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ROLE OF RHIZOBIA (*RHIZOBIUM MELILOTI*) OF ALFALFA IN THE BIOREMEDIATION OF CONTAMINATED SOIL WITH HYDROCARBONS

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Abstract

The aim of this study is to isolate the Rhizobia bacteria from the alfalfa plant and determine their efficiency in the break down and reduction of hydrocarbons from contaminated soils. The experiment was carried out in two plastic pots in order to study two factors the first factor which is the role of vaccination with the bacteria Rhizobia with alfalfa (*Medicago sativa L.*) in the repair of soils contaminated with the second factor fuel oil, the experiment was designed within the system (RCBD)) 5 replicates as the addition of Rhizobia vaccine (vaccine addition) marked by (R1) and another treatment (without addition) marked by (R0) and the addition of fuel oil within 3 levels (0%, 1%, 2%) marked by H0, H1 and H2 respectively. 30 plant pot were used and 3 harvesting were done for alfalfa. Rhizobia were obtained by isolating it from the root nodes of the alfalfa from one of the agricultural fields in the Al-Sophia area and studied its properties. The experimental vaccine was used on the same plant. The results showed a significant difference in the number of root nodes and total bacterial density and the number of Rhizobia and the wet and dry weight of the vegetative and root groups at the treatment of addition of bacterial vaccine and treatment of addition of fuel oil at the concentration of 1%. The increase in the amount of hydrocarbons in the vegetative part of the plant of the first, second and the third harvest and in the roots of the treatment of addition vaccine and the treatment of addition fuel oil at the concentration of 2% and achieved more overlap when the combination H2R1 (concentration of 2% with the addition vaccine) on the rest of the combinations. The amount of hydrocarbons in the soil is clearly reduced. The removal of fuel oil from the soil for the added quantity in the treatment R1 (90.33% and 80.7%) to the concentrations 1% and 2% compared to the treatment without adding R0 (89.2% and 80.0%) respectively. We conclude that the use of alfalfa and rhizobia to remove soil pollutants was very effective.

Key words : Rhizobia bacteria, alfalfa, fuel oil, bioremediation.

Introduction

Oil pollution is a worldwide threat to the environment, polluted soil, sediment and water. It is one of the first pollutants in the environment, posing a major threat to human life and other life (Refaat *et al*., 2008). Fuel oil pollution is a global problem in developing and industrialized countries. EL-Tarabily (2002). Fuel oil is a complex mixture of aromatic hydrocarbons, aliphatic and paraffin compounds with nitrogen, oxygen, sulfur-containing compounds, and organic and inorganic organic matter materials (Bachmann *et al*., 2014). There are harmful and negative effects on plants when polluting the soil with oil, the effect of these compounds include indirect harmful effects especially on soft parts like roots, Plant branches, it effect limited in the Wooden parts of buds and trees, these compounds effects on the cell fatty membranes (McGill *et al*., 1981).

The use of microbial vaccines is one of the most environmentally safe ways to remove oil pollution from other methods. The importance of microorganisms in the decomposition of organic pollutants is based on the use of these substances as a source of energy and carbon (Okerentugba *et al*., 2003).

Rhizobium is one of the most highly valued organisms that fix nitrogen with Leguminous crops (Tariq *et al*., 2014). It has the ecological capacity to reduce organic pollutants, making it useful for rehabilitating polluted soil. It stimulates survival and work with other pollutant soil bacteria, thus reducing their concentration, because synergistic action promotes plant growth, plant interaction and rhizobia will provide a promising option for removal (Hao *et al*., 2014)

The alfalfa plant is one of the most important fodder crops with high capacity to resist environmental conditions, in addition to having many qualities that make it the first place to alleviate the problems of environmental pollution. It is characterized by having a deep root system of up to 4.5 meters (Karimi, 2013) and have the ability to absorb heavy metals and its resistance to difficult conditions and the fixation process, as the accumulation of hydrocarbons in the leaves of the plant and adsorption by the roots, which leads to the elimination of pollutants faster, especially that can be harvested every 20 days (Putnam, 2001) Malik *et al.* (2010) mention that the herbal plants consider a preferred for use in the plant treatment than trees and shrubs because of their high and rapid growth rate and have a large biomass as well as more adaptive to environmental stresses. The state of plant and microorganism interferences in the biological treatment of soil and polluted environments has resulted in high efficiency and rapid cleaning of the contaminated site.

