the aerial biomass for crushed nodules, when considering root dry weight, there is no difference between the crushed dry and the crushed fresh nodules.

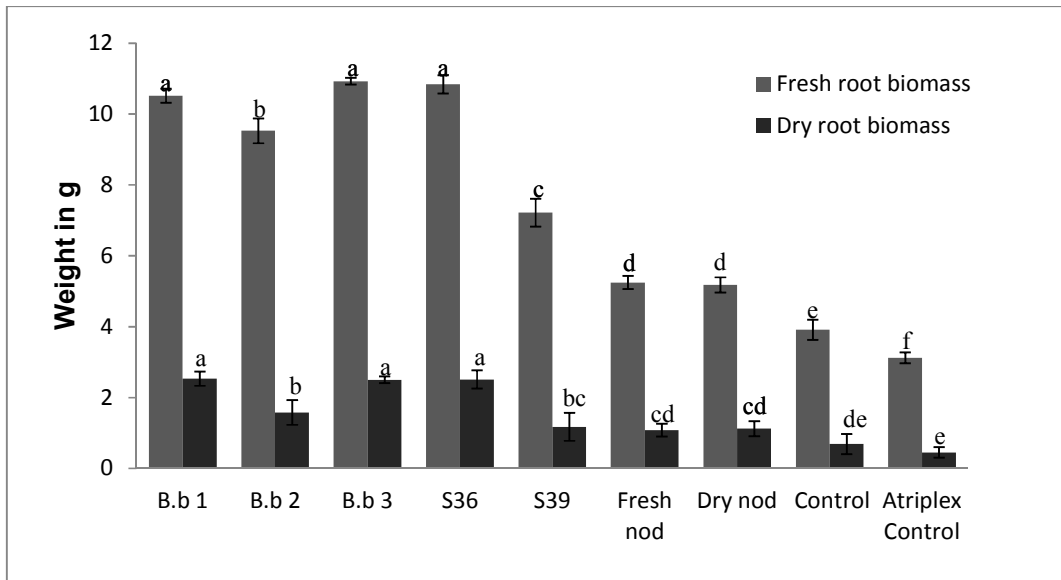


Fig. 3. Effect of the bacterial inoculation on fresh and dry root biomass of *B. bituminosa*
(Means with the same letter do not differ significantly; LSD test at $P < 0.05$)

3.4 Effect of Bacterial Inoculation on the Nitrogen Nutrition of *Bituminaria bituminosa*

The Figs. 4 and 5 show, respectively, the results of the variation of the percentage and total nitrogen in the aerial part of plants; it appears that the effect of inoculation is positive on both of the two parameters in comparison to the non-inoculated control.

For the nitrogen content, the comparison of the means at $P < 0.05$ enable us to identify five homogeneous groups between which the difference is significant; classified in decreasing order, there are: fresh nod (1.91%)/B.b1, B.b3, dry nod/ B.b2, S36/ S39 (0.39%)/control. For the total nitrogen content, the difference is significant at $P < 0.05$ between the inoculated plants and the control. While with the strain S39, the parameter reaches the lowest value ($0.014 \text{ g. plant}^{-1}$); the plants inoculated with B.b1 have the highest content ($0.092 \text{ g. plant}^{-1}$) which is similar to the N content ($0.09 \text{ g. plant}^{-1}$) of the plants inoculated with B.b3 as well as with crushed fresh nodules. Between these extreme values, those recorded with the others treatments are 0.07, 0.076, 0.08 g. plant^{-1} for B.b2, crushed dry nodules and S36 respectively.

3.5 The Efficiency

The results of infectivity and efficiency allow the screening of the strains with the best power of

infection and nitrogen fixation; thus, expressed as a percentage of the dry weight improvement of inoculated plants in comparison to the non-inoculated control, the efficiency of all strains and tested isolates is remarkable and varies between 458% for Bb1 and 319% for S39.

