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Studying of New Bio-Fertilizer Formula Composed of Streptomyces and Nitrogen Fixing Bacteria to Sustain *Vigna Radiate* (L.)

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Abstract

The recent study aimed to develop and produce bio-fertilizer as a supplement to chemical fertilizers, with the goal to reduce their application. In this study, new Streptomyces species were introduced along with nitrogen-fixing bacteria to support the growth of Mung beans in Iraq. The experiment involved the application of five strains of Rhizobium, two strains of *Streptomyces*, and high indole acetic acid-producing *Azospirillum* spp4 and *Azotobacter* spp7. These bio-fertilizers were tested in a field using a complete randomized design at al-Tuwaitha research station in Baghdad.The results indicated that the bio-fertilizer containing Streptomyces and *Azospirillum* 4 had a significantly positive impact on the growth of Mung bean crops compared to using NPK 100% (conventional chemical fertilizer). The germination percentage reached 75%, with a plant height of 73 cm and 11 branches per plant. The number of legumes per plant was 44.66, with an average legume height of 3.56 cm. The weight of 100 seeds was 142 g, and the fresh weight and dry weight were recorded as 40.33 g and 35.33 g, respectively. Moreover, the study found no statistical difference when comparing the treatment of mung bean seeds with the mono-inoculum of *Rhizobium* isolated from Phasoleus nodules and *Streptomyces* Z. This suggests that the newly developed bio-fertilizer containing Streptomyces and *Azospirillum* 4 is a promising and viable option to enhance the growth and yield of Mung beans without relying heavily on imported chemical fertilizers.

1. Introduction

Global fertilizer prices have increased by more than 200% since 2007, according to the International Centre for Fertility of Land. The use of chemical fertilizers in agriculture, which is economically costly as well as polluting environment that might lead to global rejection of agricultural products contaminated by chemical residues. All of that prompted the biotechnologists to explore and use bio- fertilizers as an alternative method [1,2]. Plant growth promoting bacteria (PGPB) were used extensively in the field as single inoculum or as a formula of more than microorganism and gave successful and effective effects to reduce and get rid of these chemicals, in addition to its

high effectiveness in increasing productivity and preventing the emergence of diseases within integrated control programs [3]. Therefore, these vital fertilizers must be extensively studied about their benefits, mechanisms of work, currency, and the economic feasibility of applying bio-inoculum and their being regarded as safe microorganisms [4]. All these topics have been highlighted since the establishment of the industrial and Environmental Microbiology department which possesses leadership in this field in terms of research and production projects. We have a pioneering project to produce these fertilizers for most legume crops, grains, and vegetables.

Most farmers abandon producing legumes because of low yields and raising production costs as well as crop losses after harvesting and storage. The most important reason for the decreased yield of legumes cultivar was soil nutritional value and nodulation of plant roots [5]. Farmers in different countries who grow legumes without any fertilizers as well as poor soil could recognize and resolve this problem by applying chemical and organic fertilizers to occupy the deficiency of macro and micro-nutrients [6,7].

The increasing requirements for essential protein with the high cost of animal protein encourage projects to raise planting legumes. They are mostly grown in winter during the rainfall season in the north of Iraq.

The most important legumes were Chick pea *Cicer arietinum* L. and Mungbean (*Vigna radiate* (L.) both were grown at different Iraqi provinces such as duhook, Qurdistan, Holy Karbala, and Anbar [7-13].

Due to the increase of chemical fertilizers cost and their negative ecological impact, this research aimed to assess the importance and efficiency of Streptomyces spp on the formulation with either inoculum of *Rhizobium* spp, *Azotobacter*, and Azospirillum spp and their impact on the grown characters of Mungbean (*Vigna radiate* (L.) grown at Baghdad province.

2. Experimental Procedure

2.1. Isolation of Plant Growth Promoting Rhizobacteria (PGPR)

Roots and rhizospheric soil from *Arachis hypogaya* (ground nut), *Vicia faba* (broad bean, *Phaseolus vulcaris* (beans), *Vigna unguiculata* (green cowpea) and *Vigna radiate* (mung bean) were collected and put in paper bags. Then samples were transferred to a laboratory for *Rhizobium* spp isolation following Boraste et al., [14] and Ogutcu *et al*., [15] techniques with some modifications.