Materials and Methods

1. isolation of Rhizobia bacteria from root nodes

The root nodes were taken from the roots of the alfalfa plant grown in Al-Sophia area. They were thoroughly washed with water and then sterilized with 3% NaOCl solution for 10 minutes, then washed with sterilized water, placed on sterile filter paper to dry and then placed on a 20 mL surface of YEMA (extract mannitol agar) in petri dishes and kept in the incubator at 28º C for 72 hours (Vincent, 1970). Nodes were selected and crush with 2 ml of the liquid YEM medium by a sterile glass rod and take the sterilized loop head from the suspension and streaked on a petri dish containing the solid YEMA medium and place in the incubator for the appearance of the bacterial colonies.

2. Vaccine preparation

The vaccine was prepared by transferring the growing bacteria on the YEM solid medium to a glass flask containing 20 mL of liquid medium. The cultures bacteria are placed in the shaking incubator at 100-150 rpm and 28º C for 24 h. The growing bacteria were precipitate using a centrifuge at 1400 cycles / minute for 15 minutes. Then add 1 mL of liquid YEM medium to the precipitated bacteria to be a bacterial suspension of 2 × 108 cells / ml (Prasad *et al*., 2000). Mix the vaccine with alfalfa seed and prepare it for planting.

3. Field experiment

The experiments were carried out at a site of the center for desert studies, Anbar University in 12/10/2017, where the plant pot s were organized with 5 duplicates per treatment. In accordance with the RCBD design (the complete random sectors) and the global experimental system of two parameters (concentration of oil and the vaccine in Rhizobia) in the experiment 30 plant pot, soil was brought from a field planted with alfalfa plants in the Al-Sophia area of Ramadi. The soil was dried with air and then the soil was fertilized with fuel oil at concentrations of 0%, 1% and 2% w \ v -1. M (0.950) and sulfur content weight (3.5%) carbon content weight (6.5%) water and sediment content (1%) according to the marketing specifications of the Al-Doora refinery.

Table 1 shows the chemical, physical and biological properties of the soil used in the study, the soil was placed in plastic pots 25 cm high and 28 cm diameter and the seeds were vaccinated with *Rhizobium meliloti* vaccine after it was moistened with a 20% sugar solution and some seeds were left without a vaccine. The pots planted with alfalfa local type (*Medicago sativa* L.) vaccinated and non-vaccinated at 40 seeds per pot. It was watering and after a week of growth, the plants were reduced by 20 plants per pot (Makki, 2017). After the last harvest, the following parameters were measured: number of nodes in the plant, soft and dry weight of the vegetative part, soft and dry weight of the root part, total bacterial count in the soil, total bacterial density and Rhizobia bacteria, quantity of hydrocarbons in the vegetative part of the first, second, third and in roots Residual in soil.

Table 1 : The chemical, physical and biological properties of soil used in the study

Character	Units	Value
EC	Decimetre m^{-1}	3.45
pH	-	7.56
$CaCO_3$	Gram- $kilogram^{-1}$	223
Organic material		6.20
sand		194
slit		436
clay		370
structure	Slit - clay	Mixing soil
CEC Cation exchange capacity	Centimole-charge $–kg^{-1}$	
N total	ml- kg^{-1}	168.40
P		7.32
K		136.60
hydrocarbons	ml- kg^{-1}	0.0
Total bacteria	cfu g^{-1} log	$6.8*10^6$
Nitrogen fixer		$3.2*10^5$
Rhizobia		$1.2*10^3$

Results

1. Effect of the *Rhizobium meliloti* vaccine and the concentration of fuel oil in the number of root nodes in the plant

Table 2 shows a significant increase in the number of root nodes of the alfalfa plant to add the *R. meliloti* vaccine compared with the treatment of non-addition vaccine, which reached 845 and 547 (node -1), and the addition of the H2 concentration to H1 and H0.

Table 2 : Effect of *Rhizobium meliloti* bacterial vaccine and the concentration of fuel oil in the rate of the number of root nodes (node-1)

Fuel oil concentration	Without vaccine R0	*Rhizobium meliloti* vaccine R1	Mean
0 %H0	450	812	631
1 %H1	560	820	690
2 %H2	632	904	768
المعدل	547	845	
L.S.D (0.05) R=21.22 , H=25.99 , R+H=36.76			

2. **Effect of the *Rhizobium meliloti* vaccine and the concentration of fuel oil in the soft and dry weight of the three harvests in total vegetative of plant.**

The results of the statistical analysis in table (3) showed a significant difference (0.05) in the soft and dry weight of the total vegetative of the three harvests. There was a significant increase in the soft and dry weight of the treatment addition vaccine compared to the treatment without vaccine, also there is a significant difference in concentrations of the H1 and gave the highest value in soft weight, while the H2 concentration was higher than the dry weight of the plant.

