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Evaluation of meliloti rhizobium activity effectiveness on quantitative properties of alfalfa by bacterial inoculation in the south-east of Iran

Evaluación de la eficacia de la actividad de Meliloti Rhizobium sobre las propiedades cuantitativas de la alfalfa por inoculación bacteriana en el sudeste de Irán

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ABSTRACT

In order to investigate the effect of bacterial inoculation on yield, chlorophyll and protein content of alfalfa to obtain economically experimental products in Shahid Zande Rouh Agricultural Training Center in Kerman as a split plot in time based on a completely randomized block design with four replications on the ground Which had not been done before, was done. Bacterial inoculation was at three levels (Rhizobium meliluti, Rhizobium leguminasarum and no inoculation as a control). Bacterial inoculation had a significant effect on all studied traits and caused an increase in chlorophyll content, yield and protein percentage. In terms of fresh forage weight, the first and third crops had the highest yield with the application of Rhizobium meliloti (6 tons per hectare). The highest percentage of protein related to inoculation of Rhizobium meliloti was observed in the second Picking. According to the results of this study, inoculation with Rhizobium meliloti bacteria increases the ability of nitrogen fixation 3 to 4 times compared to the control and improved the alfalfa traits of Bami cultivar in southeastern Iran.
Keywords: Alfaalfa, Rhizobium, Picking, Bacterial Inoculation.

RESUMEN

Con el fin de investigar el efecto de la inoculación bacteriana en el rendimiento, el contenido de clorofila y proteína de la alfalfa para obtener productos experimentales económicos en el Centro de Capacitación Agrícola Shahid Zande Rouh en Kerman como una parcela dividida en el tiempo basado en un diseño de bloques completamente al azar con cuatro repeticiones en el Terreno que no se había hecho antes, se hizo. La inoculación bacteriana se realizó en tres niveles (Rhizobium meliluti, Rhizobium leguminasarum y sin inoculación como control). La inoculación bacteriana tuvo un efecto significativo en todos los rasgos estudiados y provocó un aumento en el contenido de clorofila, rendimiento y porcentaje de proteína. En términos de peso del forraje fresco, el primer y tercer cultivo tuvieron el mayor rendimiento con la aplicación de Rhizobium meliloti (6 toneladas por hectárea). El mayor porcentaje de proteína relacionado con la inoculación de Rhizobium meliloti se observó en el segundo Picking. Según los resultados de este estudio, la inoculación con la bacteria Rhizobium meliloti aumenta la capacidad de fijación de nitrógeno de 3 a 4 veces en comparación con el control y mejoró los rasgos de alfalfa del cultivar Bami en el sureste de Irán.
Palabras clave: Alfaalfa, Rhizobium, Picking, Inoculación bacteriana.

1. INTRODUCTION

Forage plants of the legume family have been considered worldwide due to their higher protein content and the effect of these plants on soil fertility due to their ability to coexist with rhizobim (Gunes et al., 2008). One of the most important forage plants is alfalfa, forage alfalfa It is a member of the legume family. Consumption of this product is very important due to its high quality and food reserves. The development of fodder crops, especially alfalfa, in addition to providing food needed for livestock for agriculture and preservation of natural resources and economy of each region is important (Taherkhani et al., 2009). Due to the coexistence of the plant with the nitrogen-fixing bacteria Rhizobium meliloti, alfalfa is expected to receive most of its nutritional needs through bacteria. Nitrogen fixation in soil causes its cultivation to be favorable for crop rotation (Fukami et al., 2018).

Bacterial inoculation can eliminate the harmful effects of plant pathogens and living and non-living environmental stresses. In Iran, the area of soils affected by nutrient deficiencies and excessive fertilizer consumption is over 50% of the total agricultural land. Nitrogen fixation and inoculation increasing yield (Aslani et al., 2011; Abusuwar & Daur, 2015).

