Effect of interaction between inoculation with Rhizobia bacteria and sulfur addition on iron and protein content of broad bean (*Vicia faba* L.) seeds in soil treated with humic acid

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Abstract

Factorial pot experiment was conducted in the green house in the College of Agriculture / University of Basrah in the winter season 2019-2020, according to the CRD (Complete Randomized Design) with three replicates in order to study the effect of interaction between inoculation with two isolates of Rhizobia in addition to treatment without inoculation (R_1, R_2 and R_0) respectively and three levels of humic acid (0, 10 and 20 L ha^{-1}) and three levels of agricultural sulfur (0, 1 and 2 tons ha^{-1}) in iron content and protein percentage in the seeds of broad bean plant. The results showed that inoculation with isolate R_1 led to a significant increase in iron content and protein percentage in seeds amounting to 329.4 mg Fe kg^{-1} and 20.97%, respectively. The increase in humic acid levels led to a significant increase in iron content and protein percentage in the seeds , The level of 20 L ha^{-1} achieved the highest average of 369.3 mg Fe kg^{-1} and 22.33%, respectively. While the level of 1 ton S ha^{-1} significantly excelled and gave the highest iron content in the seeds (320.5 mg Fe kg^{-1}) and the level of 2 ton S ha^{-1} with the highest average of protein in the seeds (21.31%), the binary and triple interaction between the studied factors was significant.

Key words: Rhizobia, humic acid, agricultural sulfur

*** A part of MSC. thesis of the second author**

Introduction :

Biological fertilizers are one of the best methods used in agriculture because of their great importance in preparing nutrients for plants and at a lower cost to reduce the use of chemical fertilizers and reduce the pollution resulting from them (Rana *et al.*, 2013). Biofertilizers are microbial inoculation capable of adding important nutrients to the soil and converting them from an unusable form to a form available for uptake by plants through their biological processes. Biofertilizers have been widely used as an environmentally friendly approach, improving soil fertility and increasing crop production through the activity of microorganisms in the rhizosphere (Nalawde and Satish, 2015) .

Rhizobia plays an in important role in leguminous crops and maintains and improves soil fertility through its ability to fix nitrogen from the air through root nodes (patel *et al*., 2016) and Nutman (1976) showed the importance of inoculating the Broad Bean plant with Rhizobia bacteria in improving productivity in order to obtain its needs of nitrogen element through the symbiotic relationship, and thus, the biologically fixed nitrogen will reduce the amount of nitrogen fertilizers added to the soil, which leads to reducing groundwater pollution with nitrate and Reducing environmental pollution. Organic matter and humic matter are among the factors that help to increase the efficiency of biofertilizers. According to Chen *et al*., (2004) The use of humic acids led to a significant increase in photosynthesis and vegetative growth and an increase in the nutritional status of the plant and help in the transport and absorption of nutrients, which leads to an increase in the yield in many plants. Humic acids improve plant physiological processes by increasing the availability of macro and micronutrients and enhancing vitamins, amino acids, auxin and cytokinin content in plants (Vanitha and Mohandass, 2014). Also, sulfur is the fourth main nutrient for the plant, and it is one of the nutrients that legume plants require mainly and similar to what they require from the phosphorous element (Gowswamy *et al*., 1986).It is included in the synthesis of some amino acids such as Cysteine and methionine. It also plays a vital role in regulating the metabolism process, in addition to its important role in the process of photosynthesis, respiration, and symbiotic nitrogen fixation. It is responsible for the synthesis of vitamins such as biotin, thiamin, B vitamins, and Some coenzymes (Kumar and Singh, 2009) and sulfur is added to many soils to improve the properties of saline and alkaline soils that suffer from a high degree of soil reaction, where sulfur reduces the pH degree by oxidation of sulfur and the formation of sulfates (SO_4) by different types of microorganisms present in the soil (Belal et al., 2019 (El-Eweddy *et al*., 2005), The broad bean (*Vicia Faba* L.) is one of the most important types of legumes (Li *et al*., 2017) and is cultivated for the purpose of food, feed, and green manure (Di Paolo *et al*., 2015)). The broad bean seeds contain about 30% of the protein (Lizarazo *et al*., 2015) .As well as its role in increasing soil fertility and improving its qualities by biologically fixing atmospheric nitrogen in the soil. Through root nodules in symbiosis with Rhizobia, it is also used in the regulation of agricultural cycles (crop succession) (Abbas, 2012)

Materials and methods :

pots experiment was conducted in topsoil (0-30 cm) from Al-Qurna elimination farms of Basrah province in the green house affiliated to the College of Agriculture - University of Basrah according to the Completely Randomized Design (CRD), to study the effect of inoculation with two sources of Rhizobia bacteria (R_1 local isolate from the bean plant grown in the region, and R_2 Rhizobia obtained from agricultural science laboratories in the Ministry of Science and Technology) . and The effect of three levels of humic acid (0, 10, 20 Lha^{-1}) and three levels of agricultural sulfur (0, 1 , 2 tons ha^{-1}) and their interaction on protein percentage and iron content in seeds .