In this study, the roots from each sample were examined for the presence of pink root nodules and then thoroughly washed to remove any soil. The nodules were carefully removed using a cutter, and then they underwent a sterilization process using a 6% H_2O_2 solution for 15 minutes. Afterward, the nodules were washed multiple times with distilled water and homogenized using a sterilized mortar. A sample from the homogenized tissue suspension was streaked over a modified yeast mannitol agar (MYM) medium with some adjustments to its composition. The modified medium contained 10g of mannitol, 10g of yeast extract, 0.5g of K_2HPO_4, 0.2g of $MgSO_4.7H_2O$, 0.2g of NaCl, 0.06g of $FeCl_3.6H_2O$, and was made up to 1000 ml with distilled water. The pH of the medium was adjusted to 7.2. The plates were then incubated at 28°C for 48 hours.

Colonies that grew on the MYM agar were isolated and further cultivated on the same medium for further analysis and study. Rhizospheric soil samples from vegetables were screened for *Azotobacter* spp and *Azospirillum* spp. Soil suspensions of10% were prepared using distilled water then loopful was spread on two types of nitrogen free agar designed for the isolation of *Azotobacter* spp respectively (Burks medium)(15 g of mannitol, 0.5 g of $MgSO_4$,0.2 g of K_2HPO_4, 0.02 g of $CaCl_2$, 0.05g of $FeCl_3$, 0.01g of aluminium molbidate and 1000 ml of DW pH 7.2) and *Azospirillum* spp medium (5 g of Malic acid,0.5 g of K_2HPO_4, 0.02g of $CaCl_2$, 0.01g of $MnSO_4.2H_2O$, 0.01g of $FeCl_3$, 0.01g of $Na_2Moo.4H_2O$ and 4ml of 1M KOH pH 6.8). The growth of each bacterial isolate under study on the nitrogen free medium was used as indicative of their capability for nitrogen fixation [16].

To isolate *Streptomyces* spp, soil samples were collected from a depth of 10-20 in several desert areas, orchards and gardens. Gausa agar was applied as an isolation medium of Streptomyces bacteria (components were weighted grams for liter distelled water: starch 20 g, KNO_3 1g, NaCl 0.5g, $CaCO_3$ 0.3g, $MgSO_4$ 0.5g, K_2HPO_4 0.5g, $FeSO_4$ 10). Briefly, soil samples were dried at 25 C to complete dehydration, one gram of each sample was suspended by

9 ml of sterile saline and serial dilutions were prepared, the cotton swab was immersed into dilution and spread over agar medium. Plates were incubated at ambient temperature reached to 30 C for seven days. Isolated colonies were picked up by platinum wire and spread on new agar plates for maintenance and further study, as described in 2022 in the work made by Nonthakaew *et al*. [17].

2.2. Screening for the Production of Indole Acetic Acid

Each bacterial isolate designated as *Azotobacter*, *Azospirillum*, *Rhizobium*, and *Streptomyces* was inoculated into MS Medium containing 0.3% tryptophan, incubated for 24 h at 30 C using ashaker incubator at 120 rpm. Meanwhile, bacterial growths were centrifuged for 10 min at 10000 rpm, supernatants were used for screening of plant hormone the indole acetic acid. Salkowski's reagent was prepared by mixing one milliliter of 0.5M $FeCl_3$ with 49 ml of 35% perchloric acid and storage at dark bottle [18]. The formation of apink color was an indicative of a positive result due to the reaction between Salkowski's reagent and indole acetic acid. For quantitative determination only One milliliter of supernatant was mixed with two milliliters of Salkowski's reagent and incubated at dark for 30 min then absorbance was recorded at 530 nm using spectrophotometer Analytikjena specord 205. The concentration of IAA was estimated by applying standard curve to screen the best bacterial producer [19]. According to the concentration of IAA produced by test bacterial species, high producers*Azosperillum* 4 and *Azotobacter* 7 and five low producers of *Rhizobium* spp were applied in the field experiment.

