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***Research article***

# THE EFFECT OF DIFFERENT METHODS OF RHIZOBIUM BACTERIA INOCULATION ON BIOLOGICAL NITROGEN FIXATION IN BROAD BEAN

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**ABSTRACT:** The experiment was carried out as a split plot in the form of randomized complete block design with four replications. Three main treatments included different applications of Rhizobium bacteria as $E_0$: control (non-inoculation), $E_1$: syrup, $E_2$: gum Arabic. Three sub treatments included different broad bean cultivars as $V_0$: Algerian, $V_1$: Barekat, $V_{2:}$ Shami, among the methods applied by the bacterium, inoculation with syrup was superior to other methods. The highest number of nodules belonged to inoculation treatment with syrup by 338.46 and the lowest belonged to the control (non-inoculation) by 89.38. Moreover, the highest number of nodules belonged to Barekat cultivar by 253.13 and the lowest number of nodules was observed in Algerian cultivar by 204.63. The highest number of roots was observed in inoculation with syrup by 143.81 and the lowest one belonged to the control treatment by 132.44 and also the highest number of roots belonged to Barekat cultivar by 141.99 and the lowest number belonged to Algerian cultivar. The highest volume of root in broad bean belonged to inoculation with syrup by 70.20 cc and the lowest volume of root belonged to the control treatment by 43.92 cc. in inoculation with syrup the highest rate of root dry weight was reported to be 84.72 g. the highest percent of nitrogen fixation was related to inoculation treatment with syrup by nearly 14% and the lowest percentage belonged to the control treatment (non-inoculation) by about 2%. In this experiment it became clear that inoculation method with syrup and Barekat cultivar were more effective and more efficient than other methods and cultivars which were applied.

**Keywords:** broad bean, bacteria inoculation method, biological nitrogen fixation

## INTRODUCTION

What nowadays encourages the developed countries to produce and use bio-fertilizers is their serious attention to environmental side effects resulting from the unbalanced and extreme use of chemical fertilizers. Considering the annual consumption of more than 85000 tons chemical fertilizers in legume cultivating lands in Iran, it is quite necessary to provide the farmers with effective inoculants for each major crop legumes of the country through proper planning [4].one of the most important ways to take advantage of useful interaction of microorganisms and to maintain diversity in agricultural ecosystems is to use useful soil microorganisms which are living in rhizosphere environment and are capable of improving plant nutrition and soil fertility through biological fixation of nitrogen[1], phosphate solubilization, and generally enhancement of plant growth. At present, bacteria around the roots are used as bio-fertilizers [16,11,10]. Today, the use of bio-fertilizers containing crop growth enhancer bacteria is increasing. These bacteria are able to improve crop growth directly or indirectly through different mechanisms such as producing growth regulators, vitamins, amino acids, antibiotics, and siderophores [5]. The symbiotic interaction between two symbiotic components, legume and rhizobium is highly specific and is determined via signal changes between the crop and bacterium at different stages. At first, the host legume releases signal compounds (mainly flavonoids) within the rhizosphere and rhizobium reacts to these signals by producing combinations which are called nodulation factors. Then, nodulation factors are responsible for the growth of nodules in crops [15]. Nitrogen is one of the most important elements which limit crops production. The average rate of nitrogen in plants dry matter is 1-2% and sometimes it reaches 4-6% and it exist as the key element in structure and composition of such biomolecules as nucleic acids, adenosine triphosphate (ATP) molecules, nicotinamiddinucleotide (NAD) and proteins [30].

