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Survey and Distribution of Symbiotic Bacteria Nodules *Rhizobium spp* and Determine Its effectiveness in Nitrogen Fixation

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Abstract:
The nitrogen importance to plant growth encouraged researchers to study it as plants limiting factor. Nitrogen fixation by life organism Rhizobium spp. is an important to increase growth and yield. The study aimed to determine the distribution and activity of bacteria symbiotic in legume growing at Thi-Qar province- southern Iraq. Field survey studied at six locations grew with legumes to know bacteria types. Two field experiments were studied, so the first was the effect of bacteria concentration on cowpeas growth. Three bacterial concentrations were studied. The second was bacteria concentration and nitrogen fertilizer effectiveness on alfalfa dry and fresh weight yield including 3 levels from both factors. Plant vegetative qualities and nodules number affected significantly by the bacterial concentrations. The alfalfa dry and fresh yields significantly were affected by bacterial concentration and nitrogen. The inoculation of seed before planting is important to increase legume growth and yield. The biological Nitrogen fixation is the most important procedure in agriculture to reduce using of amount Nitrogen fertilizers to crop because of amounts of Nitrogen fertilizers is losing by many processes, like (soil erosion, ammonia volatilization, Denitrification, leaching NO_3*, and immobilization process). Increasing density of inoculation is causing increasing by nitrogen fixation, height of plants number of leaves, dry matter, fresh material, productivity of legumes, dry matter and fresh increasing a significantly p α 0.05 to all plants in the study. The number of nodules raised with increasing density of inoculation a significantly (p α 0.05). The bacteria that were isolated from broad been roots was R. legumninosarum. In addition, R. melioti was isolated from the alfalfa roots and the other bacteria such as R. trifolli and Brady R. sp. was isolated from clover (trefoil) and cowpeas crops respectively.*

Key words: Bacteria nodules, Nitrogen fixation, Rhizobium, Inoculation

1. Introduction
The nutrients are such one limiting factor to increase the crop yield such as nitrogen, thus natural sources of nitrogen are many such as, vital fixation, lightning and photochemical interactions, which are estimated at about 200-250 million tons every year (Dyson, 1996). On the other hand, nitrogen can be possible to produce artificially by the Haber- bush method in which it needs large amounts of energy and very high cost more than 20 billions of dollars per year (Vance, 1997). Moreover, adding the artificial nitrogen to the soil lose by volatilization, fixation and de-nitrification as well as leaching NO_3^- to the soil, which caused pollution to the ground water (Nielson, 2006; Bundick et al., 2009). The biological nitrogen fixation is the most important procedure in agriculture to reduce the use of the amount of nitrogen fertilizer to the crop field and vegetative crop and contributes in using nitrogen in agriculture by 65% (Vance, 1997). Nitrogen is the important mineral nutrient to plant. Its percentage in earth atmosphere is 78.1% as gas; therefore, the atmosphere is considered the main source of the nitrogen fixing in which it converts N_2 to NH_3 by symbiotic with leguminous plants. These microorganisms (symbiotic nitrogen fixation organisms) can format nodules

on legume plants roots such as *Rhizobia* and fix about 10-350 kg ha^{-1} $season^{-1}$ of air nitrogen (Brewin, 1992). *Rhizobium* fixes the highest amount of nitrogen as well as increases the agricultural yield in different ecological system (Bordeleau & Prevost, 1994). On other hand, other microorganisms can fix N from the atmosphere in non-symbiotic as *Azotobacter*, and some of these microorganisms can fix and convert nitrogen to NH_3 in non-aerobic conditions as *Clostridium*. In addition, *Azospirillum* fixes the N from earth atmosphere to rice, barely and corn plants, although it is aerobic bacteria compulsory (Alexander, 1982). The importance of nitrogen fixation is due to the numbers of *Rhizobium* in soil, its activation to fix N_2 and convert it to NH_3, legume genotype, and soil conditions (Barran & Bromfield, 1997; Mackersie, 1997; Issa, 1990). Nitrogen fixation method is affected by the soil and climate conditions such as temperature, moisture, salinity, soil pH, desiccation and pesticides (Al-Rashidi, 1978).