2.3. Preparation of Bio-Fertilizer Formula

Five *Rhizobium* spp isolated from different legumes, *Azotobacter* spp7, *Azospirilum* spp4, and *Streptomyces* spp strain M and strain Z were grown at their corresponding broth medium until reached to countof 10^7 CFU for the first step in bio-fertilizer application. For mixed biofertilizer, a ratio of 1:1 was prepared for each bacterial strain of *Rhizobium*, *Azotobacter,* and *Azospirillum* in combined with Streptomyces. Eleven formulas were prepared as follows:

1: Rhizobium spp isolated from (Arachis root nodule)+ Streptomyces Z; 2: StreptomycesM+Azotobacter 7; 3: Rhizobium 4 from Jack bean; 4: control 100% NPK; 5: Rhizobium from Mung bean nodules; 6:Rhizobium from Vigna unguiculata root nodule; 7:Rhizobium from Mung bean+ Streptomyces M; 8: Streptomyces M; 9: Streptomyces Z; 10:Streptomyces M+ Azospirillum 4; and 11: Rhizobium from Phasoleus nodules**.**

Local mung bean seeds were washed with tap water and then washed with sterilized distilled water. Then seeds were soaked in single or mixed bacterial growth broth for 30 minutes. Mung bean seeds were replanted as full randomized blocks design, at the field with 29m × 40m (length: width) that was prepared at al- Tuwaitha Research Station/ Baghdad. The soil was sleeked and permutated by machine, lines each 4m were chapped and prepared for culturing.

Control was managed with 100% of NPK as recommended for mung bean plants while, 50% of NPK was applied for all other treatment blocks at the beginning of the field experiment; watering and removing of brushwood were examined and removed periodically. Experimental seeds were manual-feeding in the field.

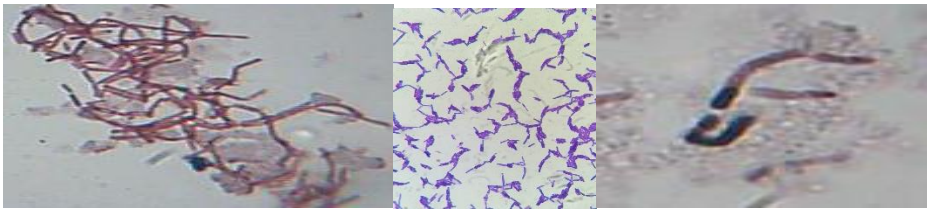
2.4. Studied Characters and Statistical Analysis

Many characters were studied and statistically analyzed, the germination percentage of seeds was examined, plants high were measured, number of branches and legumes, weight of legumes and weight of 100 seeds.Data were statically analyzed for comparison between chemical fertilizer application and bacterial bio-fertilizer, the analysis was also done among the bio-fertilizer, when using single or mixed bacterial inoculums. Gene stat program was used to evaluate differences in the experimental field which was designed in randomized block design with three replicate plots and ten plants/ treatment, Duncan, LSD, and means were analyzed using ANOVA- two-way analysis.

3. Results and Discussion

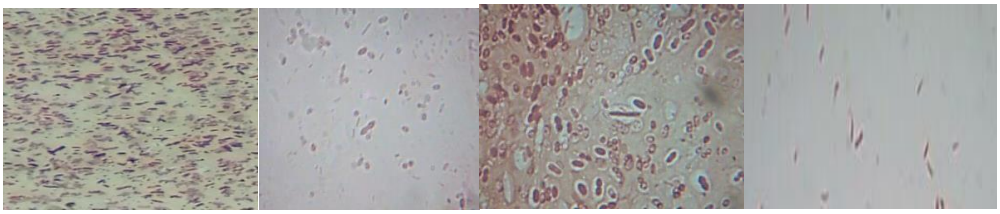
Five *Rhizobium* spp were isolated from different root nodules of lgumes; their shape under microscopic examination appeared as motile small rode, while their shape was converted to irregular rode upon sub-culturing of growth. Four strains of *Azotobaccter* spp were isolated from the rhizospheric soil of vegetables. They appeared