Table 3 : Effect of pollen *Rhizobium meliloti* and the concentration of fuel oil in the total weight of the vegetative part for the total three harvests (g/ pot-1).

Wet weight of vegetative part			
Fuel oil concentration	**Without vaccine R0**	***Rhizobium meliloti* Vaccine R1**	**Mean**
0 %H0	499.4	641.1	570.2
1 %H1	550.9	702.2	626.5
2 %H2	546.7	726.0	636.3
mean	532.3	689.7	
L.S.D (0.05) R= 13.0 , H= 15.2, R+H= 22.5			
Dry weight of vegetative part			
0 %H0	113.9	126.9	120.4
1 %H1	128.3	142.0	135.1
2 %H2	116.4	131.8	124.1
Mean	119.5	133.5	
L.S.D (0.05) R= 2.1 , H= 2.6 , R+H= 3.7			

3. **Effect of the *Rhizobium meliloti* vaccine and the concentration of fuel oil on the soft and dry weight root of the plant.**

Table (4) shows the presence of a significant effect on the addition of the bacterial vaccine in the soft and dry weight of the root of the plant compared to the treatment without vaccine. The treatment was superior to the addition of bacterial vaccine to the treatment without addition of both weights also there is significant differences in treatment addition fuel oil, the concentration 1% was superior to concentrations 0% and 2%.

Table 4 : Effect of the *Rhizobium meliloti* vaccine and the concentration of the fuel oil on total weight of root (g/ pot -1).

Wet weight of vegetative part			
Fuel oil concentration	**Without vaccine R0**	***Rhizobium meliloti* Vaccine R1**	**Mean**
0 %H0	170.7	190.4	180.5
1 %H1	178.5	205.6	192.0
2 %H2	174.7	200.2	187.4
mean	174.6	198.7	
L.S.D (0.05) R=3.7 , H=4.5 , R+H=6.4			
Dry weight of vegetative part			
0 %H0	59.8	62.8	61.3
1 %H1	57.7	65.2	61.5
2 %H2	56.2	64.2	60.2
Mean	57.9	64.1	61.3
L.S.D (0.05) R=1.4 , H=2.1 , R+H=3.0			

4. **Effect of the *Rhizobium meliloti* vaccine and the concentration of fuel oil on total bacterial density and the rhizobia bacteria in the soil.**

Table (5) shows a significant difference in the treatment of the addition of the rhizobia vaccine on the total bacterial density and the number of rhizobia bacteria in the soil. The addition of the vaccine was given the highest value in the bacterial count compared to the treatment without addition. There is a significant difference (0.05) in addition of fuel oil group for rhizobia bacteria. The highest concentration was found at 2% while the number decreased at the same concentration in total bacterial count.

Table 5: Effect of *Rhizobium meliloti* vaccine and concentration of fuel oil in total microbial density and soil rhizobia bacteria (log cfu g-1)

Total bacterial density			
Fuel oil concentration	**Without vaccine R0**	***Rhizobium meliloti* Vaccine R1**	**Mean**
0 %H0	7.83	7.67	7.75
1 %H1	7.12	7.37	7.24
2 %H2	6.88	7.29	7.08
mean	7.28	7.44	
L.S.D (0.05) R=0.26 , H=0.32 , R+H=0.45			
Rhizobia bacteria			
0 %H0	6.42	7.52	6.97
1 %H1	6.78	7.87	7.33
2 %H2	6.83	7.91	7.37
Mean	6.68	7.88	
L.S.D (0.05) R=0.06 , H=0.07 , R+H=0.10			

5. **Effect of *Rhizobium meliloti* vaccine and concentration of fuel oil on the amount of hydrocarbons of the vegetative part of all the harvests.**

The results of the statistical analysis in table (6) showed that there was a significant difference (0.05) in the amount of hydrocarbons in the total number of three harvests for the treatment of the addition bacterial vaccine. The treatment addition vaccine R1 exceeded the treatment without the R0 vaccine for the three harvests. There is a significant difference in treatment addition of fuel oil, the concentration of H2 was higher than the H1 concentration. also a significant difference was also observed for the interaction parameters (vaccine addition and fuel oil addition) the combination of H2R1 give the highest mean while the lowest mean at the combination of H1R0.

