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Study the interactional effect between various concentrations of (Fe) and (*Sinorhizobium Meliloti*) on growth, and nitrogen fixation process in Alfafa (*Medicago Sativa*) Plants.

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Abstract

This experiment studied the effect of various concentrations of Fe-chealate (Fe) in the efficacy of four isolates of *Sinorhizobium melioti*, isolated locally from root nodules of alfafa plants (Medico sativa), in nitrogen fixation process these plants gathering from different locations in Iraq (Baghdad). The rhizobiume isolates were named (R1, R2, R3, and R4).

This study divided in two parts, a laboratory experiment measured the colony forming unit (CFU) for all isolated strains under 3 concentrations of (Fe) (2mg, 4mg, 8mg)/ L, through incubation of 48, 72 hours, In 28c° The results of this experiment showed a significant increasing in a count of (CFU) ($P \leq 0.05$) for all strains, specially under the concentrations (4mg, 8mg,) Fe/L.

The farmer experiment alfafa plants were agriculture in plastic pots size (5kg) soil putted in the green house, then studied some growth parameters of alfafa plants such as: the length of shoot, dry weight, numbers of root nodules, and the concentration of nitrogen and protein in plants. The results showed that the of adding concentrations of (0.2mg , 0.4mg , 0.6mg) (Fe) / L to the soil with bacterial isolates was very effective in the efficiency of all four Sinorhizobium isolates under study, in nitrogen fixation process, and increasing their ability for fixing nitrogen when association with Medico sativa plants. The results present a significant increasing in the growth parameters were mentioned.

These parameters were increased when increased the (Fe) concentrations. The treatment (bacteria & 0.6mg Fe) showed the best results comparing with other treatments isolates. The isolates R3 present the best results comparing with other isolate but R2 strain showed the less significant results in this study.

Keywords: Sinorhizobium; (Fe); Alfafa plants**;** CFU**;** Nitrogen fixation process

1. Introduction

Biological nitrogen fixation by Rhizobium bacteria is a very important nitrogen source for plants, because it's requires less energy and causes less environment pollution. [1] .

Biofertilizing technology by using microorganisms like Rhizobium bacteria with legume crops is an important way for fixing nitrogen in legume plants, and this technology used a wildly range in different counteries, also this technology add nutrients to the soil, and stimulating plant growth through synthesis of growth – promoting substances [2].

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Biofertilizers by inoculation with rhizobium bacteria has an extra benefit of nitrogen addition to soil and to the legumes plants, also this way known as eco-friendly which avoiding the environmental pollution also it's cost-effective relative to chemical fertilizers. [3]and [4].

The rhizobium bacteria required iron for nitrogen fixation with legumes, many researches present that the Fe element enhancing the bacterial activity in fixing nitrogen in plants.

Defines in iron can effect the nodulation process, initiation and development of the nodules on roots, because iron is necessary for synthesis of iron-contaning proteins in host plant, including (Legheamoglobin), and in bacteriods for nitrogenase and cytochroms of the electrons transport chains, which are moving the nitrogen fixation process. [5].

2. Material and methods

2.1. Isolation of rhizobium strains

Four isolates were isolation and identification locally, the isolates were for Sinorhizobium meliloti isolated from root nodules of alfafa plant (Medicage sativa) that are cultured in different regions in Baghdad. The isolates named (R1, R2, R3,R4).

Alfafa plants growing in good from were selected and taken from a different area, the soil was non fertilizer, for isolation of nodules bacteria. The roots washed well by water, than root nodules that are close to the main root were removed, wash with distal water many times and sterilized according to the [6] the root nodules were washed with sterilized distalled water, then ethyl alcohol (95%) for 5 min, wash with sterilized distal water, then with (0.1%) of acidified HgCl2 for 3-6 min, then planed in glass test tube with (3ml) of NaCl (0.85%) with good scratch. Taking (1m) from bacterial suspension and cultured on manitol salt yeast extract medium (MSY) and incubated at 27°c for 2-3 day until appearance of colonies. The bacteria isolated were identification by using gram stain and with successful infected for alfafa plants again.

2.2. The purification of isolates

After appearance of white mucous colonies, a loopfull were taken from the colonies and streaking on (MSY) and incubated for 3 days. This experiment was repeated for many times unit obtain of pure cultures. Four dilutions of bacterial suspension were done by using normal saline (0.85%), and the diluted bacteria were cultured by spread (0.1)ml from last dilution on MSY in petri disc and incubated at 28°c until appearance of colonies.