In addition, adding Nitrogen fertilizer to soil affected the nitrogen fixation by bacteria, thus increasing nitrogen fertilizer amount, the ability to fix nitrogen by bacteria decreased (Muller & Pereira, 1995; Longnecker et al., 1998). Eaglesham et al. (1981) found that the dry weight of cowpeas increased by increasing the N level from zero to 100 kg ha^{-1} but the number of nodules decreased from 36.8 to 13.3 nodules. Significant increases in nodules number of alfalfa by adding 40 kg N ha^{-1} but decreased at 100 kg N ha^{-1} (Neghamish, 1985). The soil moisture and salinity are the important factors in southern Iraqi soil. Thus, Al-Rashidi and Damergi (1977) found that some of Iraqi soils considered poor to the soybean's bacteria because of lack of appropriate growth conditions for bacteria because of salinity and dry soil. The soils in mid and southern Iraq contained small numbers of bacteria nodules because of soil salinity and soil dry, thus; this type of soils needs to be inoculated with bacteria of nitrogen fixing (Al Rashidi and Fath-Alla, 1983). Neghamish (1985) found that the moisture and the desiccation had the highest effect on the growth stabile *Rhizobium* strains in Basra. In addition, he reported that the 38° C was killing to all the *Rhizobium* strains under the dry condition but not under the moisture. Mineral nitrogen that added to soil affected the symbiotic nitrogen fixation negatively, especially at the high level of mineral nitrogen (Muller & Preivo, 1995; Longnecker et al., 1998). Thus, this proposal aimed to study and find the contribution of *Rhizobium* depending on the legume plants in Thi-Qar area to see the best activation to fix N_2 by this microorganism.

2. Materials and Methods

2.1. Field Survey experiment

To determine the bacteria nodules, the field survey hold in sixth soil types in Thi-Qar area that were planted with different kinds of legume crops in order to the bacteria types and activity (As shown in Table 1).

Table 1. *Field survey, and the type of legume crops that were planted within in Thi-Qar area*.

No	Survey Area	Type of legume plant
1	Chebaish	Alfalfa, broad bean, cowpeas
2	Al-Arja	Alfalfa, broad bean, Cowpeas
3	Al-Mashtal	Alfalfa, broad bean, Cowpeas
4	Electric power station	Trefoil, Alfalfa, Broad bean
5	Science college field	Alfalfa, Cowpeas
6	AL-Shatrah	Alfalfa, Trefoil, Cowpeas

Bacteria related measurement-included numbers of nodules in each crops and the shape of the bacteria was studied as well as plant height (cm) and number of tillers per plant were calculated as crop related measurements. The bacteroids were isolated and separated in the specialized media (yeast mannitol agar) to study bacteria activation in nitrogen fixing (Burton et al., 1972). After identifying the number of nodules in each plant at each location, bacteria were transplanted in petri dishes containing the specialized media (yeast mannitol agar) and incubated at 28° C for 3-5 days then kept on slant media to use later to inoculate the soil.

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2.2. Isolation and Diagnosis of Bacteria

The bacteria that were isolated from broad been roots was *R. legumninosarum*. In addition, *R. melioti* was isolated from the alfalfa roots and the other bacteria such as *R. trifolli* and Brady *R. sp.* was isolated from clover (trefoil) and cowpeas crops respectively of the soils for the current study. Then, the bacteria of alfalfa and broad bean crops isolated and kept to prepare the inoculation concentrations to use them on soil for field experiments.

2.3. Seeds Preparing and Planting

The seeds of alfalfa and broad been brought from local plants that grew in Thi-Qar area. The good seeds have been selected and sterilized by 0.0001 $g.ml^{-1}$ of $HgCl_2$ for 5 minutes. After that the seeds have been transferred to the ethyl alcohol [75%] for 5 minutes, and then the seeds were washed by distilled water for several time to get rid of disinfectants. Sterilized seeds of broad bean were planted in 2 cm depth by using two seeds in each hole. Alfalfa seeds were planted on parallel lines by three lines for each treatment and the distance between lines was 15 cm. The area of broad bean was 2×2 m and 3×2 m for alfalfa.