In the last few decades, the use of chemical inputs in agricultural lands has caused environmental problems such as contamination of water resources, the quality loss of agricultural products, and reduced soil fertility (Ensiye et al., 2018). The use of nitrogen fertilizers to increase crop production will continue for the foreseeable future, but more attention should be given to the biological stabilization of nitrogen by microorganisms in order to reduce their use. *Rhizobium bacterium* inside the tuber or node produced on the plant root receives air nitrogen, stabilizes it, and converts it to NH_3, the product of which is used both by the bacterium itself and the host plant. Proliferation of *R. bacteria* around the root of Leguminosas can increase the absorption of elements such as phosphorus, potassium, calcium, and iron (Amri et al., 2010).

Rhizobium inoculation not only increases effective root tubers but also the grain yield and has a positive effect on increasing biological nitrogen fixation and alfalfa yield.

In a study on faba bean, it was observed that inoculation with *Azotobacter* and *Rhizobium* significantly increased total aerial part dry weight in comparison with control treatment. Of the sixteen nutrients required by plants, seven elements are iron, zinc, manganese, boron, copper, molybdenum, and chlorine. These elements play a role in increasing production after balancing

the use of nitrogen, phosphate, and potassium fertilizers. In Iran, the deficiency of micronutrients, especially zinc, manganese, and boron, is common in farms and gardens due to decreased soil organic matter content, the presence of carbonate and bicarbonate ions in irrigation water, and the high consumption of phosphorus (Yang et al., 2013).
In an experiment, the effect of *Rhizobium* was investigated on the amounts of absorbed elements. Deficiency of nutrients decreased the amounts of elements in leaves and roots of non-inoculated plants (control), but nutrient deficiency was observed only in the leaves of plants inoculated with *Rhizobium* and the elements remained unchanged in the roots. In the absence of nutrient deficiencies, the uptake of trace elements increased significantly in alfalfa inoculated by bacteria (Dordas, 2009). It seems that by performing this experiment and inoculating nitrogen-fixing bacteria, while enhancing the growth of the crop, it can also increase the yield and can also play a role in controlling the pathogens, and follow the path to organic and sustainable agriculture (Hu et al., 2013).

2. MATERIALS AND METHODS

The effects of bacterial inoculation on chlorophyll content, protein content, and the yield of alfalfa, was investigated in an experiment as a split-split plot in time based on randomized complete block design with four replications in Shahid Zandeh-Roh Agricultural Training Center of Kerman (10 km Jupar Road) located at 25° and 32′ N and 53° and 29′ E with an altitude of 1755 m in growing season in 2013. According to the soil test results, no cultivation was carried out previously in the studied land, and there was a nutrient deficiency. Hence, a foliar application was provided and implemented in order to provide the nutrient elements according to (Table 1).
Experimental factors included foliar application of micronutrient spraying using biomethane branded chelate fertilizer at five levels including spraying of iron chelate, zinc chelate, manganese chelate, iron + zinc + manganese mixture, and no spraying (as a control). At a concentration of four per thousand as the main factor, bacterial inoculation was as a sub factor at three levels (*Rhizobium meliloti*, *R. leguminosarum*, and non-inoculation or the control) and the number of alfalfa crop was considered as a sub-factor.
Seeds of alfalfa beam from Bam seed were prepared from Kerman Agricultural Jihad Organization. Seeds were washed with 70% ethanol for two minutes and then by 1% sodium hypochlorite for 5 minutes, followed by three times with distilled water. Sterilized alfalfa seeds were immersed in vacuum for one hour at ambient temperature to facilitate penetration of bacterial strains into the seeds through the pores. Control seeds were also placed under similar conditions. Bacterial inoculation was performed using *R. meliloti* and *R. leguminosarum*. Inoculation was carried out as seed. For this purpose, the seeds were impregnated in a prepared 10% solution of diluted sugar water, with an optimum concentration of of 110 ml/cfu to stimulate alfalfa growth. Bacteria were obtained from the laboratory of the Faculty of Agriculture, Shahid Bahonar University of Kerman, and inoculated seeds were immediately cultured on May 5, 2013.
Immediately after sowing, the first irrigation was carried out as leakage, and after regular germination, irrigation was continued once every seven days. Weeding was done manually. No disease or pest was observed on the farm during the growing season. Foliar application of each plot was performed individually based on experimental treatments when the plants were 10-15 cm long before flowering stage at early hours of the morning and sometimes in late hours of the