The local Rhizobia R_1 was isolated from the root nodules of the bean plant using Yeast extract mannitol agar at pH of 7 and incubated at 30° C for a week, then the colonies appeared in snowy white color. The bacteria were purified by sub-cultures by taking a pure colony and streaking it by loop on the surface of the nutrient medium (YEMA) and incubating it at 30 °C for 72 hours, and the process was repeated several times to obtain a pure isolate that was preserved in a nutrient agar (Slant) until its use (Beak *et al*., 1993) .

Humic acid was extracted by treating a specific weight of fermented cow's waste (for two months) with 0.1 *N* NaoH and getting rid of the formed precipitate (Humen) then treating the filtrate with concentrated HCl acid until the pH reached about 2 and left until the next day to form the precipitate which represents humic acid as its pH 6.5.

The Broad bean plant seeds (*Vicia Faba L.*) local cultivar were used and superficially sterilized with sodium hypochlorite (1%), then washed with ethyl alcohol (95%) three times and washed with sterile distilled water several times to remove the trace of the sterilized substance (FNCA, 2006).It was divided into three sections. The first section was placed in a clean and sterile glass container containing the liquid bacterial inoculation containing R_1 with the addition of 10% gum arabic (to increase the adhesion of bacteria with the seeds) for one hour. The same process was repeated with the R_2 bacterial inoculation, while the last part of the seeds was left without inoculation for the purpose of control.

Agricultural experiment:

The pots were filled with the studied soil after milling and passing through a sieve with 4 mm in diameter by 10 kg pot $^{-1}$ and fertilized with nitrogen at a level of 150 kg N ha^{-1} in the form of urea fertilizer (46% N) in two batches, the first at planting and the second after a month of germination, and phosphorus at a level of 50 kg P ha^{-1} in the form of concentrated superphosphate (47% P_2O_5)and agricultural sulfur was added at levels 0, 1 and 2 tons S ha^{-1} The fertilizers were mixed well with the surface layer of the potting soil and three levels of humic acid (0, 10 and 20 L.ha^{-1}) were added, mixed with irrigation water, the treatments were planted by 8 seeds pot $^{-1}$.Taking into account the cultivation of non-inoculation seeds first to avoid pollution , and the treatments were irrigated with R.O. water With the limits of field capacity and moisture compensation on the basis of weight, after germination, the plants were thinning to 4 plants .After the end of the growing season, the pods were harvested, the seeds were dried at 65 °C, crushed with a ceramic mortar and passed through a sieve with 1 mm in diameter, then 0.2 g of seed powder was digested by adding 8 ml of acidic mixture (nitric acid and perchloric acid in a ratio of 3:1). It is left for 24 hours (Kalar, 1998) and then heated until a clear solution is obtained then the volume was completed to 50 ml with distilled water . The iron content in the seeds is estimated by an atomic absorption device.

0.2 g of seed powder was digested by adding 5 ml of concentrated sulfuric acid and left for 24 hours then heating for a half an hour, then 3 ml of a mixture of sulfuric acid and perchloric acid at an average of 4% (96 ml of sulfuric acid: 4 ml of perchloric acid) (Cresser and Parsons, 1979), transfer the digested samples into 50 ml volumetric bottles, complete the volume with distilled water, estimate the nitrogen content in the seeds by steam distillation method according to Page *et al*., (1982) and calculate the protein percentage from the following equation :

protein %= % of nitrogen in the seeds x 6.25

Table (1) Some chemical, physical and biological properties of the studied soil

traits	**Units**	**Values**
pH	**—**	**7.81**
EC	**$^{1-}$dS m**	**4.1**
CEC	**Cmol $^{(+)}$ Kg-^{1}**	**12.3**
CaCO3	**g kg^{-1}**	**180.25**
Organic matter	**g kg^{-1}**	**6.9**
total nitrogen	**g kg^{-1}**	**1.2**
total iron	**g kg-^{1}**	**10.353**
available Nitrogen	**g kg-^{1}**	**0.0252**
available phosphorous	**g kg^{-1}**	**0.0552**
available potassium	**g kg^{-1}**	**0.095**
available sulfur	**g kg^{-1}**	**0.02802**
available iron	**g kg-^{1}**	**0.00597**
total bacteria	**Cfu g^{-1} Dry soil**	**72.33 × 10^{6}**
fungi	**Cfu g^{-1} Dry soil**	**13 ×10^{3}**
Soil Rhizobia	**Cfu g^{-1} Dry soil**	**0.25 × 10^{2}**
thiobacillus bacteria	**Cfu g^{-1} Dry soil**	**0.17 ×10^{5}**
soil texture	**silty loam**	
Clay	**g kg^{-1}**	**458**
silt	**g kg^{-1}**	**326**
sand	**g kg^{-1}**	**216**

