THE EFFICIENCY OF PREPARED BIOFERTILIZER FROM LOCAL ISOLATE OF *BRADYRHIZOBIUM SP* ON GROWTH AND YIELD OF MUNGBEAN PLANT

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ABSTRACT

Bradyrhizbium sp. **was isolated from 40 legumes roots and soil samples collected from various agricultural areas of Iraq by growing them on yeast extract mannitol agar (YEMA) and identification based on culture and microscopic characteristics, biochemical and physiological test and PCR technology. A split plot experiment was carried out to study the effect of biofertilizer prepared from this isolate on the growth and yield of mungbean plant in sterile and non-sterile soil. The results showed a significant increases in all studied growth and yield traits due to biofertilization. The increase percentage for the biofertilized treatments was 159.35% and 266.66% compare to the non-fertilized treatments in the number and dry weight of nodules, respectively. The increase in dry weight of root and vegetative part were 114.28% and 86.57% respectively , while the increase in the nitrogen concentration in the vegetative part was 16.94%. The results showed that the biofertilized treatment was significantly superior in number of plant pods, total yield and protein % in seeds compare with non-biofertilized, the percentage increase was 49.44%, 90.59% and 16.86% respectively. As for sterilization, the results showed no significant differences between sterilized and non-sterile soil in all growth and yield parameters of mungbean plant. The effect of interaction was significant, the biofertilized and sterilized treatment gave the highest value for all studied traits except the concentration of nitrogen in the vegetative part.**

Key words: Inoculation, Sterilization, Rhizobia, legumes

كفاءة السماد الحيوي المحضر من العزلة المحلية *Bradyrhizobium sp.* في نمو وحاصل نبات الماش

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المستخلص

عزلت بكتريا *Bradyrhizbium sp.* من 40 عينة جذور بقوليات و تربة جمعت من مناطق زراعية مختلفة من العراق وذلك بتنميتها على وسط سكر المانيتول ومستخلص الخميرة (YEMA) وشخصت مظهريا اعتمادا على الصفات المزرعية و المجهرية والكيموحيوية والفسيولوجية وجزيئيا باستخدام تقنية PCR. نفذت تجربة حقلية وفق تصميم الالواح المنشقة لدراسة تأثير السماد الحيوي المحضر من هذه العزلة في نمو وحاصل نبات الماش في تربة معقمة وتربة غير معقمة. اظهرت النتائج زيادة معنوية في جميع صفات النمو المدروسة بالنسبة للمعاملات المسمدة حيويا، اذ كانت الزيادة المئوية في المعاملات المسمدة حيويا 159.35% و266.66% بالقياس مع المعاملات غير المسمدة في عدد العقد ووزنها الجاف على التتابع، أما الزيادة المئوية في الوزن الجاف للمجموعين الجذري والخضري فقد كانت 114.28% و86.57% على التتابع، بينما بلغت الزيادة في تركيز النيتروجين في الاجزاء الخضرية 16.94%، وبينت النتائج تفوق المعاملات الملقحة معنويا في عدد قرنات النبات والحاصل الكلي ونسبة البروتين في البذور على غير الملقحة، وكانت الزيادة المئوية 49.44% و90.59% و16.86% على التتابع. اما تأثير عامل التعقيم فلم تظهر النتائج فروقا معنوية بين التربة المعقمة وغير المعقمة في جميع صفات النمو والحاصل لنبات الماش. اظهرت النتائج بان تأثير التداخل بين التسميد الحيوي والتعقيم كان معنويا واعطت المعاملة المسمدة بالسماد الحيوي والمعقمة اعلى القيم في كل الصفات المدروسة ما عدا تركيز النتروجين في الجزء الخضري.