2.3. The measurement of bacterial colonies (colony forming unit CFU) under affecting of (Fe) concentrations

Prepared a broth culture of (MSY) media, and inoculated with isolates (under study), then incubated in shaker incubator (100 rpm/sec)with 28°c [7]. After 24h of incubation, taking 1 ml from sample and inoculated with 25 ml broth culture of (MSY) media in conical flask, then incubate in a shaker incubator (100 rpm/sec) under 28°c., taking (1ml) from bacterial culture after 48h and 72h respectively. After serial dilutions until (10^{-6}), spread 1 ml broth solid culture media that contain on concentrations of (Fe) (0.2mg, 0.4mg, 0.6mg)/L, and incubated for 2-3 days under 28°c. the bacterial colonies were count for three times with control sample for each growth period.

2.4. Host plant used

The local variety seed of Medicago sativa were used. Which obtain from Iraqi seeds certificate center.

2.5. Soil analysis and preparation

The soil used in the farmer experiment was analysised to determine physical and chemical properties before using.

Silt	Clay	Sand	Organic matter	Mixture soil	pH	EC (ds/m)	CaCo3
R10/g soil	185/g soil	245/g soil	905/g soil	Loamy soil	7.5	2.30	21.3%

In this experiment we used a plastic post size (5kgm) and sterilization well by using sodium hyopchloride, and each pot filled with constant quantity (4kgm) of loamy soil, the soil was sieved with diameter (2mm) to performe homogenate and remove impure from it.

The soil was sterilized by autoclave. The concentration of (Fe) was counted on the basis soil weigh (4kgm) (0.1, 0.3, 0.6gm/4kgm) and mixed with soil by good form to distribution of element to all parts of soil. The seeds of Medicago sative were used (10) seeds in each pot. The pots put in green house under 20-25°c, and irrigated rotator.

2.6. Inculation the soil by Sinorhizobium stains

A loop full from all Sinorhizbium isolates (under study) were taking and grown in (10ml) of (MSY) broth, and incubated in shaker inocubator (100rpm/sec) in 28°c for 24h., then (2ml) from incubated in shaker incubator (100rpm/sec) in 28°c for 48h, the this bacteria broth was centrifuged in (3000rpm/sec) for 5 min, then suspensitioned the bacterial precipitate by normal saline (0.85%), and complete the volume to (50ml), then added to the pot after 3 days of agriculturing.

The harvest of plants

The plants were harvested in march/2022, these plants harvested with roots, the roots washed with water to remove the soil, then the plant's samples were transport to the laboratory and recorded the following data:

- The length of shoot system.
- The dry weight of plant.
- The number of root nodules.

2.7. Estimation of nitrogen and protein ratio in plants

The known weight of dried plant are grounded with good form according to [8] and then, estimation of N2 ration in plants according to [9]. The estimation of protein percentage was according to [10].

2.8. Statistical analysis

The statistical analysis system, [11] was used to effect of isolates and concentrations in study parameters. Least significant difference (LSD) test was used to significant compare between means in this study

3. Results and discussion

3.1. Bacterial colonies account

Table (1) showed that all four strains were effected by high concentrations of (Fe) under study. There were a significant increasing in the number of colonies ($P \leq 0.05$) comparing with control without (Fe), after 48 and 72 hours from growth.

Bacteria grow up with (4mg) and (8mg) /L present a significant result comparing with control. Strain (R3) present higher significant, and strain (R2) present the less significant increasing comparing with other strains. These results agree with other study [12] which was showed an increasing in account of rhizobium bacteria associated with Vigna radiate plants, under different concentration of (Fe), and supported with other study [13] which was present a significant increasing in account of Sinorhizobium meliloti colonies under effect of (2,4,6mg Fe)/L, another study [14] showed that an enhanced growth of rhizobium colonies isolated from (pigeopea) under effect of (0.1% to 0.3%) concentrations of (Fe) added with growth media. The result in this study maybe related to that (Fe) is necessary and essiential for synthesis of many proteins like (cytochromes) which important in electrons transport chains in the bacteria. [5]

3.2. The effect of (Fe) concentrations and rhizobium strains on growth parameters of alfafa plants

The famer experiment showed that the interaction between Sinorhizobium strains and (Fe) was very effective in increasing and promoting the growth of plants, and the adding of (Fe) to the soil enhancing the bacterial activity in nitrogen fixation in the plant grow up with rhizobium bacteria and (Fe) comparing with control plants which were (0 bacteria & 0 Fe), and (bacteria & 0 Fe) (plants inoculated byrhizobium strain without adding (Fe).