Results and discussion :

The effect of interaction between inoculation with Rhizobia bacteria and the levels of humic acid and agricultural sulfur on iron content of broad bean seeds (mg Fe kg^{-1}) .

Figure (1) shows that inoculation with Rhizobia bacteria led to a significant increase in iron content in the seeds of the broad bean plant, as it reached 329.4 and 265.5 mg Fe kg^{-1} when inoculated with R_1 and R_2 isolates, with an increase of 67.89% and 35.32%, respectively, compared to the control treatment that gave the lowest seed Iron content (196.2 mg Fe kg^{-1}) , that may be due to the ability of Rhizobia bacteria to secrete organic acids that temporarily reduce pH and thus dissolve the iron compounds and increase their availability in the soil, then increase its absorption by the plant and its transmission to the seeds (Sultan, 2011) . It is also noted from Figure (1) that isolate R_1 was significantly superior to isolate R_2 with an increase of 24.07% and this may be due to isolating R_1 as adapted to soil and environmental conditions of the region.

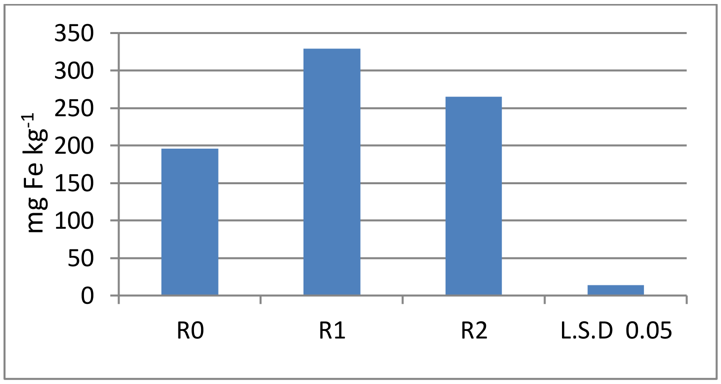

Figure (1) Effect of inoculation with Rhizobia Bacteria on Iron content in broad bean seeds (mg Fe kg^{-1})

Figure (2) shows that the addition of humic acid concentrations had a significant effect on increasing the iron content in the seeds of the Broad Bean plant. The H_2 treatment achieved the highest iron content (369.3 mg Fe kg^{-1}) compared to the control treatment which gave the lowest content (135.6 mg Fe kg-1) with an increased average of 172.35% , the level of H_2 significantly excelled on H_1 with an increase of 28.99%.These results agree with Afifi *et al*., (2010) on the Broad Bean plant, who indicated that humic acid led to an increase in the iron content in seeds, The reason for the increase is that the use of humic acid has improved the availability of nutrients and reduce the lack of nutrients. It also works to reduce the pH and dissolution of some important nutrients such as iron, then increase its absorption by the plant as well as its ability to facilitate the extension of roots in soil and increased permeability of cell membranes.

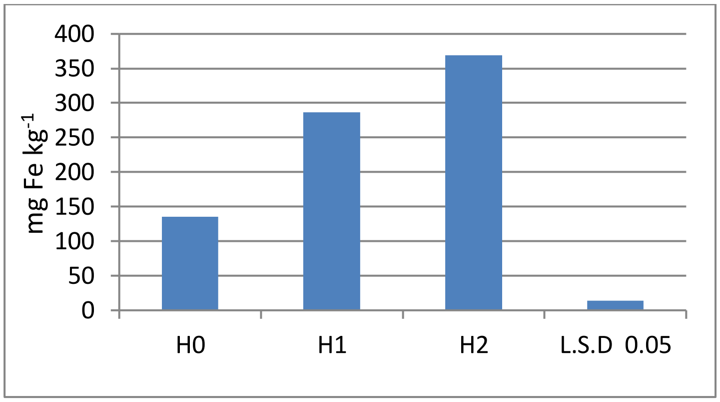

Figure (2) Effect of humic acid on iron content in broad bean seeds (mg Fe kg^{-1})