as motile in living smears and negatively stained by Gram's stain aligned as diploid big rods to irregular shape (Figure 1) and they formed cysts at prolonged incubation period. While, three isolates of *Azospirillum* developed as pinpoint translucence colonies after 72-96 h of incubation on the malic acid containing nitrogen free agar. Under microscopic examination cells appeared as red small bacilli or irregular shapes they were almost pleomorphic bacilli. The isolation of Streptomyces species revealed that isolates designated as M and K belong to the genus Streptomyces. The phenotypic characteristics of the two isolates were observed, as the isolate KSH1 was in the form of floury white colonies tending to a leaden color, and the microscopic examination showed that it was positive for Gram stain, and its cells were arranged in the form of threads with internal spores (Figure1). Some biochemical tests indicated that they produced catalase and oxidase enzymes as well as their ability to hydrolyze starch (amylase enzyme), gelatin (gelatinase) and protein hydrolysis (proteases) both strains diagnosed as belonging to Streptomyces bacteria.



(A): Gram stain of Streptomyces

(B): Growth at Gausa agar (Streptomyces)



Rhizobium cells *Azotobacter* cells *Azospirillum* cells

Figure (1): Examination of bacterial growth and cells: upper Streptomyces; lower nitrogen fixing bacteria.

Screening for IAA production indicated that the higher producer was *Azospirillum* spp 4 followed by *Azotobacter* spp7 then *Azospirillum* spp6; minor color developed for the growth of other species under study and Figure (2) declared color changed to pink for bacterial IAA production.

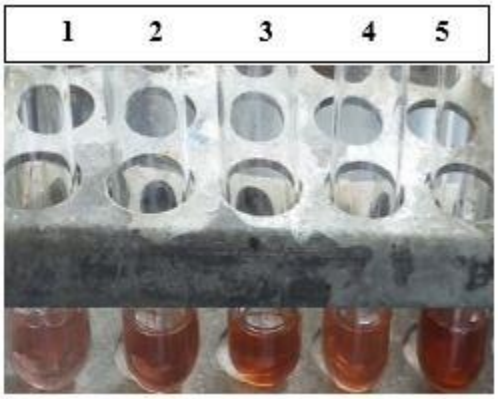


Figure (2): Determination of IAA by Salkawaski reagent.
1 = control; 2 = *Rhizobium*spp9; 3 = *Azospirillum* spp6; 4 = *Azotobacter* spp7; 5 = *Azospirillum* spp 4.

The concentration of IAA produced by nitrogen fixing bacterial species ranged from 1.955 mg/L produced by *Azospirillum* spp 4 to 0.091 mg/L by *Rhizobium* spp3 other work described the production of IAA from nitrogen fixing bacteria and the importance of IAA to reduce abiotic stress as concluded by [20, 21]. Otherwise, growth of the three *Rhizobium* spp was higher at nitrogen free medium in compared to *Azotobacter* spp and *Azospirillum* spp as indicated in Table (1).

Two isolates of Streptomyces designated as M and Z were evaluated for their significant plant-growth promoting activity to enhance the growth of Mung bean seedlings a field trial. Also, the formula of nitrogen fixing bacteria in conjunction with Streptomyces tested; biotic traits, including germination percentage, plant length and wet and dry weights, legumes number, and high.

Table (1): Indole acetic acid production from nitrogen fixing bacteria isolated from different sources.

Bacterial strain	Concentration IAA mg/L	Growth at nitrogen free medium	Sample Source
Azotobacter sp 7	**0.993**	**++**	**Onion root**
Azosperilum spp 5	**0.125**	**++**	**Tomato root**
Rhizobium sp10	**0.220**	**++++**	***Phasoleus abu* greeb**
Azotobacter sp 11	**0.223**	**++**	**Soil altoncobry**
Rhizobium sp 3	**0.091**	**++++**	***Arachishypogayae*/Hsaeba**
Azotobacter sp 12	**0.379**	**++**	**Soil/Arbil**
Rhizobium sp 17	**0.116**	**++++**	***Mung bean*/Hatemia**
Rhizobium sp 2	**0.2**	**++++**	***Vigna unguiculata*/baghdad**
Rhizobium sp 9	**0.231**	**++++**	**Phasoleus/Baghdad**
Azosperilum sp6	**0.519**	**+**	**Rice/Holly Najaf**
Azosperilum sp13	**0.119**	**+**	**Soil/Hatmea**
Azosperilum sp4	**1.955**	**+**	**Soil/ Latefea**