At present, a large amount of agricultural products is produced by consumption of chemical fertilizers particularly nitrogen which unfortunately has adverse effects on soil, environment, and groundwater and causes high costs in production, as well. Studying and examining biological nitrogen fixation by prokaryotes such as rhizobium bacteria in soil have been recently emphasized by scientists. The most important and effective bacterium which is known is Rhizobium Leguminosarum which has different strains [17]. By replacing chemical fertilizers with bio-fertilizers it is possible to increase production of agricultural products and to reduce the costs of production and environmental pollution and ultimately achieve sustainable production in agriculture. Nitrogen is a key element which is highly required for plant growth and thus achieving an optimal yield greatly depends on it.As a result, inappropriate and extensive use of it as a chemical fertilizer causes a lot of economic losses and environmental harm. Therefore, a more appropriate alternative to these fertilizers should be found. Molecular nitrogen fixation which is a biological method to change atmospheric nitrogen into a usable form for plants can undertake this important task [6,34]. That is why nowadays the use of symbiotic Rhizobium-Leguminose is considered very essential in planning sustainable agriculture systems [34]. Application of nitrogen fertilizer and rhizobium for nitrogen fixation required by plant due to the increase of plant growth and photosynthesizing surface [6,37] leads to the increase of leaf area [23,37] and consequently the increase of dry weight of leaf and stem [26] and total dry matter of plant [20,21,23,33] and thus vegetative and reproductive traits will increase [26], so that the application of such fertilizers will lead to the increase of sub branches, grain and pods per square meter and plant height [26] and crop yield [6,25,20,22,19]. Undoubtedly, biological nitrogen fixation is the best and most important way to enrich soil with nitrogen naturally. During this biological process which is done by various species of prokaryote microorganisms and with the help of nitrogenase enzyme system large amounts of atmospheric nitrogen (about 170 million tons) will naturally enter natural ecosystems each year. This nitrogen is mainly organic which doesn't cause any economic and environmental problems made resulting from unbalanced consumption of nitrogen fertilizers. In this regard legume-rhizobium symbiosis is highly important because it is responsible for about 50% of global nitrogen and the economic value of this amount of nitrogen is estimated to be over 85 billion dollars annually [8,27,28,30]. Studies conducted by many scientists indicate that the potential of molecular nitrogen fixation is legumes is highly affected by two factors of bacterium race and crop cultivar as well as environmental factors such as soil, climate, and crop management and if these two factors are selected and used appropriately, the symbiotic system will have the optimal efficiency in terms of nitrogen fixation [7,18,27]. Various effects of different plant genotypes and bacteria strains on traits related to biological nitrogen fixation such as the number and weight of root nodules and the activity of nitrogenase enzyme system for legumes such as normal peas, peanuts, Indian beans, soybeans, beans, and green beans have been identified before the 1980s. According to Somerton et al the interactive effects of legume and symbiotic bacterium strain on ability of symbiotic system in molecular nitrogen fixation is so specific that accurate evaluation of biological nitrogen fixation of a legume and a rhizobium strain is impossible without considering environmental conditions and identifying the genotypes of symbiotic party [13].

## MATERIALS AND METHODS

This experiment was carried out in the field of Shahid Salemi at latitude 30°54´north and longitude 48°41´east and 12 m above the sea level in 2011-2012.

### Experiment Treatments

The treatments examined in this experiment included practical application of rhizobium as the main treatment and broad bean cultivars as the sub treatment. In this study, three levels of rhizobium application, i.e. without rhizobium application (control), rhizobium application with sugar syrup, and a method of rhizobium inoculation with gum Arabic were exercised as the main treatments represented as $E_0$, $E_1$, and $E_2$ in the following for convenience.

### Using Rhizobium Bacteria with Sugar Syrup

In order to inoculate the seeds with rhizobium bacteria, at first the seeds were placed in a plastic with the volume of at least twice as much as their volume and then the solution of 20% sugar was added. After adding the bacteria powder to the seeds the bag contents were well shaken. Then the inoculated seeds were kept on a clean surface in the shadow and finally cultivation was done immediately. It should be noted that for every 1 kg of broad bean seed 7 mg bacteria was used for inoculation and no pesticides or herbicides were used before or after planting [1].

### Using Rhizobium Bacteria with Gum Arabic

Powder inoculants containing bacteria strains were weighted as much as it was recommended one day before sowing and then were kept in refrigerator. For better adhesion of bacterial strains to seeds, 20 mm of Gum Arabic (20%) was added to every 1 kg of seeds and stirred. Then 7 g of powder inoculums of each strain was added to the seeds (at least containing $2\times10^8$bacteria per gram) and well stirred.

## METHODS

### Specifications of Experiment Plan

In this research, the effect of three inoculation methods on three broad bean cultivars were studied in a split plot experiment as randomized complete block design with four replications. Each replication included 9 plots and the total experiment included 36 plots. After the land preparation, planting operation was done manually. In order to ensure the desired density, in each hole two seeds were planted in appropriate depth of land and after germination the crops were thinned and seeds used had 97% viability and purity degree of 100.