الكلمات المفتاحية: التلقيح، التعقيم، بكتريا الرايزوبيا، البقوليات

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INTRODUCTION

Vigna radiate is one of the important seed legumes grown in tropical and subtropical regions. The edible plant seeds are rich in proteins 20-25%, fatty acids 1.0-1.2%, vitamins A, B1, B2, C, sugars, organic and amino acids and minerals for example potassium and phosphorus (19 ,15). Farmers suffer from low yields of Mungbean, especially in the summer. This may be due to severe environmental conditions such as drought, low soil fertility or low nitrogen fixation by indigenous rhizobial bacteria, which play a role in supplying plants with optimal nitrogen through the symbiotic relationship (26). So farmers always tend to use excessive chemical fertilizers, including chemical nitrogen fertilizers that might have adverse effects due to environmental pollution, as well as large amounts are could be lost by leaching and volatilization. Researchers are looking for effective and safe alternatives which contribute to the preservation of human health and the environment in which they live as well as its inexpensive price, such as bio-fertilizer. Biofertilizer can defined as a solid or liquid substance containing beneficial organisms that is added with seeds, or to soil or plant seedlings in order to improve plant growth and increasing its yield by supplying the plant with nutrients ,hormones and siderophores or by increase the nutrients absorption efficiency, resistance to adverse environment conditions and diseases, and ultimately for clean environment and sustainable agriculture. The symbiotic relationship between *Rhizobia* and legumes has a significant impact in the success of leguminous crops cultivation of and was found that these bacteria have the potential to convert nearly 20 million tons of N_2 to NH_3, 50-70% of the world's bio-fixed nitrogen, and the high bio-fixed nitrogen can determine the success of the symbiotic relationship between Rhizobia and legumes (20).The arid and semi-arid regions of the world suffer from a lack number of indigenous rhizobia in the soil with poor efficiency of nodulation and nitrogen fixation(2). Researchers in an Australian study of Mungbean found that it did not nodulate when not inoculated with *Bradyrhizobium* bacteria (14). Therefore the treatment of legume seeds with bio-fertilizer containing root nodules bacteria is economically necessary of whether it relates to soil fertility and productivity or yield.The objective of this study was to test the effect of bio-fertilizer prepared from Bradyrhizobium sp. Which was diagnosed according to protocol including morphological, microscopical, biochemical and molecular methods on growth and yield of mungbean.

MATERIALS AND METHODS

Forty roots of legumes and soil samples were collected from different agricultural sites in Iraq: Samara, Kirkuk and Sulaymaniyah. Pure culture of isolated bacteria were identified on the Yeast Extract Mannitol Agar(YEMA) by several cultural phenotypic tests including color, shape and colony height according to Collins et al. (7) and Microscopic tests using a Gram stain to identify its shape, arrangement and response to the dye under oil lens of the microscope as well as to determine its movement with the hanging drop method**,** and biochemical tests using Congo Red stain to distinguish the Rhizobia species from the genus Agrobacterium. Bromothymol blue was also used to identify the genus of the root nodule bacteria, whether it is fast or slow-growing such as Rhizobium, Bradyrhizobium, Sinorhizobium etc., as well as physiological tests (pH and heat), then molecular identification, was done depending on extracted DNA (6) with some modifications, and multiply them using the initiators of nolXWBTUV gene, namely the (Primer 5'-TGGGAAGCGACGCTGCCGG-3 ') and the primer (R primer 5'-GGCGGAGAAATAGGCGCCGT-3') with polymerase chain reaction to distinguish between Rhizobia species as reported by Videira et al.(24).

Preparation of biofertilizer

An appropriate amount of peat moss was sieved, this peat moss was produced by the FAO as a good carrier in accordance with the specifications (22). Sieved peat moss was then distributed by 250g in high temperature resistant plastic bags, and moisturized with water by 20% of its weight, the bags were sterilized at 121°C and 1500 kPa for 30 minutes, sterilization was repeated three times for three days. Randomized samples of the

carrier were tested to ensure that they were free of microorganisms by taking 1 ml of the first dilution and cultured on the YEMA medium, carrier was examined for its safety according to (12). After the injection area was sterilized in the wall of each bag filled with peat moss. The liquid inoculum was then injected into the logarithmic growth phase (1.5×10^9) as demonstrated by Vincent (25), using a 20 ml medical injection in the middle of the mass of the carrier under sterile conditions, the size of the added inoculum was 200 ml to make the moisture up of sterile and inoculated carrier of 50-60% of its weight, After the injection was completed, the injection hole was closed directly using a sticky paper tape with the recorded biomass data. The inoculated carrier was incubated at 30 ° C for 7 days with daily flipping in several directions (17), The number of living bacterial cells in carrier by dilution and counting in dishes method, The total density of living bacterial cell must be (4.6×10^9) CFU per gm carrier , which proved the results of the diagnosis that it belongs to the type Bradyrhizobium sp.

Implementation of experiment

Field experiment was conducted in 15-5-2017 in Salahhuddin Governorate samara district, Table 1 show some physical, chemical and biological characteristics of soil, experiment carried out by use split plot, the land was divided into two parts representing the sterilization factor (without sterilization, with sterilization of soil), each part of the land was divided in to three replicates the distance between each replicate was 1m, and each replicate was divided in two plots representing the biofertilization factor (without biofertilizer, with biofertilizer) the area of each plot was 1m x 1 m and the distance between plots was 1m.