3.6 Biological Nitrogen Fixation

To estimate the biological nitrogen fixation by *B. bituminosa*, the classic method known as the total nitrogen difference method is used. The legume uptake the nitrogen from two sources (soil and atmosphere) while the control (Uninoculated *B. bituminosa* or *Atriplex nummularia* plants) have only access to the nitrogen from the soil; so, any difference in nitrogen content between the two types of plants would be due to nitrogen fixation.

The Fig. 6 shows the percentages of symbiotically fixed nitrogen; in comparison to the non-inoculated control, it appears that almost all the nitrogen comes from the atmosphere, with values ranging from 97% for all isolates and strains, except for S39 where the estimated value is 86%. The difference is significant mainly between S39 and all the others strains and isolates whose are statistically similar to each other.

However, on the basis of *A. nummularia* total nitrogen, the values of the percentage of fixed

nitrogen estimated by the difference method are lower and do not exceed 55% for B.b1, the most efficient strain, and are even quite low in S39,

with only 15%. Here, the difference is significant between the four homogeneous groups Bb1, Bb3, fresh nod/ S36, S39, dry nod/ B.b2/ S39.

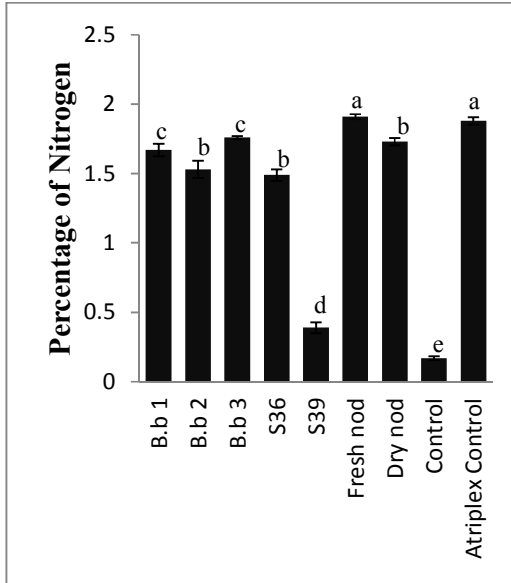


Fig. 4. Nitrogen Percentage in the aerial part of *B. bituminosa* according to the inoculation treatments
(Means with the same letter do not differ significantly; LSD Test at $P < 0.05$)

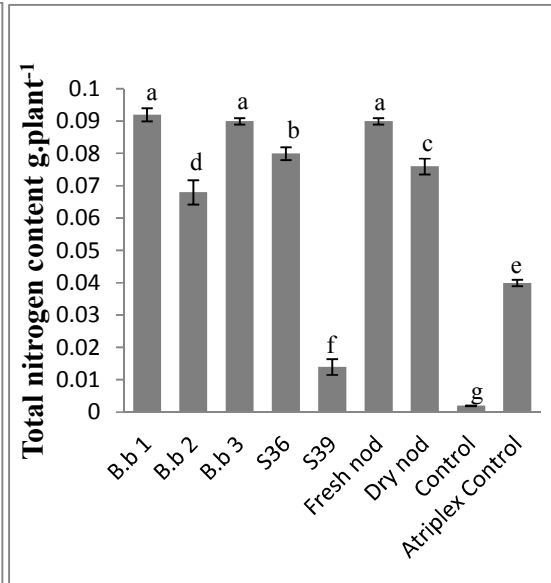


Fig. 5. Total Nitrogen content in the aerial part of *B. bituminosa* according to the inoculation treatments
(Means with the same letter do not differ significantly; LSD Test at $P < 0.05$)