The strain R3 present the highest significant increasing for all growth parameters, and R2 present the less increasing in all growth parameters comparing with other strains.

3.3. Plant length, and dry weight.

Table [2], showed a significant increasing in the length of alfafa plants that inoculated by Sinorhizobium strains, comparing with control plants, also the table showed no significant increasing in length of plants growth with (0.2gm Fe/4kg pot soil), but a simple increasing presented in plants grown with (0.4gm Fe) and (0.6gm Fe)/4kg pot soil (without bacteria) comparing with control groups (0 bacteria & 0 Fe). A significant increasing in the lengths of plantswas shown that inoculated by Sinorhizobium bacteria and the (Fe), specially the concentrations (0.4gm Fe) and (0.6gm Fe)/4kg pot soil.

Table (3) also showed the same significant increasing in dry weight of alfafa plant that inoculated by Sinorhizobium and (0.2gm, 0.4gm, 0.6gm) Fe/4kg pot, comparing with the control groups which were (0 bacteria & 0 Fe), and (bacteria & 0 Fe).

These results supported by other studies [15, 16].

3.4. The number of nodules

Table (4) showed a significant increasing in numbers of root nodules in the alfafa plants inoculated by Sinorhizobium strain specially plants grown with (0.4gm) and (0.6gm) Fe/4kg pot.

comparing with control groups (bacteria & 0 Fe). These results may be related to the (Fe)is an essential element for nodulation process, and necessary for synthesis of leyheamoglobiun, and nitrogenase enzyme. [5] [17].

3.5. The ratio of nitrogen and protein in plants

Table (4), and table (5) showed the alfafa plants which were grown up with Sinorhizobium strains and (Fe) concentrations gives the best results in ratio of nitrogen and protein comparing with control groups (0 bactria & 0 Fe) and (bacteria & 0 Fe).

The plants grown with all Sinorhizobium stain and with (0.2gm Fe / 4kg pot) present no significant increasing, the plants grown with (0.4gm) and (0.6gm) Fe/4kg pot, present significant increasing in ratio of nitrogen and protein for all strains, comparing with control groups. These results supported and agree with another studies [13] [18] [19] and [20] these experiments presented a significant increasing in numbers of root nodules, and in concentrations of nitrogen and protein (Symbiotic nitrogen fixation) in legumes plants when added a different concentrations of (Fe) to the soil of agriculture with the plants.

Table 1 Effect of Fe concentrations and Sinorhizobium isolates in Number of cfu

Isolate	**Concentration Fe (mg/L)**				**LSD value**
	0	**2**	**4**	**8**	
R1	7 ± 0.35	8 ± 0.19	21 ± 0.13	33 ± 0.40	6.592 *
R2	9 ± 0.50	11 ± 0.36	28 ± 0.72	35 ± 0.29	5.713 *
R3	12 ± 0.42	19 ± 0.64	30 ± 0.53	43 ± 0.75	6.985 *
R4	11 ± 0.56	13 ± 0.39	24 ± 0.44	38 ± 0.38	6.746 *
LSD value	3.188 *	3.752 *	4.067 *	4.974 *	----

* (P<0.05).Control (0/0)without bacteria and without Fe.

Table 2 Effect of Fe concentrations and Sinorhizobium isolates in Plant length (cm)

Isolate	**Concentration of Fe (gm/4kg soil)**				**LSD value**
	0	**0.2**	**0.4**	**0.6**	
0	16.80 ± 0.38	20.33 ± 0.43	24.66 ± 0.63	31.33 ± 0.74	6.294 *
R1	24.50 ± 0.93	32.33 ± 0.77	46.33 ± 1.28	56.33 ± 1.19	6.704 *
R2	18.83 ± 0.62	25.00 ± 0.46	37.00 ± 0.95	43.33 ± 0.85	5.926 *
R3	31.66 ± 0.59	41.66 ± 1.15	58.33 ± 2.09	67.33 ± 2.37	7.016 *
R4	28.66 ± 0.48	32.00 ± 0.94	48.33 ± 1.26	59.33 ± 1.93	6.833 *
LSD value	5.179 *	5.883 *	6.074 *	5.821 *	----

* (P<0.05).Control (0/0)without bacteria and without Fe.