Table 1. Some Physical , Chemical and Biological Characteristics of Soil

Characters	Unit	Value
EC	$ds.m^{-1}$	2.18
pH	-	7.83
O.M	$g.kg^{-1}$	15.57
Available N		26.35
Available P	mg kg^{-1}	8.33
Available K		89.5
Sand		516.0
Silt	g. kg^{-1}	190.0
Clay		294.0
Soil texture	Loam	
Total number of Fungi	CFU. g^{-1}	5×10^3
Total number of Bacteria	CFU. g^{-1}	102×10^6

Soil sterilization

The soil was taken at a depth of 30 cm from surface of soil and was sieved with 2 mm metal sieve. Then it was transferred to the laboratory and air dried in laboratory. Soil samples were sterilized by autoclave at a temperature 121 ° C and 1500 kPa for one hour, the sterilization was repeated 3 times to ensure the elimination of the microorganisms, and then the soil was returned with bags to the site of the experiment to be distributed to the experimental units. Urea fertilizer (46% N) at 40 kg N ha^{-1} was added in two batches before planting and at flowering (17), triple super phosphate(21% P) at 160 kg P ha^{-1} ,and potassium sulphate (41.5% K) at 160 kg k ha^{-1}, which were added once before planting(1).

Seed surface Sterilization

The seeds were washed several times and placed in sterile glass conical flasks and submerged in a sufficient amount of Sodium hypochlorite at 2.5% concentration for 3 minutes then washed with sterile water for at least 6 consecutive times to get rid of the effects of the sterilization material

Seeds treatment with bio-fertilizer and planting: The seeds were treated with bio-fertilizer according to the method described by Somasegaran and Hoben (22). The arabic gum (40%) solution was prepared and used as an adhesive materials, then the sucrose solution was prepared to increase the vitality and efficiency of the bacteria in the formation of the root nodules by dissolving 15 g sucrose in 100 ml sterile distilled water, and added to inoculum on peat moss in ratio of 1:3 respectively before use . mungbean seeds were processed for cultivation by mixing 100 g of seeds with 4 g of bio-fertilizer (10), then the inoculated seeds were spread on blotter paper

in order to dry them in the shade away from the sun light. The mungbean seeds were planted on 15/5/2017 in lines at a distance of 15 cm between lines and depth of 2-3 cm , and after planting the experiment unit was irrigated directly. After 10 days of germination, the seedlings were thinned in densely areas. The distance between one plant and another was 15 cm and by 5 plants in the line .All management of the crop, such as hoeing, weeding, irrigating and others were conducted as required.

Studied characteristics

Number of root nodules (nodule plant $^{-1}$), dry weight of the nodules (gm), dry root weight (gm plant $^{-1}$), Dry vegetative weight (gm plant^{-1}) , Number of pods in the plant, Seed yield (tons ha $^{-1}$), Nitrogen concentration in the vegetative and seed parts (8). The experimental results were statistically analyzed using ANOVA, and the averages was compared with the least significant difference of LSD at the level of 0.05 based on the program (18).

RESULTS AND DISCUSSION

Table 2 shows the effect of biofertilization with *Bradyrhizobium sp.*, the sterilization and interaction in the number of root nodules of the mungbean during the flowering stage. Results show that the biofertilization has had a significant effect on the number of root nodules of the mungbean, and significantly exceeded of biofertilizer compare with non-fertilizer. Biofertilizered treatments gave average of nodules number 19.40 nodule plant $^{-1}$, compared with non-biofertilized which gave 7.48 nodule plant $^{-1}$ with increase of 159.35%.The increase in the number of root nodules of biofertilizered plants may be due to the fact that biofertilization has a significant effect on increasing the number of Bradyrhizobium in the soil, which contribute on infection and formation of root nodules and then increase the number of nodules size and weight . This result is in agreement with Majeed and Abdul-Bagi (13) and Sipai *et.al.* (21) in their studies on the mungbean.

Table 2. Effect of inoculation with *Bradyrhizobium sp.*, sterilization and interaction on the number of root nodules plant^{-1} during flowering stage

Inoculation / Sterilization	I_0 Without *Bradyrhizobium sp.*	I_1 With *Bradyrhizobium sp.*	The average
Non sterile soil S_0	14.97b	14.38b	14.68a
Sterile soil S_1	0.00c	24.42a	12.21a
The average	7.48b	19.40a	
LSD 0.05	Inoculation	Sterilization	Interaction
	6.2207	N.S	8.7974