Table 6 : Effect of *Rhizobium meliloti* vaccine and the concentration of fuel oil in the amount of hydrocarbons absorbed in the vegetative part of the plant (ml/ pot -1).

First harvest			
Fuel oil concentration	**Without vaccine R0**	***Rhizobium meliloti* Vaccine R1**	**Mean**
1 %H1	0.25	0.29	0.27
2 %H2	0.28	0.32	0.30
mean	0.26	0.31	
L.S.D (0.05) R=0.0013 , H=0.0013 , R+H=0.0018			
Second harvest			
1 %H1	0.16	0.21	0.19
2 %H2	0.19	0.25	0.22
Mean	0.17	0.23	
L.S.D (0.05) R=0.0013, H=0.0013, R+H=0,0018			
Third harvest			
1 %H1	0.13	0.15	0.14
2 %H2	0.13	0.17	0.15
Mean	0.13	0.16	
L.S.D (0.05) R=0.0013, H=0.0013, R+H=0,0018			

6. **Effect of *Rhizobium meliloti* vaccine and concentration of fuel oil in the amount of hydrocarbons absorbed to the root mass.**

Table 7 shows a significant difference in the amount of hydrocarbons in the root of the plant for the treatment addition of rhizobia vaccine. R1 treatment was given the highest treatment rate without the R0 vaccine. The significant difference was also observed for the addition of fuel oil, which high at H2 concentration compared to H1 concentration, And the treatment of overlap between the two parameters was a significant difference at (0.05) as the combination H2R1 gave the highest rate for the amount of oil absorbed by the roots and the lowest rate was in the combination of H1R0.

Table 7 : Effect of *Rhizobium meliloti* vaccine and fuel oil concentration in the amount of Hydrocarbons absorbed in the root mass of alfalfa Plant (ml/ pot -1).

Fuel oil concentration	**Without vaccine R0**	***Rhizobium meliloti* Vaccine R1**	**Mean**
1 %H1	0.20	0.25	0.22
2 %H2	0.24	0.28	0.26
mean	0.22	0.27	
L.S.D (0.05) R=0.0045 , H=0.0045 , R+H=0.0064			

7. **Effect of *Rhizobium meliloti* vaccine and concentration of fuel oil on the remaining hydrocarbons in soil**

Table 8 showed statistically significant difference in the amount of hydrocarbons remaining in the soil after the last harvest of the treatment addition vaccine. The treatment of the additive exceeds the treatment without the vaccine which contains the minimum remaining amount of oil in the soil. A significant difference was also observed for the addition of oil in the H1 concentration compared to H2, the difference in the treatment was also observed in treatment of the interaction between the parameters. The H1R1 combination gave the lowest amount of residual hydrocarbons in the soil, while the more hydrocarbons were obtained in the soil at the H2R0 combination.

Table 8 : Effect of *Rhizobium meliloti* vaccine and concentration of fuel oil in the amount of hydrocarbons remaining in the soil (ml/pot -1 soil).

Fuel oil concentration	**Without vaccine R0**	***Rhizobium meliloti* Vaccine R1**	**Mean**
H1 %1	10.8	9.6	10.2
H2 %2	40.0	38.5	39.2
mean	25.4	24.0	
L.S.D (0.05) R=1.4 , H=1.4 , R+H=2.0			

Table (9) shows the percentage of added fuel oil and decomposed oil removed by the plant and remaining in the soil and the percentage of total removal using the alfalfa and *Rhizobium meliloti* bacteria.

Table 9 : percentages of added oil and fuel oil and removed and remaining in the soil and total removal.