### Calculating Biological Nitrogen Fixation

In order to examine biological nitrogen fixation, every 14 days three plants were completely removed from each plot and the roots were taken out of soil in a cylindrical form. After separating the roots from plants, they were washed and the number of nodules on roots, dry weight of nodules, number of sub roots, dry weight and volume of roots were measured. The roots and nodules were placed in an oven for 48 h at $75^{\circ C}$ and then their dry weight was measured. In order to calculate the volume of roots after counting the roots, the number of sub roots and dry weight of roots, via Archimedes Principle and the difference of the volume of water in column, the volume of roots per cc was calculated. Some of the nodules which had remained intact were carried to the laboratory of science and research and the nitrogen percentage was calculated through Micro Kendal method.

## RESULTS AND DISCUSSION

### Number of Root

The ANOVA results of the number of roots showed that there was a significant difference between different levels of inoculation methods at 1% probability level (Table 1). Application of inoculation with syrup represented the highest number of roots in this experiment (Table 2). Moreover, there was a significant difference between different levels in terms of the number of roots at 5% level, so that the highest number of roots belonged to Barekat cultivar and the lowest number was found in Algerian cultivar. The interactive effect of different levels of inoculation method and cultivars on this trait was significant at 1% level (Table 4-2).the highest number of roots belonged to inoculation with syrup and Barekat cultivar and the lowest number belonged to control treatment (non-inoculation) and Algerian cultivar (Table3). The results of the mean comparison of measured traits are illustrated in Table (2). The mean comparison of the interactive effect of inoculation method and cultivar on the number of roots shows that the highest number of roots in all cultivars belongs to the inoculation treatment with syrup and Barekat cultivar and the lowest number is related to control treatment without inoculation and Algerian cultivar. The number of roots in inoculation treatment with syrup and Barekat cultivar was significantly more than the others. The results showed that in the treatment without inoculation, the number of roots showed a decreasing trend. The highest number of roots belonged to inoculation with syrup and the lowest one belonged to the treatment without inoculation.

**Table 1.The ANOVA results of number of nodules, root dry weight, root volume, number of roots, and percentage of nitrogen fixation in broad bean nodules at different levels of inoculation method and cultivar**

| Mean squares | | | | | | |
|---|---|---|---|---|---|---|
| Percentage of nitrogen fixation in nodules (%) | Number of roots | Root volume (cc) | Root dry weight (g) | Number of nodules | Degree of freedom | Variation sources |
| 399.39 ** | 456.25 ** | 2356.05 ** | 2728.84 ** | 188383.7 ** | 2 | Inoculation method |
| 0.88 | 33.47 | 3.88 | 12.41 | 131.0 | 9 | Error (a) |
| 75.08 ** | 3525 * | 668.42 ** | 327.76 ** | 8849.4 ** | 2 | cultivar |
| 350.34 ** | 2786.62 ** | 154.70 ** | 456.68 ** | 34696.1 ** | 4 | Inoculation method ×cultivar |
| 0.51 | 6.12 | 1.21 | 1.99 | 31.67 | 18 | Error (b) |
| 9.08 | 1.81 | 2.02 | 2.01 | 2.54 | | Coefficient of variations % |
| 7.84 | 136.75 | 54.37 | 70.46 | 221.82 | | Traits mean |

Ns, *, ** respectivly indicate non-significant difference, and significant difference at 5% and 1% levels.

## Root Volume

The ANOVA results of this trait showed that there was a significant difference between different levels of inoculation methods at 1% level. The application of inoculation method with syrup provided more root volume for the crop (Table 1). Moreover, there was a significant difference between different cultivars in terms of root volume at 1% probability level. The best cultivar among the studied cultivars in this experiment was Barekat cultivar (Table 2). The interactive effect of different levels of inoculation method and cultivar on this trait was significant at 1% level (Table 3), so that the highest root volume belonged to inoculation treatment with syrup and Barekat cultivar. The mean comparison results showed that the highest volume of root belonged to inoculation treatment with syrup and Barekat cultivar and the lowest volume of root belonged to control treatment (non-inoculation) and Algerian cultivar (Table 2). [29] Reported the increase of root branches of Maize hybrid 704 due to the seed inoculation with azospirillum bacteria. The increase of root volume indicates further development of root which increases the absorption capacity of water and more nutrition from a wider volume of soil.