2.4. Soil samples

The samples of soils were collected from the areas of this study at depth of 0 to 30 cm to determine the chemical and physical properties such as soil pH in which it determined by pH meter. Chloride was measured in soil by titration with silver nitrate, and calcium and magnesium were measured using the method of EDTA titrimetry, In addition; organic matter identified depending upon Walkley and Black method as explained in (Black, 1965), and ammonium was extracted with (2N HCl) as explained in (Black, 1965). Soil texture was also determined as mentioned in (Bouyoucos, 1936).

2.5. Field Experiments

Two field experiments carried out in order to study the level of bacterial inoculation and density on cowpeas in which it planted in the field of Science College as the first experiment. Thus, the treatments of the first experiment were bacterial concentration (0, 2×10^{-4}, 4×10^{-8}, and 6×10^{-12}) in which they punctuated (inc0, inc1, inc2, and inc3) respectively. These treatments were arranged in randomized complete block design (R.C.B.D) with three replicates for each treatment. Plot area was 2×2 m, and each plot had 10 lines. Five kg N d^{-1} broadcasted on the soil surface. Two seeds were planted in each hole, and the distance between holes was 20 cm in each line. The plants were collected at the flowering to measure the weight and number of bacterial nodules, plant tillering, leaf area, fresh and dry weight of the plant, and plant height.

The second experiment was about the effect of nitrogen fertilizer and bacterial inoculation on alfalfa plant in the Science College field. The experiment was factorial (3×3×3) in which it had three levels of N fertilizer (0, 15, and 30 kg N/ d) and three levels of bacterial inoculation concentration (0, 2.8×10^{-4}, and 5.7×10^{-7}) with three replications. The bacterial inoculation was prepared from the broth in age 3-5 days. The symbols (b0, b1, and b2) were used to refer to the bacterial inoculation concentration respectively. The plot area was (3*2 m), and each plot was divided to three lines for each treatment. These lines had separated by alley to prevent the bacterial inoculation moved from treatment to other treatment. Thus, each plot had three lines planted with local alfalfa seeds that inoculated with bacterial concentration. Alfalfa 100 seeds weight was 0.23 g, and the seeds germination percentage was 90%. Plots were replicated three times to each treatment. Alfalfa plant was grazing in first year after the flowering stage as well as at the second year to measure the fresh weight, and the plant was dried in oven (70°C for 48 h) to measure dry weight. Effect of the bacterial inoculation density, nitrogen fertilizer levels, and their combinations on the fresh and dry weight of alfalfa plants was studied.

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3. Results and Discussion

3.1. Soils, Presence and Forms of Bacteria Nodules Results

The physical and chemical soils characteristics results appeared that all the soil of this scenario closed to the basal and had a tendency to alkaline because all the soils at Iraq are limestone soils in which lime prevents soils oriented to acidic (As shown in Table, 2).

Table 2. *Physical and chemical characteristics of studying soils in Thi-Qar area*.

Studying area	Physical characteristics								
	pH	EC ds m^{-1}	FC %	BD g cm^{-3}	OM %	Sand %	Clay %	Silt %	ST
Science college	7.7	3.0	31	1.25	1.05	54.6	10	30.4	Sandy loam
Chibayish	7.5	5.18	27	1.24	1.2	55.8	7.2	37	Sandy loam
AL-Shatrah	7.8	4.94	36.4	1.32	2.13	6.6	51.6	41.8	Clay loam
Al-Arja	7.9	13.0	34	1.30	1.2	20	50	30	Clay loam
Al-Mashtal	7.5	3.5	29	1.26	2.67	39.6	8	52.4	Silty loam
Electric power station	7.8	13.5	36	1.30	1.3	40	20	40	Clay loam

Studying area	Chemical characteristics						
	Cations				Anaions		
	Ca^{++}	Mg^{++}	Na^{+}	NH_4^{+}	Cl^{-}	$SO_4^{=}$	HCO_3^{-}
Science college	304	60.5	130	0.08	470	500	130
Chibayish	2620	240	240	0.03	690	450	260
AL-Shatrah	270	170	260	0.09	470	510	120
Al-Arja	1880	140	285	0.09	2140	1970	270
Al-Mashtal	210	150	225	0.03	510	490	150
Electric power station	1750	300	247	0.07	2440	2200	185