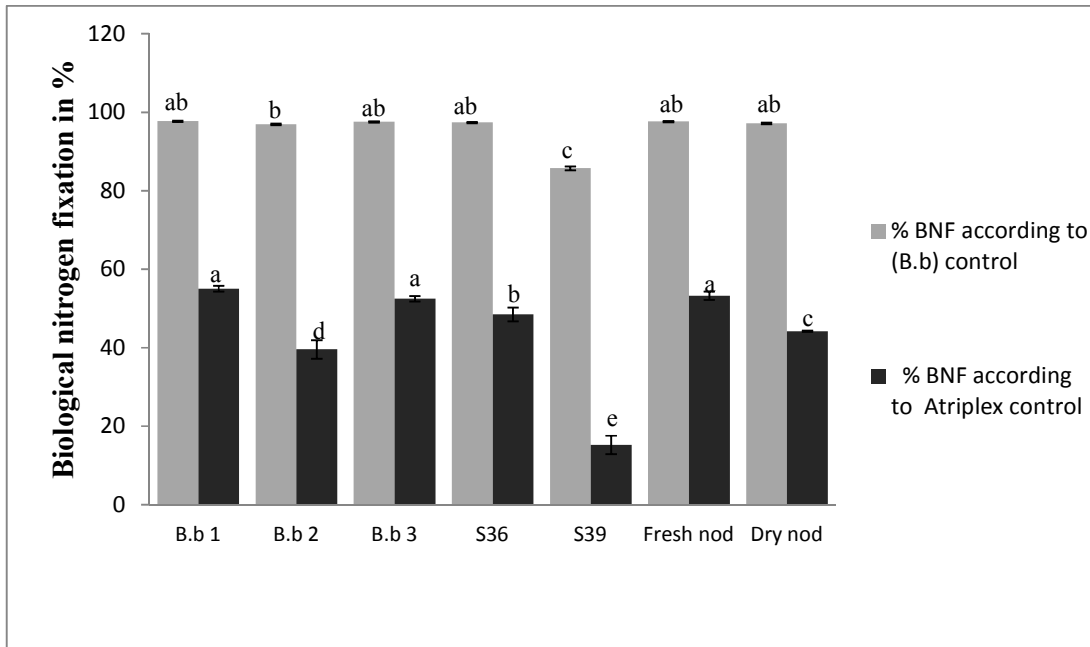


Fig. 6. The percentage of fixed nitrogen according to the type of control used: *B. bituminosa* non-inoculated and *Atriplex nummularia* a non-nitrogen fixing species
(Means with the same letter do not differ significantly; LSD Test at $P < 0.05$)

4. DISCUSSION

After the harvest, the determination of the main biometric traits of the plants, in particular, the shoot fresh and dry weights, the dry root weight and the nodules number and fresh weight enable us to investigate the comparison between inoculated plants and non-inoculated ones.

The results show that *Bituminaria bituminosa* is not infected by all strains and isolates involved in this study; in fact, it doesn't respond to any of the strains isolated from the bean, neither to one strain isolated from the adenocarp and none of the isolates from Todgha clover. This would be due to the specificity of these strains to their respective hosts; or, for the Todgha clover isolates, until they have been identified, it could not be confirmed that they are rhizobia.

The infective strains are derived from *Bituminaria bituminosa* and *Adenocarpus boudyi*. Nodules shredding also induced nodulation; the superiority of fresh nodule shredding over nodule dehydrated shredding can be due to the fact that in dried and stored nodules, the rhizobia have lost their viability and infectivity. For the control, the absence of any nodules is a proof, on the one hand, that the soil sterilization was successful and destroyed any indigenous strain; this proves that no cross-contamination took place between the inoculated and non-inoculated pots.

In addition, infectivity and efficiency are commonly used to assess the ecological and evolutionary relationships between rhizobiums and their hosts [21,22]. Although all the retained strains show an ability to induce nodule formation on the roots of the host plant, the degree of infectivity is clearly different between treatments; so, S39 "*Rhizobium radiobacter* (MF767518)" is the least efficient in terms of nodule number and fresh weight; then comes the group formed by Bb1, Bb2, Bb3 and S36, corresponding respectively to *Rhizobium tibeticum* (EU256404), *Rhizobium radiobacter* (AJ389904), *Rhizobium nepotum* (FR870231) and *Rhizobium pusense* (MF767516). The nodule crushed are the most efficient, both in number and in fresh weight of nodules; this could be due to multiple infection; each nodule crushed contains more than one rhizobial strain whose infectivity is confirmed since they come directly from a nodule, considered to be the site of symbiotic nitrogen fixation.