From the previous data we selected *Azospirillum* spp 4, *Azotobacter* spp7**,** the five*Rhizobium* spp and *Streptomyces* spp M and *Streptomyces* spp Z to formulate the Bio-fertilizer; data results described in Figure3. Results showed that the best formula was *Streptomyces* sppM combined with *Azospirilum* spp4 that would enhance more than vital parameters such the percentage of germination, legumes number, wet and dry weight as well as the weight of 100 seeds.

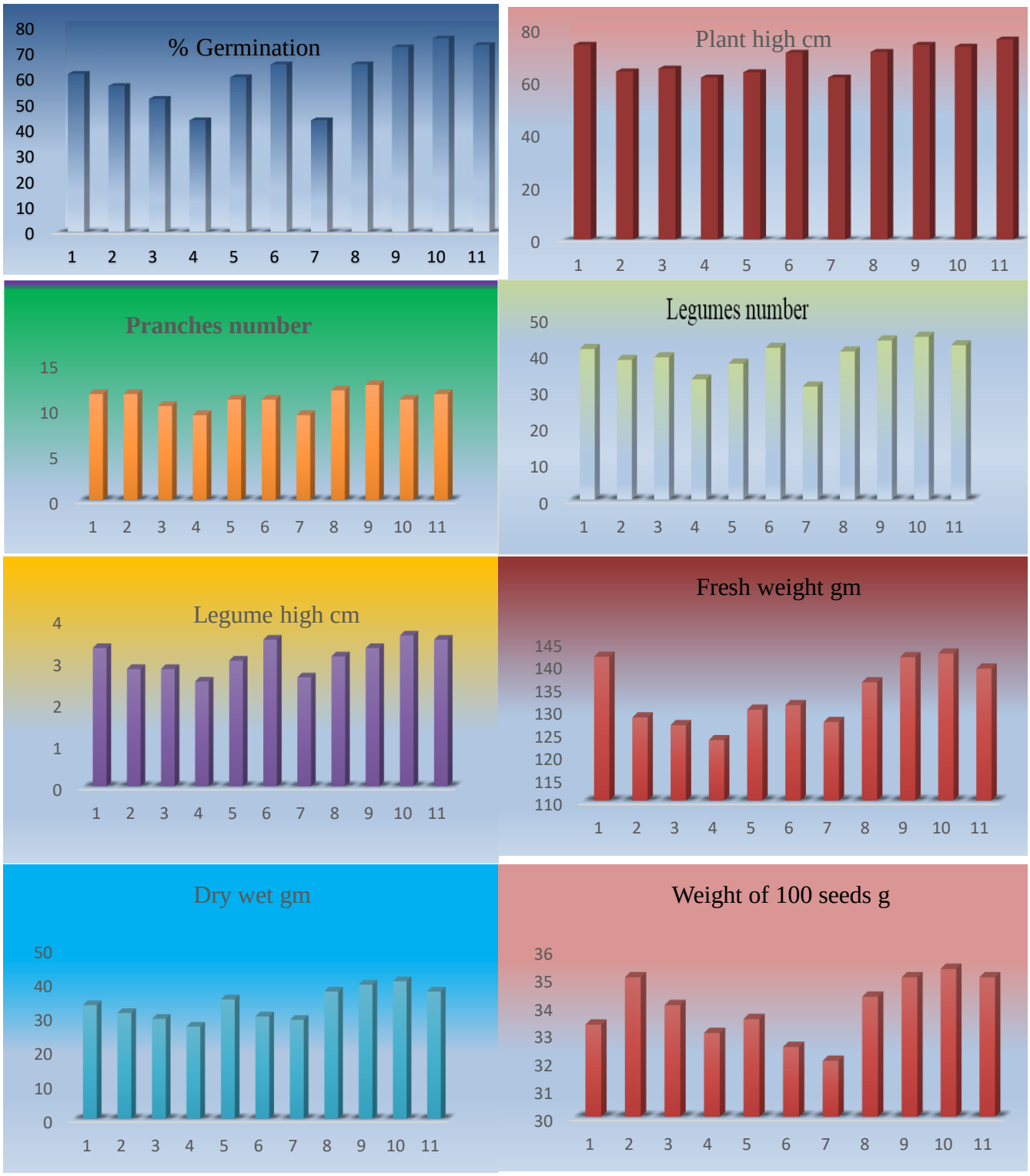


Figure (3): The effect of bio-fertilizer formula on the biological parameters of Mung bean crop.
1: Rhizobium spp isolated from (Arachis root nodule)+ Streptomyces Z; 2: StreptomycesM+Azotobacter 7; 3: Rhizobium 4 from Jack bean; 4: control 100% NPK; 5: Rhizobium from Mung bean nodules; 6: Rhizobium from Vigna unguiculata root nodule; 7: Rhizobium from Mung bean+ Streptomyces M; 8: Streptomyces M; 9: Streptomyces Z; 10: *Streptomyces* M+ *Azospirillum* 4; and 11: Rhizobium (Phasoleus nodules**.**

Other formulas as *Rhizobium* spp at formula11, *Streptomyces* sppM,and *Streptomyces* Z gave no statistical difference with treatment 10. The treatment that contained amixture of *Streptomyces* and *Rhizobium* gave lower

enhancement for plant growth and legume development. However, the control treatment gave the lowest results in response to all studied characters. This encourages to develop a vital bio- fertilizer from mixture of soil born and safe microorganisms relying on plant growth promoting bacteria with low cost and no- chemicals dispended to the environment.

The main objective of a recent study was to evaluate local soil born isolates of Streptomyces to develop bio-inoculants that might control the cause of destructive disease of mung bean crop. This is the first mention of Streptomyces as PGPR applied in the field alone or as a formula with other nitrogen fixing bacteria to produce green fertilizer. Different parameters engaged with the rhizosphere soil that furnished plant growth as nutrient and their availability to plant, roots (length and strength), soil type and aeration, microorganisms with their behavior as plant growth promoting or deleterious effects [22]. Streptomyces would give mutualistic activity with other rhizosphere microorganisms as well as secreted natural compounds and enzymes that proven plant defense against pathogens such as fungi [23] (Suárez-Moreno et al., 2019). Moreover, Behie *et al.*, [24] explained that *Streptomyces* had a positive impact and critical sustained for plant health through competitive hot spots. Also, [17] described the antimicrobial compounds that are secreted from *Streptomyces* and their behavior in Rhizosphere soil to promote the growth and health of mung bean.

On the other hand, nitrogen fixing bacteria used extensively as plant growth promoting bacteria and substituting almost 50% chemical fertilizer as NPK; *Rhizobia legomenosorum formulated as a mixed culture* with *Pseudomonas florescence* and *Bacillus megaterium* and then applied to sustain the plant mung bean in Anbar/ Iraq [25]. Other research showed that Chickpea (*Cicer arietinum* L.) grown on Erbil and inoculated with biological fertilizer displayed higher biological and economical yield (ton.ha^{-1}), harvest index, protein and fiber ratio increased with Bio-fertilizer treatment [9]. The plant *Vigna radiate* was grown in Holly Karbal during 2017 [11] and sustained using *R. legomenosorum* and *Glomus mosseae* as fertilizer with a low irrigation ratio.

4. Conclusion:

We concluded that Streptomyces spp isolated from Iraqi soil could maintain mung bean growth while, mixing Streptomyces spp with *Rhizobium* spp gave lower enhancement for plant growth and legume development growth in comparing with commercial NPK which gave the lowest result at field.

Conflict of Interest: The authors declare that there are no conflicts of interest associated with this research project. We have no financial or personal relationships that could potentially bias our work or influence the interpretation of the results.

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