day before complete darkness. Forage was harvested at a time when 5-10% of the bushes were flowering in the field. For sampling in each experimental plot, first two rows of side and 0.5 m from the top and bottom were considered as margins. The evaluated traits were chlorophyll content, protein percentage, and forage yield (ton/ha), in aerial parts. The experimental alfalfa produced four crops during the crop year and sampling was performed to evaluate traits in all the four crops.

To measure chlorophylls a and b and total at flowering stage, 0.5 g of the leaf was developed and 80% acetone solvent and spectrophotometer were used. The following equations were used to calculate chlorophyll concentrations (mg/g fresh leaf) (Delić et al., 2016):

Chl.a = (12.7 (A663) -2.69 (A645)) V / W × 1000

Chl.b = (22.9 (A645) -4.68 (A663)) V / W × 1000

Chl.total = Chl.a + Chl.b

In these relationships, A663 and A645 are absorbance at at 663 nm and 645 nm, respectively, V is soluble volume, and W is leaf fresh weight (mg).

To measure the protein, the leaf powder was boiled in concentrated sulfuric acid in the presence of boiling copper ion so that nitrogen turns into ammonia. For each mole of hydrochloric acid, there was 14 g of nitrogen in the primary tissue. Protein content was measured using a coefficient of 6.25 (Ahemad & khan Khan, 2011).

In order to obtain the dry weight after chopping the plants and mixing them, two hundred samples were selected and placed in a dryer (ironing machine) at a temperature of 75 ° C until reaching a constant weight. The fresh weight of alfalfa after harvest, It was immediately identified in the field by a digital scale with an accuracy of 0.01 g (Ahemad & khan Khan, 2011). Data were analyzed using SAS statistical software and mean values were compared using Duncan's multiple range test at at 5% level of probability.

3. RESULTS AND DISCUSSION

Chlorophyll a

The results showed that the highest amount of chlorophyll a was related to Mn foliar application and *R. leguminosarum* inoculation with 45.25 mg/g and the lowest one belonged to inoculation with *R. meliloti* and Mn foliar application of 26.24 mg/g (Table 3).

The results of the mutual effects of micronutrient and crop in each crop showed that the highest (47.17 mg/g) and the lowest (27.94 mg/g) amounts of chlorophyll a were related to the lack of foliar application and foliar application of iron in the second crop and the first crops, respectively (Table 4).

The increase in chlorophyll a content was higher due to the use of micronutrients, especially manganese. Increased plant chlorophyll content as a result of using micronutrients was also reported by Aslani *et al*. A decrease in chlorophyll concentration under nutrient deficiency appears to be due to the effects of chlorophyllase peroxidase and phenolic compounds resulting from the degradation of chlorophyll (Delic et al., 2016). Application of *Pseudomonas* bacteria in beans in the absence of nutrient deficiencies increased the level of chlorophyll a. (Dordas et al., 2009). In this experiment, an increase in chlorophyll A content was observed as a result of using micronutrients, in particular manganese. Increased plant chlorophyll content as a result of using micronutrients was reported by Daneshian (2012).