Figure (3) shows that there were significant differences for adding sulfur levels in the iron content in the seeds of the Broad Bean plant, and the highest average of iron content was (320.5 mg Fe kg-1) at the S_1 level, which was significantly excelled on S_2 level and the control treatment, which each amounted to 285.9 and 184.8 mg Fe kg^{-1} with an increase of 12.1% and 73.43% , respectively. Radwan *et al*. (2017) explained the increase in iron content to the effect of sulfur in the soil indirectly through its transformation into sulfuric acid, which dissolves many important micronutrients. These results also agree with Choudhary *et al.* (2017) on Mung bean plant and Togay *et al.*(2008) on chickpea plant, who found that the addition of sulfur led to a significant increase in iron content in the grain.

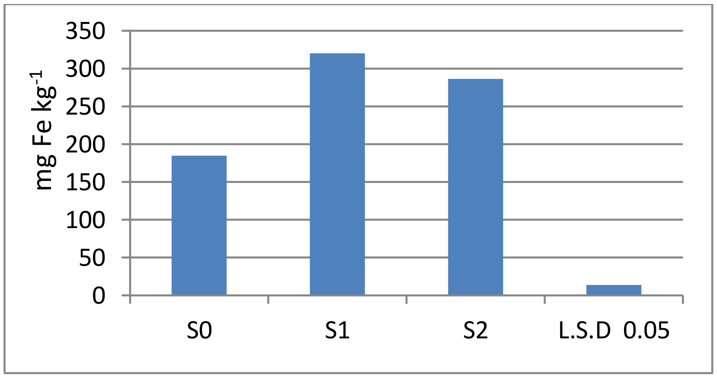

Figure (3) Effect of agricultural sulfur on iron content in broad bean seeds (mg Fe kg^{-1})

Figure (4) shows a significant increase in iron content in broad bean seeds as a result of the interaction between inoculation with Rhizobia bacteria and the addition of humic acid and it was the highest rate 419.3 mg Fe kg^{-1} in the treatment R_1H_2 compared with control (96.8 mg Fe kg^{-1}) with an increase of 333.16%. It is also noted from Figure (4) that there is no significant difference between the two treatments R_1H_1 and R_1H_2, this means that it is possible to use a half of the amount of humic acid with inoculation with isolate R_1 in order to reduce the cost of production. Whereas, inoculation with isolate R_2 was highly significant between levels H_1 and H_2, which indicates to the need for isolate R_2 to a greater amount of humic acid to activate it and make it more capable of secreting organic acids in the rhizosphere , which increases the availability of iron in the soil and increase its absorption by plant.

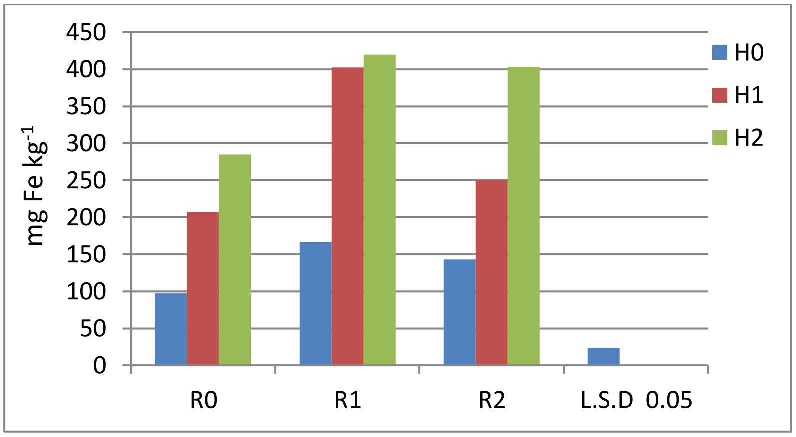

Figure (4) Effect of binary interaction between inoculation with Rhizobia bacteria and addition of humic acid on iron content in broad bean seeds (mg Fe kg^{-1})

Figure (5) shows that there were significant differences in the content of iron in the seeds of the bean plant at the interaction between inoculation with Rhizobia bacteria and the addition of agricultural sulfur. The treatment R_1S_1 achieved the highest iron content of 428.7 mg Fe kg^{-1},While the lowest average was 120 mg Fe kg^{-1} at treatment R_0S_0 with an increase of 257.25%, as well as inoculation with isolate R_2 with the addition of level S_1 gave a higher iron content than that obtained at treatment R_2S_2 and this indicates that the addition of level S_1 was better in increasing iron availability and absorption by plants. Where Lindsay (1979) showed that a decrease in pH by one unit leads to the dissolution of micronutrients and their compounds a hundred times, including iron, and thus increase their availability and absorption by the plant.