Table 3 Effect of Fe concentrations and Sinorhizobium isolates in Dry weight (gm)

Isolate	**Concentration of Fe (gm/4kg soil)**				**LSD value**
	0	**0.2**	**0.4**	**0.6**	
0	3.13 ± 0.07	4.10 ± 0.15	5.33 ± 0.09	6.32 ± 0.25	1.644 *
R1	5.80 ± 0.12	7.36 ± 0.19	9.45 ± 0.23	11.46 ± 0.44	2.497 *
R2	4.10 ± 0.07	5.20 ± 0.08	6.73 ± 0.12	7.77 ± 0.30	1.426 *
R3	6.50 ± 0.04	8.30 ± 0.11	10.53 ± 0.42	13.33 ± 0.57	2.052 *
R4	6.11 ± 0.18	7.76 ± 0.29	9.50 ± 0.39	12.23 ± 0.31	2.869 *
LSD value	1.842 *	1.796 *	2.053 *	2.916 *	----
* (P<0.05).					

Control (0/0)without bacteria and without Fe.

Table 4 Effect of Fe concentrations and Sinorhizobium isolates in Number of roots nodules

Isolate	**Concentration of Fe (gm/4kg soil)**				**LSD value**
	0	**0.2**	**0.4**	**0.6**	
0	8 ± 0.15	11 ± 0.37	17 ± 0.42	22 ± 0.61	5.409 *
R1	33 ± 1.46	48 ± 2.52	67 ± 1.79	82 ± 2.68	7.629 *
R2	12 ± 0.42	20 ± 1.25	30 ± 1.04	40 ± 1.19	7.842 *
R3	46 ± 2.09	55 ± 1.84	75 ± 3.62	93 ± 3.73	9.256 *
R4	40 ± 1.37	54 ± 2.06	70 ± 2.59	86 ± 2.16	7.883 *
LSD value	6.724 *	8.629 *	8.733 *	10.315 *	----

* (P<0.05).Control (0/0)without bacteria and without Fe.

Table 5 Effect of Fe concentrations and Sinorhizobium isolates in Nitrogen percentage (%)

Isolate	Concentration of Fe (gm/4kg soil)				LSD value
	0	0.2	0.4	0.6	
0	3.50 ± 0.14	4.20 ± 0.07	5.10 ± 0.19	6.20 ± 0.35	2.275 *
R1	6.96 ± 0.22	9.20 ± 0.52	11.76 ± 0.48	14.60 ± 0.74	3.416 *
R2	5.70 ± 0.09	7.66 ± 0.16	9.46 ± 0.33	11.13 ± 0.52	2.861 *
R3	7.70 ± 0.36	10.46 ± 0.47	12.93 ± 0.59	16.43 ± 0.64	4.094 *
R4	7.33 ± 0.41	10.11 ± 0.52	12.16 ± 0.41	15.10 ± 0.56	3.417 *
LSD value	2.072 *	2.556 *	3.163 *	2.945 *	----

* (P<0.05). Control (0/0)without bacteria and without Fe.

Table 6 Effect of Fe concentrations and Sinorhizobium isolates in Protein percentage (%)

Isolate	Concentration of Fe (gm/4kg soil)				LSD value
	0	0.2	0.4	0.6	
0	22.75 ± 0.84	27.30 ± 1.03	33.15 ± 1.72	40.30 ± 1.89	8.015 *
R1	45.24 ± 1.78	59.80 ± 2.52	76.44 ± 3.08	94.90 ± 4.16	7.442 *
R2	37.05 ± 1.09	49.79 ± 1.66	61.49 ± 2.53	72.34 ± 2.85	9.671 *
R3	50.05 ± 2.37	67.99 ± 2.48	84.04 ± 3.94	106.79 ±4.69	11.825 *
R4	47.64 ± 1.85	65.71 ± 2.51	79.04 ± 3.07	98.15 ± 3.86	9.558 *
LSD value	7.573 *	9.187 *	7.662 *	7.902 *	----

* (P<0.05).Control (0/0)without bacteria and without Fe

4. Conclusion

The treatment (bacteria & 0.6mg Fe) showed the best results comparing with other treatments isolates. Compliance with ethical standards.

Disclosure of conflict of interest

Authors confirmed no conflict of interest to be disclosed.

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