Chlorophyll b

The results of ANOVA for the effects of test factors on the traits examined are presented in (Table 2). The highest (44.2 mg) and the lowest (20.07 mg) amounts of chlorophyll b were recorded in the foliar application of Mn and iron, respectively. Application of bacteria and micronutrients had no significant effects on chlorophyll b contents (Table 4). Decreased membrane proteins and increased activity of chlorophyllase and peroxidase enzymes are among the factors affecting chlorophyll reductions in lack of insemination conditions. A decline in leaf vegetation under long-term nutrient deficiency is partly due to a decrease in nitrogen flow into the tissue and a change in nitrate reductase activity. As reported by Hadi (2009), concentrations of chlorophylls A and B decreased in wheat by 38% and 35% on average, respectively, in the first year. One of the most important reasons for the reduction of chlorophyll B is its degradation by reactive oxygen species (ROS). A decrease in photosystem II activity, reduction of Rubisco enzyme activity, and inhibition of ATP synthesis result in increased free oxygen formation in chloroplasts, and further degradation of chlorophyll B reduces its concentrations relative to chlorophyll A, which was also observed by (Hadi et al., 2009).

Total chlorophyll

The highest amount total chlorophyll (53.99 mg) was found in Mn foliar application and non-inoculation and the lowest level (37.90 mg/g) was measured in Mn foliar application with *R. meliloti* inoculation (Table 3). This result is in line with the results of a study on wheat by Monez in 2006. Studies show that the bacteria play an important role in improving the uptake and growth of the root, which in turn absorbs most of the elements involved in chlorophyll such as magnesium, manganese, and zinc through the root adsorption process. Previous studies show an important role of bacteria in improving the root uptake and growth, which in turn increases more absorption of such elements as iron and manganese involved in chlorophyll synthesis. The present study also demonstrated that nutrient depletion and deficiency stimulate senescence (leaf yellowing), resulting in a decrease in total chlorophyll, which is consistent with a research on wheat conducted by Eltegani & Rahman (Abdel-Rahman, 2013).

Protein percentage

The highest protein percentage was observed in Mn and *R. meliloti* in the fourth crop and the least (22.60%) level was detected in Zn spraying and inoculation in the first crop (Table 6). The results showed that protein levels were higher in second and fourth crops. The reason for this can be explained by the fact that more *Rhizobium* immobilization and more nutrients are available to the plant, and the bacteria present in biofertilizers increase the absorption of other nutrients and dissolve minerals. Similar results were reported by Khalil et al.

Salter 2014 stated that an increase in nitrogen content significantly increased wheat grain protein content. In addition to the leaf area index, bacterial inoculation and further stabilization of the plant in nutrient uptake associated with leaf production seem to be involved in protein elevation in the second and fourth crops of this research. In fact, it can be speculated that improvements in quantitative indices (e.g. fresh forage yield) lead to increases in qualitative traits, such as protein content, as the plant is provided with more nutrients along with more nitrogen fixation as a result of inoculation, which is in line with that of Ardakani (Khalil et al., 2010).

Function

The results showed that the highest function (71.25 g/m^2) was related to the fourth crop and no foliar application, and the lowest one (42.17 g/m^2) was found in the foliar application of Zn + Mn in the second crop (Table 4).
According to the present results of inoculation, the highest level (67.75 g/m^2) was observed in foliar application of *R. leguminosarum* in the third crop, and the lowest amount (65.45 g/m^2) belonged to inoculation with *R. meliloti* in the first crop (Table 5). There were significant differences between the control and the other treatments in the foliar application. The increase in height can be attributed to the positive role of organic fertilizers, increased nitrogen efficiency, and greater nutrient availability. Over time, cytokinin production appears to stimulate plant height growth with increase cell division. Temperature changes have a direct impact on the amount of forage in different crops, and can also reduce forage production by reducing nutrients in early crops.
The highest rate of forage production was measured in the fourth crop, which is apparently because the plant spent longer growth period and the temperature conditions was favorable for the crop during this period resulting in the maximum production of forage. Loss of dry weight is probably a consequence of a decrease in the net crop yield, which decreases the share of different organs of juice, and consequently reducing the plant weight. A decrease in photosynthesis reduces the amount of chlorophyll and the nitrogen metabolism pathway in the synthesis of compounds, such as proline, used for osmotic regulation, which causes nutrient imbalance and consequently diminishes function. In an experiment on alfalfa, it was found that nutrient restriction at flowering time reduced photosynthetic material transfer and thus affected the function (Tiedje et al., 1982).