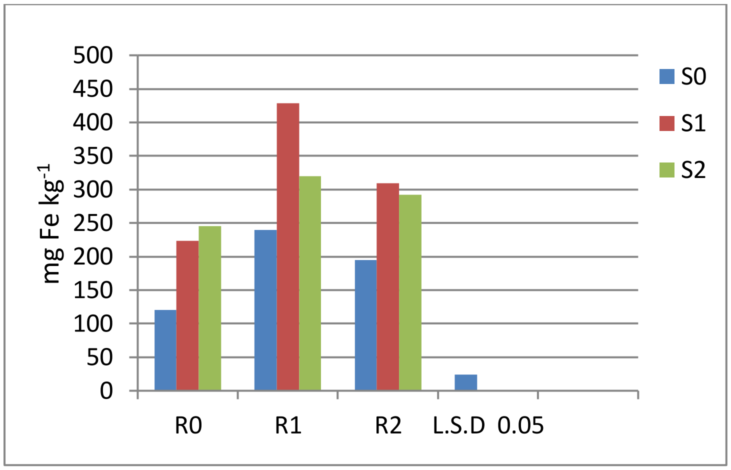

Figure (5) Effect of binary interaction between inoculation with Rhizobia bacteria and the addition of agricultural sulfur on iron content in broad bean seeds (mg Fe kg^{-1})

Figure (6) shows a significant increase in iron content as a result of the binary interaction between the levels of humic acid and agricultural sulfur, and the highest average was 471.3 mg Fe kg^{-1} by the effect of the H_2S_2 treatment compared to the control treatment (H_0S_0), which gave the lowest iron content average of 102.1 mg Fe kg $^{-1}$ with an increase of 361.61%.The reason may be due to the oxidation of sulfur by many soil microorganisms to sulfuric acid (H_2SO_4) , which leads to a decrease in the pH and the possibility of sulfuric acid interacting with calcium carbonate in the soil, forming calcium sulfate $CaSO_4$, which ionizes to Ca^{+2} and $SO4^{-2}$. This also contributes to reducing the pH and increasing the availability of some nutrients in the soil, including iron, and then increasing its absorption by the plant (Awadalla *et al.*, 2003).In addition, humic acid contains many nutrients, so its addition improves the vital properties of the soil and encourages the release of nutrients from the original soil sources in their availability forms, as well as the ease of movement towards the roots of the plant, thus easy absorption by the plant (Mahmoud *et al.*, 2011). These results agree with El- Galad *et al.*, (2013) who found a significant increase in iron content in the seeds of the bean plant when humic acid was added with agricultural sulfur.

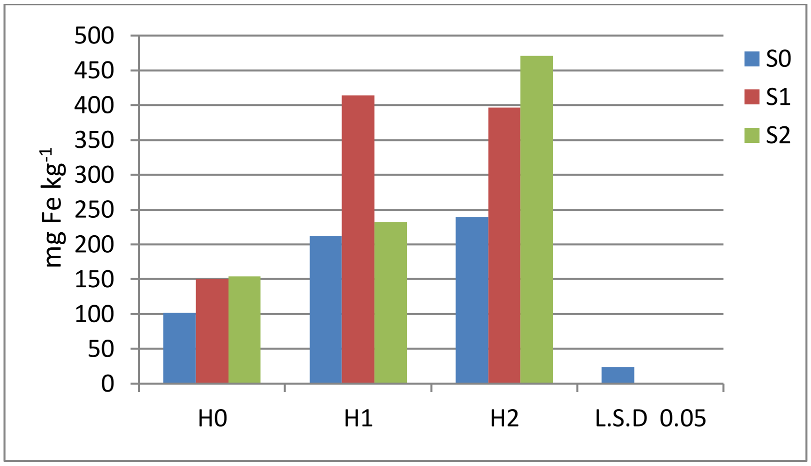

Figure (6) The effect of a binary interaction between the levels of humic acid and agricultural sulfur on iron content in broad bean seeds (mg Fe kg^{-1})

The triple interaction between the studies factors (inoculation with Rhizobia bacteria, levels of humic acid and agricultural sulfur) showed a significant effect on iron content in seeds (Table 2), and the highest average was 650.1 mg Fe kg^{-1} with the effect of treatment $R_1H_1S_1$, while the lowest average of iron content was 71.8 mg Fe kg^{-1}at the control treatment ($R_0H_0S_0$) with an increase of 805.43%, When the treatment $R_2H_2S_1$ achieved a higher iron content (495.1 mg Fe kg^{-1}) , which means that the R_2 isolate needs a greater amount of humic acid because it contains many nutrients, which contributes to providing the necessary nutrients for bacteria, and thus increases its effectiveness in fixing nitrogen and increasing its availability, which is reflected in the increase in the availability of the necessary nutrients for the plant, and agricultural sulfur plays a very important role in reducing the pH, which leads to an increase in the availability of some nutrients in the soil, including iron, and its absorption by the plant.