Sterilization did not effect on the number of root nodules. The effect of interaction between inoculation and sterilization was significant in the number of root nodules of Mungbean, the biofertilized and sterilized treatment was significantly higher in root nodules number. The biofertilized and sterilized treatment was superior on other three interaction treatments, and this treatments gave the highest average of nodules number with 24.42 nodules plant^{-1}, and this can be due to the effectiveness of introduced bacteria strains in infection and formation of nodules, this result agree with Gupta *et al.* (9) in their study on Mungbean. Table 3 shows that the inoculation has a significant effect on the dry weight of root nodules, as the biofertilized treatments exceeded the non-biofertilized and the fertilized treatments giving average dry weight of 0.11 g nodule^{-1}, while the non-biofertilized which gave 0.03 g nodule^{-1}, with an increase of 266.66%. This increases may be attributed to the ability of the introduced isolate to compete with the microorganisms, which is reflected in its ability to cause infection and the formation of the largest number of root nodules, as indicated by Rahima *et al.* (16). As for the sterilization treatments, no significant differences were found in the dry roots nodules. The interaction effect was significant, the biofertilized and sterilized treatment being superior to the other interaction treatments.

The biofertilizered and sterilized treatment gave an average for dry root nodules weight reached of 0.09 g nodule $^{-1}$ compared with the non-biofertilized and sterile treatment with 0.00 g nodule $^{-1}$.

Table 3. Effect of inoculation with *Bradyrhizobium sp.*, sterilization and interaction on dry weight of root nodules(g nodule $^{-1}$) during flowering stage

Inoculation / Sterilization	I_0 Without *Bradyrhizobium sp.*	I_1 With *Bradyrhizobium sp.*	The average
Non sterile soil S_0	0.06b	0.07b	0.07a
Sterile soil S_1	0.00c	0.09a	0.05a
The average	0.03b	0.11a	
LSD 0.05	Inoculation 0.0185	Sterilization N.S	Interaction 0.0262

Table 4 shows that the biofertilized treatment significantly exceeded the non-biofertilized treatment, and the biofertilized treatments gave the average dry root weight of 1.20 g plant^{1}, while the non – biofertilization gave the average 0.56 g plant^{-1}, with an increase of 114.28%, these results came in agreement with Kaur and Khanna (11) in their studies on mungbean. As for the sterilization factor, there was no significant effect on the weight of the root weight of the mungbean plant, the results are agreement with the Alkurtany and Ali (3) in their studies on soybean, there was no significant difference between the sterilization treatments and non-sterilization treatment. The interaction between the bio fertilization of *Bradyrhizobium sp*. and soil sterilization was significantly higher for the dry root weight, the fertilized and sterilized treatment was significantly higher than the other of the interaction treatments, the biofertilized and sterile treatment gave an average dry root weight of 1.50g plant^{-1}, compared with non-fertilized and sterile which gave average of 0.43 g plant^{-1}, with a percentage increase of 248.83%..

Table 4. Effect of inoculation with *Bradyrhizobium sp*., sterilization and interaction on dry weight of root (g plant^{-1}) during flowering stage

Inoculation / Sterilization	I_0 Without *Bradyrhizobium sp.*	I_1 With *Bradyrhizobium sp.*	The average
Non sterile soil S_0	0.68b	0.89b	0.79a
Sterile soil S_1	0.43b	1.50a	0.97a
The average	0.56b	1.20a	
LSD 0.05	Inoculation 0.381	Sterilization N.S	Interaction 0.5388

Table 5 show the effect of inoculation with *Bradyrhizobium sp.*, sterilization and interaction in the dry weight of vegetative part during flowering stage. Results indicate that the inoculation has a significant effect on the dry weight of vegetative part, the biofertilizered treatments gave dry weight reached 12.65 g plant $^{-1}$,while the non-biofertilized treatment gave 6.78 g plant $^{-1}$, with an increment of 86.57%. This increase may be attributed to the fact that the use of nitrogen-fixing bacteria leads to an increase in the accumulation of nitrogen and converting it into amino acids and thus protein compounds that benefit the plant in the formation of different tissues (17). These results agreed with Al-jourany and abass (13) and Bhushan *et al.* (5) in their studies on Mungbean.

Table 5. Effect of inoculation with *Bradyrhizobium sp.* sterilization and interaction in the vegetative dry weight (g plant^{-1}) during flowering stage

Inoculation / Sterilization	I_0 Without *Bradyrhizobium sp.*	I_1 With *Bradyrhizobium sp.*	The average
Non sterile soil S_0	7.44b	9.59ab	8.52a
Sterile soil S_1	6.11b	15.71a	10.91a
The average	6.78b	12.65a	
LSD 0.05	Inoculation 5.61	Sterilization N.S	Interaction 7.93