## Number of Nodules

The ANOVA results of the measured trait are shown in Table (1). The results illustrated that the number of nodules at different levels of inoculation method were significantly different at 1% level, so that the maximum and minimum number of nodules respectively belonged to inoculation treatment with syrup and control treatment (non-inoculation) (Table 1). Moreover, there was a significant difference between different cultivars in terms of the number of nodules at 1% level. The highest number of nodules belonged to Barekat cultivar (Table 2). The interactive effect of different levels of inoculation method and cultivar on this trait was significant at 1% level (Table 3), so that the highest number of nodules belonged to inoculation treatment with syrup and Barekat cultivar (Table 3). In a research on the interactive effect of rhizobium strains and different cultivars of soybean, [12] referred to significant difference between cultivars, so that Williams cultivar grew more nodules because of more period of growth. In an experiment on the rate of nodulation in different cultivars of lentil by different strains of rhizobium leguminosarum, Hafiz et al (2000) showed that strains and cultivars had significant effects on the number of nodules.

By comparing the means via Duncan's multiple tests, the means were separated so that the number of nodules in inoculation method with syrup and Barekat cultivar was higher than other treatments (Table 3). In inoculation of mean cultivars as seed inoculation or soil inoculation and in combination with fertilizer treatments, [35] reported that inoculated treatment along with 15 kg/ha fertilizer had the highest rate of number of nodules per plant, root length, plant organ and biological yield in comparison to inoculation treatments along with 30 and 45 kg/ha nitrogen fertilizer. In a similar experiment, the inoculation of legume crops with nitrogen fixation bacteria lead to prematurity, increase of dry matter and increase of the number of nodules in plant [35]. The use of inoculums in bean led to significant increase of the number and weight of nodules in comparison to non-inoculation treatment, so that the two strains of L-51 and L-100 were recognized as the best strains among 10 races of strains collected from different regions of Iran by producing the highest number of nodules as 62.5 and 58.8 respectively [2].

**Table 2. The mean comparison of simple effects of different levels of inoculation method and cultivar on number of nodules, root volume, number of roots, and percentage of nitrogen fixation in broad bean nodules**

| Traits mean | | | | | Treatments |
|---|---|---|---|---|---|
| Percentage of nitrogen fixation in nodule (%) | Number of roots | Root volume (cc) | Root dry weight (g) | Number of nodules | Inoculation method |
| 1.88 c | 132.44 b | 43.92 c | 54.67 c | 89.38 c | Non-inoculation |
| 13.40 a | 143.81 a | 70.29 a | 84.72 a | 338.46 a | Syrup |
| 8.24 b | 133.99 b | 48.89 b | 72.01 b | 237.63 b | Gum Arabic |
| | | | | | |
| | | | | | Cultivar |
| 4.98 c | 130.01 b | 46.30 c | 65.84 c | 204.63 b | Algerian |
| 9.61 a | 141.99 a | 61.02 a | 76.13 a | 253.13 a | Barekat |
| 8.93 b | 138.29 ab | 55.78 b | 69.39 b | 207.71 b | Shami |

The means of treatments with similar letters are not significantly different from each other at 5% according to Duncan's multiple tests.