Total fresh weight of the plant

The highest amount (75 g per plant) of Mn foliar application was recorded in inoculation with *R. meliloti* in the first crop (Table 7).
R. meliloti bacteria were reported to increase the aerial part fresh weight compared to control treatment.
In this study, it seems that the lack of water in Kerman region and consequently absorption of nutrients result in the weight loss. Water deficiency disrupts the electron transport system and induces antioxidant activities in chlorine, platelet, and mitochondria. In this study, fresh weight elevated further in the fourth crop due to better balance of nutrients and increased leaf development, as well as elevated turgescence and photosynthesis. Temperature fluctuations seem to have a direct effect on fresh forage yield in different crops. Also, the deficiency of root nutrients in the first crop and its increase in the final crops could reduce forage production by creating nutritional stress. The first harvest was reported to have the lowest fresh forage production.

Stem fresh weight

The highest amount (89.44 g) of stem fresh weight was found in Zn foliar application and *R. leguminosarum* inoculation with and the lowest level (33.25 g) was recorded in Fe spraying and *R. meliloti* inoculation with (Table 3).
The highest amount (1267 g/m^2) of non-foliar application occurred in the fourth crop and the lowest level (672 g/m^2) was obtained in Mn foliar application in the first crop (Table 4). In this study, the effect of fresh weight in the fourth crop appears to be due to better nutrient balance and increased leaf development as well as increased turgor to increase photosynthesis.

The highest (78.18 g) inoculation of *R. leguminosarum* was detected in the second crop and the lowest (35.10 g) was noticed in *R. meliloti* in the fourth crop (Table 5).
In this study, water absorption resulted in better nutrient uptake, and nutrient deficiency was inhibited by the growth and development, resulting in decreased cell growth and reduced body weight due to reduced photosynthesis. The growth and differentiation of cells depends on the absorption of nutrients and water (McKenzie et al., 2015; Delic et al., 2016).

Aerial part (leaf) fresh weight

The highest effect (25.77 g) of foliar application was on inoculation of *R. Leguminosarum* and the least effect (8.24 g) was observed in iron and *R. meliloti* (Table 3). The results showed that *R. leguminosarum* inoculation had the highest effect in the third crop and the least impact belonged to *R. meliloti* inoculation in the first crop (Table 5). It seems that the increased leaf weight is due to its length and width justification. This conclusion can be explained by the effect of dietary Zn on cell division by increasing auxin, which increases leaf length and width. In this experiment, leaf weight reached its maximum after germination until pollination, thereby resulting in increasing photosynthesis and total dry weight of the plant, which restricted leaf growth and development under lack of insemintaion.

Table 1: Field soil characteristics

Soil	pH	EC (ds/m)	K (ppm)	P (ppm)	Total %N	Zn	Fe	%OC	Depth (cm)
						mg/kg			
(Sandy-Loam)	7.5	1.64	100	6.5	0.31	0.65	2.6	0.48	0-30
(Sandy-Loam)	7.6	2.01	175	4.2	0.39	0.92	4.2	0.8	30-60