Sterilization did not show a significant effect on the dry weight of the plant, these results agreed with Alkurtany and Ali (3), who confirmed there was no significant difference between sterile and non-sterile treatments in the dry weight of the soybean plant. The interaction between biofertilization and sterilization was significant, the biofertilizerd-sterilized treatment gave the highest vegetative dry weight of 15.71 g plant^{-1}, while the non-biofertilized and sterile treatment gave the lowest mean of 6.11 g Plant $^{-1}$. The results in table 6 show that the bacterial inoculation has a significant effect on the nitrogen concentrations(%) with biofertilized treatments gave the average of 4.90%, while the non-fertilized treatments gave 4.19% with un increment of 16.94%. This may be due to the increase in the number of *Bradyrhizobium* that came from inoculation and its efficiency in the incidence of infection on the root and the formation of active root nodules, which increases the amount of fixed nitrogen and thus increases the concentration of nitrogen in the plant. These results in agreement with Badar and Qureshi (4).

Table 6. Effect of inoculation with *Bradyrhizobium sp.*, sterilization and interaction on the nitrogen% in vegetative part of plant during flowering stage

inoculation / Sterilization	I_0 Without *Bradyrhizobium sp.*	I_1 With *Bradyrhizobium sp.*	The average
Non sterile soil S_0	4.34bc	4.72ab	4.53a
Sterile soil S_1	4.04c	5.08a	4.56a
The average	4.19b	4.90a	
LSD 0.05	Inoculation 0.3008	Sterilization N.S	Interaction 0.4254

Table 7 shows the result of the number of pods at maturity stage, the bacterial inoculation significantly affect the number of pods giving 9.46 pod plant^{-1}, compared to non-biofertilizered treatment with 6.33 pod plant^{-1} with an increase of 49.44%.

Table 7 Effect of inoculation with *Bradyrhizobium sp.*, sterilization and interaction on the number of pods

Inoculation / Sterilization	I_0 Without *Bradyrhizobium sp.*	I_1 With *Bradyrhizobium sp.*	The average
Non sterile soil S_0	5.50b	9.83a	7.67a
Sterile soil S_1	7.17ab	9.08a	8.12a
The average	6.33b	9.46a	
LSD 0.05	Inoculation 2.4031	Sterilization N.S	Interaction 3.3985

The effect of interaction between the inoculation and sterilization was significant on pods number and the biofertilizered-non sterile treatment gave the highest value of number of

pods. Table 8 shows the grain yield of mungbean at the maturity stage. Results show that the inoculation has a significant effect on the total plant yield , the biofertilizered treatments gave 2.23 Mg ha^{-1}, while the non-fertilized treatments gave 1.17 Mg ha^{-1}, with an increase of 90.59%, the results are in agreement with Stajković-srbinovic *et al.* (23). The sterilization factor did not show a significant effect on the total yield of the plant, and the interaction was significant as mentioned above (Tables 6+7).

Table 8. Effect of inoculation with *Bradyrhizobium sp.*, sterilization and interaction on the grain yield (Mg ha $^{-1}$)

inoculation / Sterilization	I_0 Without *Bradyrhizobium sp.*	I_1 With *Bradyrhizobium sp.*	The average
Non sterile soil S_0	0.96c	2.14ab	1.55a
Sterile soil S_1	1.38bc	2.32a	1.85a
The average	1.17b	2.23a	
LSD 0.05	inoculation 0.6277	Sterilization N.S	Interaction 0.8878

The results of Table 9 shows that the inoculation significantly affected on the protein % in the seeds, the biofertilized treatments gave average protein of 24.46%, while the average of protein ratio for the non-biofertilized treatments was 20.93% with percentage increase of 16.86%. The biofertilized treatments showed an average protein content of 22.83% while19.47% for the non-biofertelizerd, this result may due to the increase of nitrogen concentration in plant tissue (table 5) because of inoculation with *Bradyrhizobium* .The sterilization showed no significant effect on the protein content of the seeds. The effect of binary interaction between the inoculation with Bradyrhizobium sp and Sterilization was significant, the biofertilized and Sterilized treatment gave the highest average in protein ratio of 25.36%, while the non-fertilized and sterile treatment gave the lowest value of 20.18., with increase of 25.66%.

Table 9. Effect of inoculation with *Bradyrhizobium sp.*, sterilization and interaction on the percentage of protein% in the seeds of plant after full maturity

inoculation / Sterilization	Without *Bradyrhizobium sp.* I_0	With *Bradyrhizobium sp.* I_1	The average
Non sterile soil S_0	21.67bc	23.55ab	22.61a
Sterile soil S_1	20.18c	25.36a	22.77a
The average	20.93b	24.46a	
$LSD_{0.05}$	Inoculation 1.5245	Sterilization N.S	Interaction 2.156

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