Moreover, studying soils differed in electrical conductivity extended from 3 to 13.5 ds m^{-1}, in addition; soils ranged from low salt such as Science college soils and Al-Mashtal soil to very high salt such as such Al-Arja (13 ds m^{-1}) and Electric power station soils (13.5 ds m^{-1}). The field capacity of soils was synchronized with soil texture and relying on the distribution of soil granules. Thus, it clears to identify that the fields of Al-Shatrah, Al-Arja, and Electric power station possess convergent values of field capacity because they have the same soil texture (loam clay). Due to the existence of correlation between the bulk soil density and the separating soil properties (clay, sand and silt), thus increasing in clay percentage more than 20% the bulk density will increase. OM increases either with increasing the clay percentage or with increasing the plant density. Thus, clay percentage was 51.6, 50 and 20 % at Al-Shatrah, Al-Arja and Electric power station respectively, therefore; OM on those fields increased. On the other hand, the OM increased at AL-Mashtal Field because of increasing in plant density.

The values of nutrition elements (Ca^{++}, Mg^{++}, Na^{+}, Cl^{-}, $CO_3^{=}$, HCO_3^{-}, and $SO_4^{=}$) might connect or indicate to the salt of soils because the summation of cations and anions equal to EC. Thus, cations increased with increasing in salt, therefore it will affect the bacteria and plants growth. Furthermore, the bacteria presence and activity for fixing nitrogen are highly correlated to soil physical and chemical characteristics as well as affecting on nodulation. Diatloff (1970) mentioned that the lake in soil moisture, high temperature and alkaline soil lead to minify the nodulation. Increasing the organic matter percentage in soil from 0.3% to 2-3% had led to raised survival of *R. phaseoli* and *R. meliloti* (Chao and Alexander, 1982).

Thus, the results showed differences in existence of bacteria number depending upon the plant type and location of study (As shown in Table 3). The highest finding for the bacteria was on the alfalfa plant at all locations of which means that these bacteria can survive and withstand in difficult soil conditions such as salty. In the same time, the growing bacteria on the bean roots were very sensitive to salty soil, and its existence varied depending upon location, therefore; its numbers were (4, 0, 2, and 3) at the locations of Chibayish, Al-Arja, Al-Mashtal, and Electric power station fields respectively. Therefore, the results of number of bacteria might affect by the EC as the results showed that. So, there was no record to any presence of bacteria in the Al-Arja soil because of the high salt (130 ds m^{-1}), thus Gaballah and Gomaa (2005) mentioned that the increasing in the soil salinity to 6000 ppm caused to decrease in number of *Rhizobium* nodules for the salinity tolerant and sensitive varieties. These results agreed with previous studies (Flowers, 2004) when he mentioned that salty soil curb the growth of bacteria in legume plants.

3.2. Legume Plants Results

The results of a survey about the legume plant such as plant height and tillering number explained in (As shown in Table, 3), thus observing from the results that broad bean plant recorded the highest plant height (95-120 cm) at the Science college field.

Table 3. Studying areas planted with legume plants.

Studying area	Legume type	No of nodules	Plant height cm	Tillering No
Science college	Cowpeas Alfalfa	14 22	95-120 22-30	3 4
Chibayish	Broad bean Cowpeas Alfalfa	4 18 15	20-35 45-72 27-45	4 5 3
AL-Shatrah	Cowpeas Alfalfa Trefoil	12 13 15	30-35 25-33 30-40	3 2 3
Al-Arja	Cowpeas Alfalfa Broad bean	8 14 0	20-27 20-33 ------	2 2 ----
Al-Mashtal	Cowpeas Alfalfa Broad bean	12 11 2	30-40 33-47 30-35	3 3 2
Electric power station	Trefoil Broad bean Alfalfa	12 3 16	40-42 32-34 22-27	2 2 2