Otherwise, all the biometric parameters measured were significantly improved by inoculation and the strains B.b1, B.b3 and S36 were the most effective.

The biomass improvement by the application of nitrogen fixing bacteria is attributed primarily to biological nitrogen fixation, considered to be one of the first qualities of rhizobacteria promoting plant growth [23]; whereas, in the present experiment, non-inoculated plants have only a washed and poor substrate, unable to supply their nutrient requirements.

Rhizobial inoculation increases the biomass, nitrogen and phosphorus content of plants [24]. Also, the most successful strains in this trial are phosphate-solubilizing bacteria (BSP) [25,26]. Others advantages can be associated with inoculation with rhizobacteria like rhizobiums, notably the direct stimulation of the synthesis of phytohormones such as indole acetic acid [27,28,29], the induction of systemic resistance in legumes [30] or the reduction of the effect of pathogens [31].

The difference is also significant between inoculated and non-inoculated plants in terms of nitrogen content; the percentage of nitrogen in inoculated plants with crushed fresh nodules is significantly higher, followed by B.b1, B.b3 and crushed dry nodules. The link is to be made directly with the efficiency of the inoculated strains in nitrogen fixation as the percent estimated by difference from the non-inoculated control, is about 97% for all treatments, except for S39 where it is 86%. With *Atriplex nummularia* as a control, the estimated values of nitrogen fixation are lower (15% for S39 and maximum 55.07% for Bb1); this shrub, even if it is not of the same species or family as *B. bituminosa*, it has developed the same root biomass as the non-inoculated control, but its above-ground biomass and nitrogen content are much higher, which is probably due to a higher growth rate. However, even if the value of *Atriplex nummularia* as a control for growth estimation is debatable, the value reported for the strain S39 seems reasonable in view of the nodule number formed, which is too low.

Otherwise, plant yield is not necessarily correlated with the nodulation parameters investigated; indeed, the authors of references [32,33] reported that a good yield could be observed with a lower number of nodules, whereas a high number of nodules could give a low yield. Hence, it is necessary to distinguish

between two characteristics of fixing bacteria; it's about infectivity and effectiveness. While the first relates to the number of nodules induced, the second reflects the ability to give red leghemoglobin-rich nitrogen-fixing nodules. It is obvious when we compare the Bb1 and the crushed fresh nodules treatments; in fact, although the number and fresh weight of nodules formed on the roots of the plants inoculated with crushed fresh nodules are the highest (79 nodules with fresh weight equal to 1.5 g), the efficiency of this inoculum, the shoot dry weight and the percentage of nitrogen fixed in the corresponding plants are statistically similar to the values recorded with the Bb1 strain whose nodulation parameters are statistically very low as it develops only 24 nodules weighing 0.49g. It means that not all root nodules induced by crushed fresh nodules are effective or efficient in nitrogen fixation; according to the author of reference [34], sometimes there is a lack of the penetration of bacteria into the nodules due to a lack of release from the infection cord or due to the fact that the bacteroids are not nitrogen-fixing.

5. CONCLUSION

This study reveals that the strains Bb1, Bb3 and S36, previously identified as *Rhizobium tibeticum* (EU256404), *Rhizobium nepotum* (FR870231) and *Rhizobium pusense* (MF767516) can be considered the best candidates to produce an effective inoculum for *Bituminaria bituminosa*. Indeed, on the basis of the good biomass production of the inoculated plants, in relation to the infectivity, effectiveness and efficiency, these strains can be used to promote the establishment of *B. bituminosa*. So, these symbiotic complexes would be an effective tool for rehabilitating degraded sylvo-pastoral lands, particularly in the Central Middle Atlas region while ensuring both good fodders for livestock and improving soil fertility.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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