Table (2) The effect of the triple interaction between inoculation with Rhizobia bacteria and levels of humic acid and agricultural sulfur on iron content in broad bean plant (mg Fe kg^{-1})

Sulfur			**Humic**	**Inoculation**
S2	**S1**	**S0**		
114.8	**103.8**	**71.8**	**H0**	**R0**
191.6	**311.5**	**117.2**	**H1**	
428.7	**255.9**	**170.9**	**H2**	
186.3	**196.7**	**117.5**	**H0**	**R1**
257.2	**650.1**	**298.7**	**H1**	
517.2	**439.2**	**301.6**	**H2**	
160.2	**152.2**	**117.1**	**H0**	**R2**
249	**279.9**	**221.2**	**H1**	

468	**495.1**	**246.8**	**H2**	
41.72				**L.S.D 0.05**

The effect of interaction between inoculation with Rhizobia bacteria and the levels of humic acid and agricultural sulfur on the protein percentage of broad bean seeds (%) .

Figure (7) shows that the inoculation with Rhizobia bacteria led to a significant increase in the protein percentage in the seeds of the broad bean plant. Inoculation with isolates R_1 and R_2 gave the highest percentage of protein in the seeds (20.97% and 19.2%, respectively), with an increase of 19.62% and 9.53% compared to the control treatment that gave the lowest average (17.53%) This may be due to the activity of Rhizobia bacteria in fixing atmospheric nitrogen and preparing it for the plant, which led to an increase in the proportion of protein in the seeds. These results agreed with of Al-Hasnawi (2017) and Al-Khafaji (2018) results on the bean plant, and isolate R_1 significantly excelled isolate R_2 with an increase of 9.22%.

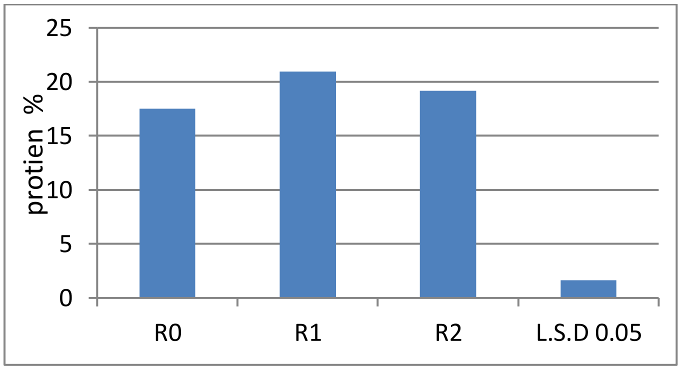

Figure (7) Effect of inoculation with Rhizobia bacteria on protein percentage in broad bean (%)

It is noticed from Figure (8) that there are significant differences in the protein percentage in the seeds of the bean plant when humic acid is added. The H_2 level achieved the highest rate of protein percentage (22.33%) compared to the control treatment which gave the lowest rate (15.64%) and an increased average of 42.77% . The level of H_2 significantly excelled at the level of H_1 with an increase of 13.12%. The reason may be due to the fact that the humic substances increased the efficiency of the plant in absorbing water and improving the mineral nutrition and the proportion of protein in the grains (Morard *et al*., 2011 and Daur and Ahmed , 2013) .

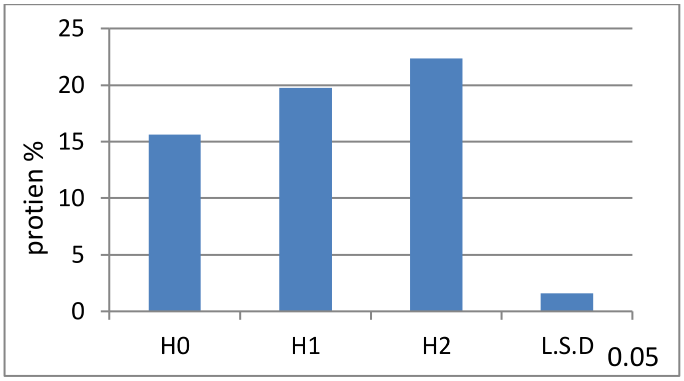

Figure (8) Effect of humic acid on protein percentage in broad bean seeds (%)

Figure (9) showed significant differences in protein percentage in the seeds of the broad bean plant when adding agricultural sulfur, where the highest average was 21.31% at the S_2 level , while the lowest rate was 17.22% when the control treatment (S_0) with an increase average of 23.75%. The S_2 level significantly excelled on the S_1 level with an increase of 11.16% , that may be due to the fact that the addition of sulfur led to a significant increase in the nitrogen and sulfur content in grains, as both nutrients are closely related to the protein metabolism process.