At the Al-Chebaish field the cowpeas height was (42-72 cm), and the lowest height of crop was (20-27 cm) at Al-Arja field. In addition, the same trend of alfalfa crop observed accordingly, plant height differed among the locations and the tallest alfalfa crop was at the Al-Chibayish field (27-45 cm) compared to the shorter alfalfa crop at the Electric power station field (22-27 cm). Bean crop as well as recorded the highest crop height at the Al-Chibayish and Al-Mashtal fields (35 cm). All the locations of study showed no different in the height clover crop. In addition, crop tillers differed among the locations and were (3, 4, and 5 tillers $plant^{-1}$) at the Al-Chibayish field to the alfalfa, bean, and broad bean crops respectively, and the lowest tillers for all crops observed at the Al-Arja field (2 tillers $plant^{-1}$). However, it clears to clarify and understand the effect of the soil EC in the trend and response of those two related measurements of crop because of the EC effect on the nodulation as well as physiological impact on plants. From the soil analysis result, Electric power station and Al-Arja fields recorded the highest EC. These results agreed with the previous finding about alfalfa yield decreasing to more than half if it grew in salty solution compared to other plant that grew in non-salty solution (Bower et al., 1969; Pessarakli & Huber, 1991).

3.3. Effect of Bacteria Inoculation Concentrations at the first study

The results of the first experiment appeared a significant superiority to the bacteria inoculation concentration in the related measurements of the broad bean plant (As shown in Table, 4), where it was found that the inc3 concentration resulted the best number of the root ganglia (83) compared to the control concentration inc0 (6).

Table 4. *Effect of broad bean bacteria inoculation concentration on the nodules number and weight (g) as well as plant height (cm), tillering number, leaf area (cm^2), and dry & wet weight of broad bean plant (g) at the first experiment*.

Treatment	**Nodule Number**	**Nodule weight g**	**Plant height cm**	**Tillering No/ plant**	**Leaf area cm^2**	**Dry weight of plant g**	**Wet weight of plant g**
inc 0	6 d	5 d	27.64 d	3 a	17.1 d	3.47 d	17.44 d
inc 1	31 c	10.3 c	38.36 c	4 a	22.8 c	7.48 c	22.52 c
inc 2	51 b	12.5 b	59.3 b	2 a	39.9 a	8.58 b	26.71 b
inc 3	83 a	13.4 a	70.16 a	3 a	25.65 b	9.87 a	39.27 a

Generally, the weight and number of nodules significantly increased at the high level of the inoculation of bacteria concentration. The weight of nodules was (5, 10.3, 12.5, and 13.4 g) to the treatments (inc0, inc1, inc2 and inc3) respectively. Furthermore, traits of plant height and leaf area significantly responded to the bacterial inoculation concentration and increased at the highest concentration (inc3), and were (70.16 cm) and (25.65 cm^2) respectively. This, trend of traits might be to the increasing in fixing nitrogen by the nodules in which it led to increasing the crop growth (Burten, 1972; Neghamish, 1985). Dry weight of the crop significantly affected by the inoculation, and it was (3.47, 7.48, 8.58, and 9.89 g plant^{-1}) to the treatments of inc0, inc1, inc2 and inc3 respectively. Moreover, fresh weight of the crop rose with increasing the inoculation concentration, and the values were (17.44, 22.52, 26.71, and 39.29 g plant^{-1}) to the same treatments respectively. This trend of results is depending upon the activity and population of nodules, which it has been decreased with increasing the soil salt, especially of broad bean crop (Van Hoorn et al., 2001). These results also coincided with antecedent studies that mentioned to increase the dry and fresh weight of plant with increasing the bacterial inoculation (Burton et al., 1972; AbulRedda, 1984; Neghamish, 1985).

3.4. Results of the second experiment

3.4.1. Dry Weight of Alfalfa Crop

Analysis of variance of the second experiment showed a significant effect of treatments on the traits of alfalfa plant at at $p \geq 0.05$ of confidence level. Thus, alfalfa dry weight (g) significantly responded to nitrogen fertilizer at both grazing in the first and second year (As shown in Table 5).

Table 5. *Effect of the interaction of nitrogen fertilizer and inoculation concentration on the alfalfa-dry weight (g unit area^{-1}) at the first grazing and at the second grazing of the second experiment*.