Table 2: Analysis of variance of studied traits in alfalfa

Source of variation	**df**	**Chlorophyl a**	**Chlorophyl b**	**Total Chlorophyl**	**Protein (%)**	**Yield**	**Total shrub**	**Aerial Fresh Weight**	**Stem Wet Weight**	**Leaf Fresh Weight**
Replication	3	5.11 ns	1577.93**	25.766 ns	34.075 ns	1267.53**	613.67 ns	174.745 ns	296.636 ns	0.497 ns
Nutritious	4	1009**	4864.5**	373.09**	3566.08**	922.42**	1973.64**	92.946 ns	1860.876*	1.786*
Error A	12	2.220	163.266	13.922	11.620	159	2008.16	177.857	520.992	0.611
Bacteria (B)	2	131.77**	1077.75 ns	531.43**	1350295**	665.1**	1240.41**	2341.662**	22607.298**	24.508**
AB Nutritious × Bacteria	8	38**	732.97 ns	230.83**	28.915**	89.3 ns	570.10*	312.371*	2070.657**	0.632 ns
Error B	30	9.168	405.008	7.350	0.573	1046.697	115.92	132.136	428.968	0.482
Picking	3	188.8**	928.15 ns	1042.5**	91.0592**	2755.1**	226.5 ns	114.162*	810.689**	0.207**
AC Nutritious × Picking	12	41.5**	456 ns	0.84 ns	34.804**	177.31**	360.59 ns	65.617 ns	894.872**	0.14 ns
BC Bacteria	6	6.81ns	276.18 ns	0.08 ns	2.680**	205.55*	278.27 ns	196.474**	1350.692**	0.007 ns

× Picking										
A×B×C Nutritious × Bacteria ×Picking	24	4.1 ns	450.8 ns	0.91 ns	1.916**	48 ns	1245.71 **	33.720 ns	209.578 ns	0.018 ns
Error C	135	3.658	409.315	3.164	0.981	81.306	249.079	41.185	139.226	0.017
(%CV)		6.54	24.74	4.83	2.81	16.76	5.53	12.34	26.80	9.29

ns, *, and ** non-significant and significant at 1 and 5% probability levels, respectively.

Table 3: Mean comparison of the effect of nutrient foliar application and bacteria for some measured traits of alfalfa

Nutrients	Bacteria	Chlorophyll a (mg/g)	Total Chlorophyll mg/g	Stem wet weight	Areal fresh weight
Fe	Rhizobium 1 Rhizobium 2 Without Rhizobium	30.47c 31.07c 34.30b	49.14bc 50.27b 45.50de	33.25e 80.68ab 47.90ce	8.243d 24.75 ab 12.51 cd
Zn	Rhizobium 1 Rhizobium 2 Without Rhizobium	30.47c 40.92a 32.11c	51.72b 41.93f 42.74f	55.49c 89.44a 37.39de	12.65 cd 25.77 a 10.40 d
Mn	Rhizobium 1 Rhizobium 2 Without Rhizobium	25.20d 41.24a 31.77c	36.90g 41.62f 53.98a	41.50ce 45.25ce 54.50ce	17.87 abcd 12.97 cd 11.68 cd
Fe+Zn+Mn	Rhizobium 1 Rhizobium 2 Without Rhizobium	30.90c 39.05a 28.80c	50.97b 47.67f 45.11e	43.63ce 71.81b 43.75ce	9.422 d 20.50 abc 10.74 d
Without Spraying	Rhizobium 1 Rhizobium 2 Without Rhizobium	29.88c 35.84b 29.85c	46.54de 47.36cd 46.07e	47.30ce 76.72ab 51.55cd	11.61 cd 23.43 ab 15.61 bcd

Means in each column followed by similar letter(s) are not significantly different using Duncan's multiple range tests.

Table 4: Mean comparisons for the effect of nutrient foliar application and picking on performance for some measured traits of alfalfa.