## Root Dry Weight

In this experiment the effect of different levels of inoculation method on trait dry weight of root was significant at 1% level. Inoculation method with syrup was higher than inoculation with gum Arabic and non-inoculation method in terms of this trait. Moreover, there was a significant difference between different cultivars at 1% level in terms of root dry weight (Table 1). Barekat broad bean among the studied cultivars had the highest dry weight of root and the lowest dry weight of root was seen in Algerian cultivar. Accordingly, the application of rhizobium bacteria with syrup lead to significant increase of dry weight of root in comparison to application of bacteria with gum Arabic or control treatment (non-inoculation). In an experiment, [9] investigated the effect of different strains of Pseudomonas, Agro bacterium, and Azospirilhum on colza (rapeseed) and reported the increase of dry weight of colza root about 11-52%. Moreover, the inoculation of rhizobium lead to increase of nitrogen absorption and consequently better growth of plant and increase of plant height, root dry weight, and the shoots. The ANOVA results of interactive effect of inoculation method and cultivar indicated a significant difference at 1% level) Table 1), so that the highest dry weight of root belonged to inoculation method with syrup and Barekat cultivar (Table 3). Inoculation with syrup increased the dry weight of root in comparison to non-inoculation method, and the seeds of Barekat cultivar in inoculation treatments with syrup had higher root dry weight.[14] reported that rhizobiums in different compounds such as auxin, cytokinin, riboflavin and vitamins lead to induction of growth enhancement by penetrating into the roots of legumes and non-legumes[36] reported that the length and dry weight of maize increased due to application of growth enhancer bacteria producing auxin. In addition, the mean comparison of the interactive effect of different levels of inoculation and cultivar on dry weight of root via Duncan’s multiple test at 5% level as shown in Table (3) indicated that Barekat cultivar in inoculation method with syrup had the highest root dry weight (Table 3). The comparison mean of date illustrated that rhizobium groups with syrup and gum Arabic produced higher root dry weight rather than control group. The probability of having desirable genetic characteristic to adapt more to symbiotic bacteria and taking better and more advantage of environmental conditions can also be effective in enhancing the yield of Barekat cultivar.

## Nitrogen Fixation Percentage in Nodules

The ANOVA results of nitrogen fixation percentage in nodules showed that there was a significant difference between different levels of inoculation method at 1% level (Table 1). The results probably indicate the fact that nodule formation and nitrogen fixation stages at nodules are essential stages for plants and in case of lack of inoculation the percentage of nitrogen fixation in nodule will decrease significantly. There was also a significant difference between different cultivars in terms of this train at 1% level. The ANOVA results of the interactive effect of different levels of inoculation method and cultivar represented a significant difference at 1% level (Table 3). The mean comparison results showed that the highest percentage of nitrogen fixation belonged to inoculation method with syrup and Barekat cultivar (2). However, the mean comparison of the interactive effect (Table 3) indicated that inoculation method with syrup and Shami cultivar had the highest percentage of nitrogen fixation. In examining the rate of nitrogen fixation in harvester cultivar of bean inoculated with 10 different rhizobium isolates in 3 northern, central, and southern regions in Jordan, [31] concluded that JOVI isolate from southern region had higher rate of nitrogen fixation than other isolates and the control treatment. He also observed that this isolate could increase the production of dry matter in been shoots in comparison to other treatments due to its high capability of nitrogen fixation.

**Table 3. the mean comparison of interactive effects of different levels of inoculation method and cultivar on number of nodules, root volume, number of roots, and percentage of nitrogen fixation in braod bean nodule**

| Traits mean | | | | | Treatment | |
|---|---|---|---|---|---|---|
| Percentage of nitrogen fixation in nodule( %) | Number of roots | Root volume (cc) | Root dry weight (g) | Number of nodules | cultivar | Inoculation method |
| 3.22 e | 146.29 c | 36.45 f | 49.13 f | 94.73 g | Algerian | Non-inoculation |
| 1.62 f | 105.20 f | 52.62 c | 50.61 f | 5.78 h | Barekat | |
| 0.81 f | 145.85 c | 42.69 e | 64.29 e | 167.62 f | Shami | |
| 0.55 f | 135.40 d | 55.43 b | 85.61 b | 309.97 c | Algerian | Syrup |
| 14.55 b | 166.60 a | 77.54 b | 96.82 a | 408.98 a | Barekat | |
| 25.09 a | 129.44 e | 77.91 a | 71.73 d | 296.45 d | Shami | |
| 11.16 d | 108.20 f | 47.03 d | 62.80 e | 209.20 e | Algerian | Gum Arabic |
| 12.66 c | 154.19 b | 52.91 c | 80.96 c | 344.63 b | Barekat | |
| 0.91 f | 139.74 d | 46.75 d | 72.16 d | 159.06 f | Shami | |

The means of treatments with similar letters are not significantly different from each other at 5% according to Duncan’s multiple tests.

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