	First grazing			Second grazing	
	Inoculation concentration			Inoculation concentration	

N fertilizer kg d^{-1}	b0	b1	b2	Mean		b0	b1	b2	Mean
0	234.40 h	238.89 h	305.90 d	259.73 b		274.20 d	344.27 b	278.70 d	299.05 c
15	292.82 e	369.20 a	336.28 c	332.76 a		316.13 c	346.84 b	356.90 a	339.69 b
30	305.82 d	342.71 b	345.93 b	331.48 a		358.10 a	346.94 b	353.17 a	352.74 a
Mean	277.78 c	316.93 b	329.36 a			316.15 c	379.35 a	329.60 b	

The values were (277.78, 316.93, and 329.36 kg/plot area) in the first crazing for the treatments (b0, b1, and b2) respectively, and were (316.15, 379.35, and 329.60 kg/plot area) for the same treatments respectively at the second crazing. Dry weight of the crop increased significantly with increasing in the concentration of bacteria at both cuttings, and the highest yield was (329.36 and 379.35 kg/plot area) for the treatments b2 at first year and b1 at the second year respectively. Furthermore, traits of alfalfa plant appeared highly responsive to the interaction between nitrogen fertilizer and bacterial inoculation concentration. Thus, the treatment (N2×b2) recorded the highest dry weight of the crop (345.93 kg/plot area) compared to other interactions among the treatments at the first crazing, but at the second crazing, the highest dry weight was (358.10 kg/plot area) at the interaction of the treatments (N2×b0). Although alfalfa crop is classified as moderate salt tolerant (Munns and Tester 2008), the increasing in nodule number of bacteria on the alfalfa roots was observed. Our field recorded the lowest EC (3 ds m^{-1}) as mentioned in (As shown in Table. 2), therefore this case raises the nitrogen fixing in which supply a good condition of nitrogen to the crop growth. The soil salinity has been negatively affected on the activity of nodules and their population in alfalfa root, especially at the highest (150 mM NaCl) level of salt (Nabizadeh et al., 2011). In addition, increasing the bacteria inoculation led to increase the alfalfa dry weight significantly. This scenario agreed with the other finding in previous (AbdulRedda, 1984; Neghamish, 1985).

3.4.2. Fresh Weight of Alfalfa Crop

Fresh weight of the crop (kg/plot area) significantly increased by raising both nitrogen fertilizer and bacterial inoculation at both crazing (As shown in Table. 6).

Table 6. *Effect of the interaction of nitrogen fertilizer and inoculation concentration on the alfalfa-wet weight (g unit area^{-1}) at the first grazing and at the second grazing of the second experiment.*

N fertilizer kg d^{-1}	First grazing					Second grazing			
	Inoculation concentration					Inoculation concentration			
	b0	b1	b2	Mean		b0	b1	b2	Mean
0	1176 i	1194 h	1547 e	1036 c		1371 h	1721 c	1393 g	1495 c
15	1405 g	1772 a	1614 d	1597 b		1517 f	1699 e	1713 dc	1643 b
30	1467 f	1679 b	1695 c	1614 a		1745 b	2190 a	1730 c	1888 a
Mean	1349 c	1548 b	1618 a			1544 b	1870 a	1612 c	

In the first crazing, the results showed that the fresh weight of the treatments N2 and b2 conferred the highest weight (1614.10 and 1618.90 kg/plot area) respectively compared to the other treatments. On the other hand, at the second crazing of crop the highest fresh weight recorded at the treatments N2 and b1 (1888.74 and 1870.29 kg/plot area) respectively compared to other treatments.

The interaction among the treatments affected significantly in fresh weight at both crazing, and the significant highest fresh weight for the first crazing noticed at the interaction of N1×b1 (1772.16 kg/plot area) and was (2190 kg/plot area) for the interaction of treatments N2×b1 at the second crazing compared to the other interactions. The increasing in N fertilizer might be raise the number and weight of bacteria, and that lead to increase in growth of crop (Burton et al., 1972).

4. Conclusions

It is clear to understand that all legumes crop is not being apple to attack by the nodules roots bacteria depending upon different effects such as soil conditions. However, in this scenario, the bacteria attacked all legumes plants in all the soils of study. Thus, our conclusions mentioned that all the soil of this study have the bacteria of *Rhizobium* that attacked the legumes plant in different percentages. The inoculation by bacteria is necessary to guarantee the finding and effectiveness of bacteria to increase the legumes yield. On the other hand, the increasing in nitrogen fertilizer will inhibit the activity of bacteria to fix nitrogen in soils.

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