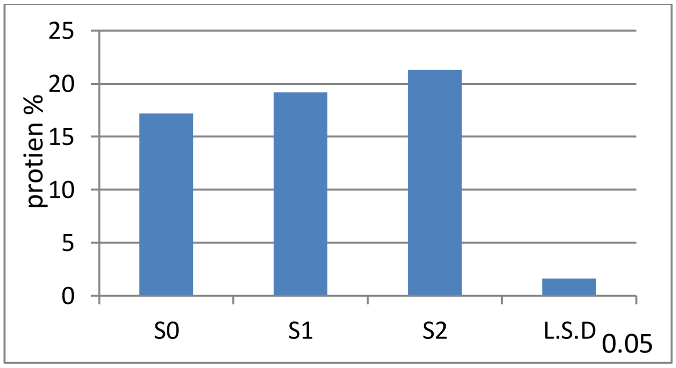

Figure (9) Effect of agricultural sulfur on protein percentage in broad bean seeds (%)

Figure (10) shows that the binary interaction between inoculation with Rhizobia bacteria and the addition of humic acid had a significant effect on protein percentage in the seeds. The treatment R_1H_2 achieved the highest average of 24.84%, while the lowest average reached 13.42% in the control treatment (R_0H_0) with a percentage of 85.1% increase. The reason may be due to the fact that inoculation with Rhizobia bacteria led to better development of root nodules, and then increased atmospheric nitrogen fixation and its use in protein synthesis as a result of better availability of nutrients, including nitrogen (Singh *et al.*, 2018). Humic acid also has a role in increasing the availability of nitrogen and increasing its absorption by the plant, and thus increasing its content in the seeds, where nitrogen enters the formation of amino acids, which represent the cornerstone in protein synthesis ,Which led to an increase in protein percentage in the seeds of the bean plant (Al-Azi and Karim, 2019). It is noted from the same figure that there is no significant differences between treatment R_0H_2 and R_1H_1 and R_2H_1 treatment which means that inoculation with Rhizobia bacteria suffices for half the amount of humic acid.

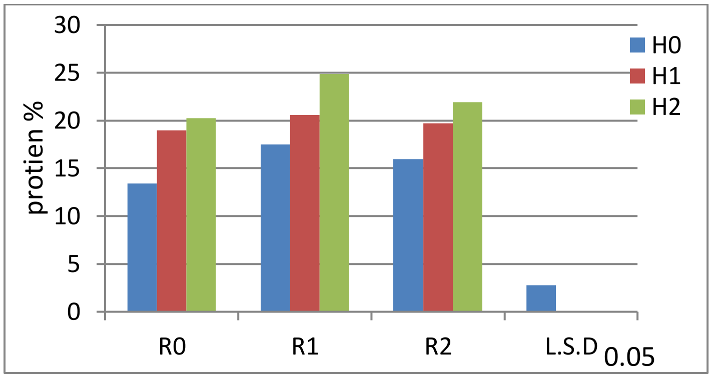

Figure (10) Effect of the interaction between inoculation with Rhizobia bacteria and the addition of humic acid on protein percentage in broad bean seeds (%)

Figure (11) shows that the binary interaction between inoculation with Rhizobia isolates and the addition of levels of agricultural sulfur gave a significant increase in protein percentage in the seeds of the broad bean plant, and the highest average was 23.28% in treatment R_1S_2 compared to the control treatment, which gave the lowest seed content of 15.36% an increase of 51.56%.The reason may be mainly due to the increase in nitrogen content in the seeds as a result of inoculation with Rhizobia bacteria and the addition of agricultural sulfur, noting that there is no significant difference when inoculation with the two studied isolates and the addition of S_1 and S_2 levels, and this average that half of the amount of agricultural sulfur can be used in order to reduce the cost , and also It is noticed from the same figure that there is no significant difference between treatment R_0S_2 and both treatments R_1S_1 and R_2S_1 , this means that half the amount of agricultural sulfur can be dispensed with by inoculation with one of the two studied isolates to obtain the same protein percentage in seeds.