Added fuel oil ml /10kg^{-1}	**Removed by plant ml/ pot^{-1}**		**Remaining in soil ml/10g^{-1}**		**Decomposed ml/10kg^{-1}**		**Percentage of total removal %**	
	R0	**R1**	**R0**	**R1**	**R0**	**R1**	**R0**	**R1**
100 H1	34.92	50.90	10.80	9.65	54.28	39.45	89.20	90.35
200 H2	36.59	50.52	40.00	38.60	132.41	110.88	80.00	80.70

Discussion

The study showed a significant increase in the treatment of the addition of rhizobia vaccine and in the number of root nodes. This is due to the increase in the number of rhizobia bacteria in the soil which has the ability to penetrate the roots and form the root nodes, especially with the presence of rhizobia vaccine (Al-Shujairi *et al*., 2017 and Elsaadony, 2015) Increasing the level of addition of the concentrations of fuel oil from 0-2% due to the increase in the amount of secretions resulting from the roots because of the presence of oil in the soil, which leads to attract the roots of rhizobia and increase its infects to plant (Teng *et al*., 2010).

The results showed an increase in plant biomass due to the processing of nitrogen and other nutrients of the plant through the bacteria interacting with it through the bio-stabilization of nitrogen, which has an important role in the synthesis of amino acids and proteins of plant and thus increase its weight (Al-Assafi *et al*., 2017) the cause of superior treatment of addition oil to increase the secretions of the roots that are working to supply the plant essential nutrients such as nitrogen, phosphorus and carbon, which affects the increase of biomass of the plant as well as the study showed the significant difference in the treatment of the addition of rhizobia vaccine in root weight due to the improvement of plant growth and increase the absorption of nutrients by the roots in the presence of the vaccine and the ability of bacteria to produce the Geberlins and stimulants to absorb the essential nutrients of the plant and thus increase the weight of roots (Makki, 2017)

Also there is a significant difference in the treatment of addition fuel oil as a result of the availability of hydrocarbons in the soil, a large amount of sugars resulting from the process of photosynthesis will be transferred to the roots under the stress conditions resulting in increasing the biomass of the roots (Taiz, 2002). The study showed the significant difference in the treatment of vaccine addition in the presence of bacteria in the soil due to the fact that rhizobia bacteria encourage soil bacteria to grow. The proportion of substances needed by bacteria in their growth, especially the organic acids and peptides, increases with the presence of rhizobia (Hammadi, 2015). significant difference in fuel oil addition treatment due to the microorganisms in soil consume hydrocarbons in the soil as a source of energy and carbon and thus increase their numbers (Al-Qargouli and Jubouri, 2013),

As well as the increase in the number of rhizobia bacteria caused by the support of rhizobia vaccine for soil rhizobia bacteria, and the reason for the increase in the treatment addition of oil levels due to the availability of carbon energy source of these microbes and increasing the source of increasing numbers (Biro *et al*., 2000). The results showed significant differences in the experimental parameters for the amount of hydrocarbons absorbed in the vegetative section of the three plants and because of the high ability of rhizobia in the biological treatment, especially the presence of clover plant, which has a strategic symbiotic relationship as they played a dominant role in the repair of soils contaminated with PAHs (Teng *et al*., 2011),

In the treatment of the addition of fuel oil, the increase is attributed to the efficiency of the biological treatment and the root secretions that support the rhizosphere plant treatment. Hydrocarbons are the source of energy and carbon for the microbes in the region of the rhizosphere (Wenzel, 2008). The interference treatment is due to the symbiotic relationship between the rhizobia and the legumes which support Plant efficiency and its role as Rhizoremediation for hydrocarbon contaminants (Johnson *et al*., 2005).

The results showed the amount of hydrocarbons absorbed by the roots of the alfalfa. The significant increase in the treatment of the bacterial vaccine is due to the symbiotic role of the rhizobia with the clover. The rhizobia consume carbon easily in oil and facilitate its absorption by the roots and its storage (Zhou *et al*., 2013). in the fuel oil addition the reason for the increase is due to the stimulation of the roots with the presence of oil to increase their secretions and activate the microbes in the soil to carry out the biological decomposition of oil absorption by the roots and stability in them, as well as the treatment of interference between the vaccine rhizobia and the addition of oil rhizobia has an important role in the positive changes to microbial society in the Rhizosphere region as well as the role of the alfalfa in contaminated soil with hydrocarbons a conversion occurs of organic compounds through the plant and stored in the plant (Teng *et al*., 2010).

The results of the study also showed a significant decrease in the amount of hydrocarbons in the soil in the treatment addition of vaccine due to the presence of rhizobia bacteria that work on the consumption of oil and increase its break down as a source of energy and carbon, and the significant difference in the treatment addition of oil levels caused by the process of evaporation volatile substances in addition to the process of photo oxidation and biodegradation which was stimulated by plant growth that absorbed hydrocarbons (Alber, 1995).

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