Nutrients	Picking	Chlorophyll a (mg/g)	Chlorophyll a (mg/g)	Total chlorophyll (mg/g)	Protein (%)	Yield g/m^2	Stem wet weight
Fe	Picking 1	27.94 j	32.48 c	50.18 bc	24.01 e	47.17 efg	62.49 ab
	Picking 2	31.92 fh	32.08 c	51.27 b	26.65 e	53.42 cdef	45.39 de
	Picking 3	37.83 c	35.32 b	66.50 de	40.67 b	53.92 cdee	45.39 de
	Picking 4	36.10 d	35.30 b	50.90 b	32.25 c	66.33 ab	62.49 ab
Zn	Picking 1	30.38 ghi	32.48 c	51.73 b	24.59 e	56 bcde	58.30 bc

	Picking 2	32.42 ef	41.94 a	42.94 f	21.03 e	61.75 abc	63.24 ab
	Picking 3	41.50 b	33.12 c	43.75 f	47.67 a	59.50 bcd	58.30 bc
	Picking 4	28.97 ij	42.01 a	50.70 b	24.49 e	59.83 bcd	63.24 ab
Mn	Picking 1	30.65 gh	26.24 d	37.90 g	24.01 e	53.67 cdef	39.92 e
	Picking 2	33.88 e	42.25 a	42.63 f	24.65 e	66.42 ab	480.25 cde
	Picking 3	32.71 ef	32.78 c	53.99 a	29.44 d	44.75 fg	39.92 e
	Picking 4	31.44 fg	26.17 d	37.20 g	24.18 e	53.67 cdef	48.25 cde
Fe+Zn+Mn	Picking 1	32.63 ef	32.91 c	50.98 b	24.59 e	55.50 bcdef	53.75 bcd
	Picking 2	40.50 b	41.06 a	41.69 f	40.67 b	47.17 g	52.38 bcd
	Picking 3	36.54 cd	30.82 c	46.12 e	32.25 c	52.92 cdef	52.75 bcd
	Picking 4	31.72 fg	42.01 a	45.80 e	24.49 e	66.25 ab	52.38 bcd
Without Spraying	Picking 1	29.63 hi	30.90 c	46.57 de	24.03 e	42.17 efg	70.51 a
	Picking 2	47.17 a	35.85 b	48.38 cd	47.67 a	50.25 defg	43.88 de
	Picking 3	33.70 e	30.85 c	46.08 e	29.44 d	62.92 abc	70.51 a
	Picking 4	33.80 e	34.84	48.10 cd	24.18 e	71.25 a	43.88 de

Means in each column followed by similar letter(s) are not significantly different using Duncan's multiple range tests.

Table 5: Comparison of mean interactions between bacteria and picking for some measured traits of alfalfa

Mn (%)	Picking	Stem Wel Weight	Yield g/m^2	% Protein	Areal fresh weight
Rhizobium 1	Picking 1	50.96 c	45.63 e	29.92 b	15.59 bc
	Picking 2	35.10 d	58.15 bc	31.38 a	8.330 e
	Picking 3	50.96 c	64.55 ab	28.65 c	15.59 bc
	Picking 4	35.10 d	48.90 de	29.26.bc	8.330 e
Rhizobium 2	Picking 1	68.18 b	58.25 cd	31.38 a	19.75 ab
	Picking 2	78.18 a	49.90 de	28.65 c	23.22 a
	Picking 3	68.18 b	67.75 a	31.36 a	19.75 ab
	Picking 4	78.76 a	58.40 bc	27.02 d	23.22 a
Lack of Inculation	Picking 1	51.84 c	56.15 cd	29.92 b	13.89 de
	Picking 2	38.60 d	55.60 cd	31.36 a	10.50 de

	Picking 3	51.84 c	49.10 de	27.05	13.88 cd
	Picking 4	38.60 d	65.50 ab	29.26 bc	10.50 de

Table 6: Mean comparisons for the effect of foliar application of nutrients, bacteria and picking on Alfalfa protein percentage