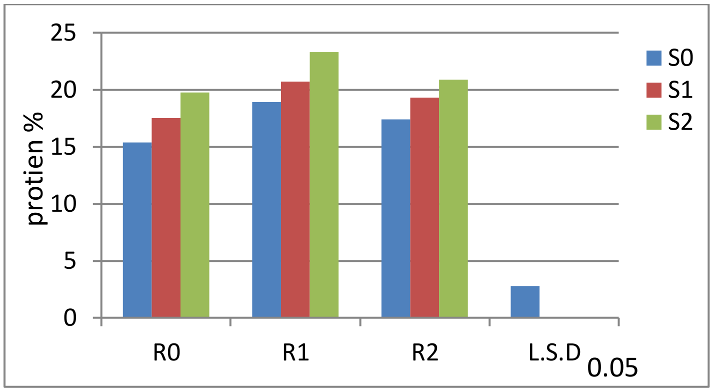

Figure (11) Effect of the interaction between inoculation with Rhizobia bacteria and the addition of agricultural sulfur on protein percentage in broad bean seeds (%)

Figure (12) showed that the binary interaction between the levels of humic acid and agricultural sulfur addition showed a significant effect in increasing protein percentage in the seeds, the highest percentage of protein was 23.92% when treated with H_2S_2, while the control treatment (H_0S_0) gave the lowest protein percentage in seeds, it reached 12.88%, with an increase of 85.71%.The reason may be due to the fact that humic acid contains many elements, including nitrogen, so its content in the plant increase and its transmission to the seeds and then increase the percentage of protein, as well as improving the soil structure and facilitating the extension of the roots to obtain nutrients and water. Also, sulfur helps in the synthesis of amino acids (cystine, cysteine, methionine), which is a source of protein (Kaisher et al.,2010) and it is noted from the same figure that there is no significant difference between the two treatments H_2S_1 and H_2S_2, and thus it is possible to reduce the amount of agricultural sulfur In order to reduce production cost, these results are in agreement with El-Galad et al., (2013) on the broad bean plant.

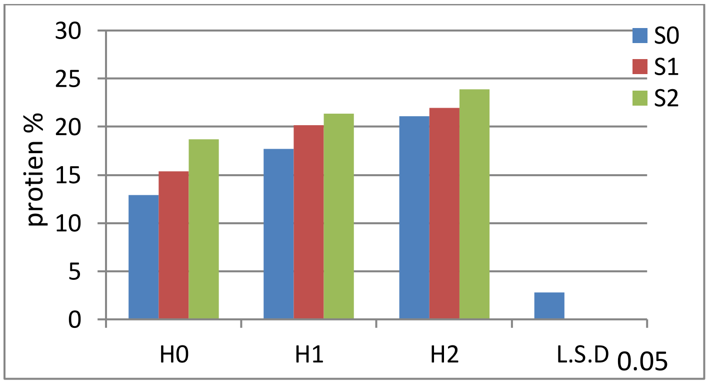

Figure (12) Effect of the interaction between the levels of humic acid and agricultural sulfur on protein percentage in broad bean seeds(%)

It is noted from Table (3) that the triple interaction between the studied factors led to a significant increase in the protein percentage in the seeds, and the highest average was 27.71% when treated with $R_1H_2S_2$, while the lowest average was 9.63% when compared to $R_0H_0S_0$ with an increased average of 187.75 % , The reason may be due to the positive effect of the studied factors in increasing the availability of nitrogen in the soil and increasing its uptake by plant, then increasing its content in the seeds, which is reflected on protein percentage in the seeds of the broad bean plant, it is noted from the same table that there is no significant differences between treatment $R_0H_2S_2$ and treatments $R_1H_1S_1$ and $R_2H_1S_1$ in their effect on the percentage of protein in seeds, which indicates that it is possible to use half the amount of humic acid and agricultural sulfur with inoculation with one of the two studied isolates, meaning that the cost can be reduced through inoculation.

Table (3) The effect of the interaction between inoculation with Rhizobia bacteria and the levels of humic acid and agricultural sulfur on the protein percentage in broad bean seeds (%)

Sulfur			**Humid**	**Inoculation**
S2	**S1**	**S0**		
16.92	**13.71**	**9.63**	**H0**	**R0**
20.85	**18.96**	**17.06**	**H1**	
21.44	**19.83**	**19.4**	**H2**	
19.98	**17.35**	**15.17**	**H0**	**R1**
22.17	**21.29**	**18.23**	**H1**	
27.71	**23.48**	**23.33**	**H2**	
19.1	**15.02**	**13.85**	**H0**	**R2**
21	**20.27**	**17.79**	**H1**	
22.6	**22.6**	**20.56**	**H2**	
4.84				**L.S.D 0.05**

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