Nutrients	**Bacteria**	**Picking 1**	**Picking 2**	**Picking 3**	**Picking 4**
Fe	Rhizobium 1	24.08 i-n	24.60 i-m	24.08 i-n	24.60 i-m
	Rhizobium 2	25.02 i-l	25.60 i	25.02 i-l	25.60 i
	Without Rhizobium	22.92 mn	23.58 k-n	22.92 mn	23.58 k-n
Zn	Rhizobium 1	24.23 i-n	24.90 i-l	24.23 i-n	24.90 i-l
	Rhizobium 2	25.27 i-n	25.35 i-l	25.27 ijk	25.35 ij
	Without Rhizobium	22.60 n	23.70 j-n	22.60 n	23.70 j-n
Mn	Rhizobium 1	45.50 c	50 a	45.50 c	50 a
	Rhizobium 2	38 d	47.75 b	38 d	47.75 b
	Without Rhizobium	38.50 d	45.25 c	38.50 d	45.25 c
Fe+Zn+Mn	Rhizobium 1	31.20 f	33.25 e	31.20 f	33.25 e
	Rhizobium 2	29.38 g	32.88 e	29.38 g	32.88 e
	Without Rhizobium	27.75 h	30.63 fg	27.75 h	30.63 fg
Control	Rhizobium 1	24.58 i-m	24.13 i-n	24.58 i-m	24.13 i-n
	Rhizobium 2	25.58 i	25.25 ijk	25.58 i	25.25 ijk
	Without Rhizobium	23.33 lmn	23.17 mn	23.33 lmn	23.17 mn

Means in each column followed by similar letter(s) are not significantly different using Duncan's multiple range tests.

Table 7: Comparison of mean interactions between micronutrients, picking, and bacteria on total shrub

Nutrients	**Bacteria**	**Picking 1**	**Picking 2**	**Picking 3**	**Picking 4**
Fe	Rhizobium 1	76.07 abc	77.22 abc	76.38 ab	79.26 a
	Rhizobium 2	67.39 j-m	62.55 nop	69.53 h-m	71.56 d-j
	Without Rhizobium	74.46 b-f	73.85 c-g	75.97 abc	75.45 a-d
Zn	Rhizobium 1	68.32 i-m	69.38 h-m	70.47 f-k	71.18 e-k
	Rhizobium 2	70.51 f-k	71.31 e-j	73.05 c-h	75.88 abc
	Without Rhizobium	55.38 s	56.97 rs	57.42 rs	60.17 o-r
Mn	Rhizobium 1	37 y	39.25 wxy	40.75 v-y	46 u

	Rhizobium 2	37.5 xy	40 wxy	44.50 uv	50.75 t
	Without Rhizobium	38 xy	45.75 u	41.25 vwx	42.25 uvw
Fe+Zn+Mn	Rhizobium 1	60.70 o-r	62.13 n-q	63.25 no	65.63 mn
	Rhizobium 2	58.75 p-s	57.97 rs	62.75 nop	66 lmn
	Without Rhizobium	55.95 s	58.80 p-s	58.50 qrs	59.25 p-s
Control	Rhizobium 1	73.69 c-g	69.43 h-m	75.68 a-d	77.25 abc
	Rhizobium 2	65 d-r	75.63 a-d	73.13 c-h	75.13 a-e
	Without Rhizobium	67.06 klm	67.85 i-m	67.83 i-m	70.10 g-l

Means in each column followed by similar letter(s) are not significantly different using Duncan's multiple range test.

4. CONCLUSION

In general, the results of this study indicate, Bacterial inoculation has a significant effect on chlorophyll content and yield and also affects protein percentage. Increasing growth parameters following rhizobium inoculation is one of the important mechanisms of the plant for greater resistance to stress, more root growth than The aerial part after stabilization means more distribution of photosynthetic material to the underground organs and as a result more root growth can help absorb water and nutrients, inoculation as a helper bacterium and compatible with rhizobia improves nodes and better stabilization Nitrogen is converted by rhizobia. Overall, it was observed that inoculation with Rhizobium Melilloti and China II had the highest yield in terms of forage in southeastern Iran.

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