Bacillus Licheniformis BS12 AS BIOFERTILIZER TO IMPROVE MAIZE GROWTH

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Abstract

The Bacillus was isolated from Rhizosphere of different plants. All the isolates have the ability to produce Indole Acetic Acid (IAA)and the BS12 has the efficient strain in the IAA production with 8.4 µg/ml, this strain is able to produce Ammonia, solublize phosphate and fix the Nitrogen and it belongs to Bacillus licheniformis. The BS12 has able to improve the maize growth by enhance to germination percent, shoot high, root high, dry mass and nutrient concentration comparing to control.

استخدام بكتريا *Bacillus Licheniformis* BS12 كمحفز حيوي لتحسين نمو نبات الذرة

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الخلاصة

عزلت بكتريا *Bacillus* من المناطق المحيطة بجذور مختلف انواع النباتات واختبرت قدرة هذه البكتريا على انتاج الهرمون النباتي حامض الخليك الاندولي IAA، اذ وجد ان جميع العزلات لها قابلية على انتاج هذا المركب ووجد ان العزلة BS12 امتلكت اعلى انتاجية وبتركيز بلغ 8.4 مايكروغرام/ مل. وقد وجد ان لهذه العزلة قابلية على اذابة الفوسفات وانتاج الامونيا وتثبيت النتروجين. شخصت هذه العزلة على انها *Bacillus licheniformis* اختبرت قابلية هذه العزلة على تحسين نمو نبات الذرة اذ استخدمت هذه البكتريا على شكل لقاح سائل ولقاح جاف وتم اضافة البكتريا الى التربة وجد ان للعزلة BS12 قابلية على تحسين النمو وذلك من خلال زيادة طول الساق وطول الجذر والوزن الجاف ونسبة المغذيات مقارنة بمعاملة السيطرة.

Introduction

Agriculture is increasingly dependent on the use of chemical fertilizers, growth regulators and pesticides to increase yield. This dependency is associated with problems such as environmental pollution, health hazards, interruption of natural ecological nutrient cycling and destruction of biological communities that otherwise support crop production. Hence, crop improvement and disease management have to be achieved in shorter intervals of time with fewer detrimental inputs. The use of bioresources to replace chemical pesticides, growth regulators and fertilizers is growing [1]. Biofertilizers are therefore gaining importance as they are ecofriendly, non hazardous and nontoxic. Biofertilizer refers to products consisting of selected and beneficial living microbes, which provides all the nutrients required by the plants and helps to increase the quality of the soil [2, 3].

Plant growth promoting rhizobacteria (PGPR) are known to influence plant growth by various

mechanisms such as produce Indoleacitic acid, Ammonia, phosphate solubilizing, Nitrogen fixation, promoting other beneficial plant-microbe symbiosis, Heavy metal resistance and plant disease control by Hydrogen cyanide and Siderophore production [4, 5]. Different bacteria that have been reported as PGPR belong to the following genera: *Pseudomonas, Bacillus, Azospirillum, Agrobacterium, Azotobacter, Arthrobacter, Alcaligenes, Serratia, Rhizobium, Enterobacter, Burkholderia, Beijerinckia, Klebsiella, Clostridium, Vario-vovax, Xanthom-onas,* and *Phyllobacterium.* Among these, *Pseudomonas* and *Bacillus* are the most widely reported PGPR. The ability of *Bacillus* to form heat and desiccation resistant endospores, they may be relatively more versatile than *Pseudomonas* since such spores can retain viability for long periods either in storage or in the soil [6].

The objective of this study is to isolate the *Bacillus* isolates and detect some PGPR traits such as IAA production, phosphate soloubilizig, nitrogen fixation and ammonia production, then test the bacterial ability to improve the maize growth with different type of inoculums.

Materials and methods

Bacterial isolation

34 rhizospheric samples of different plants in Baghdad University/ Al-jaderya were collected. They are put in nylon sacks and transited to the laboratory. Roots remains are removed, then 1 gram of sample was suspended in 9 ml of sterile distilled water and shaken vigorously for 2 min. The samples were heated at 60 C for 60 minute in a water bath. Then the soil suspensions were serially diluted in sterile distilled water, and plated on nutrient agar medium. The plates were incubated at 28 °C for 24-48 hours [7]. The cultural and microscopic properties of the isolates were studied.

Screening of bacterial isolates for IAA production

Bacterial strains of *Bacillus* were screened for IAA production. Bacterial culture was inoculated in nutrient broth with tryptophan 0.1% (w/v) sterilized by filtration. After 72 hours of incubation at 28°C, cultures were centrifuged at 6000 rpm. Then 1 ml of the supernatant was mixed with 2 ml of Solawaski's reagent (50 ml of 35% perchloric acid and 1 ml of 0.5 M FeCl3). The O.D. was measured at 530 nm using spectrophotometer [8]. The level of IAA produced was estimated by standard curve of IAA which prepared previously. The efficient strain was selected to achieve the other tests of this study.

Production of Ammonia

Bacterial isolates was examined for the production of Ammonia in Peptone water. The isolate inoculated in peptone water and incubated for 72 hours at 28°C then some drops of Nessler's reagent were added. Development of brown to yellow color indicates positive result for ammonia production [4].

Nitrogen fixation

Bacterial isolates was examined for nitrogen fixation. Fresh culture was inoculated in 100 ml of N2-free medium in a bottle and incubated for 72 hours at 28°C in a horizontal position [9].

Bacterial identification

Bacterial isolate was identified according to Bergey's Manual of Systemic Bacteriology [10] included the following tests:

Gram stain, spore stain, catalase, listhenase production, an aerobic growth and utilization of citrate, glucose, arabinose, hydrolysis of starch gelatin and Growth in 7% NaCl.

Preparation of Bacterial suspension

The bacterial suspensions were adjusted to approximately 5×10^8cfu/ml), with a sterile 0.5% NaCl solution and by using standard curves relating numbers of bacteria (cfu) to optical densities measured with a spectrophotometer at 570 nm [11].

Phosphate solubilizing

The phosphate solubilizing test was done using Pikovskaya's medium (10 gm glucose, 5 gm tricalcium phosphate, 0.5 gm ammonium sulphate, 0.2 gm potassium sulphate, 0.1 gm magnesium sulphate, 0.5 gm yeast extract, trace amount of manganese sulphate and ferrous sulphate, 20 gm agar, 1000 ml distilled water). Agar plates were prepared and the bacterial strains were spot inoculated at the centre of the plates and incubated for 5-6 days. The plates were observed for a clearing zone around the colony [1].

Pot experiments

Maize seeds (Ipa'a 2001) from State Board for Seeds Testing and Certification/ ministry of agriculture were surface sterilized for 2 minute with 70% ethanol then soaked for 20 minute with 1% sodium hypochlorite and rinsed four times in sterile distilled water [12].

3 kilograms of soil were put in sterilized pots with 20 cm/diameters and 14 cm height. The soil prosperities were the following: sand 64%, silt 16% and clay 20%.
The following treatments with three replicates were investigated:-

Control
Maize seeds were soaked with distilled water for 1 hour before plating.

Liquid inoculums
Maize seeds were soaked in BS12 bacterial culture with concentration 10^8 cell/ml for 1 hour.

Dry inoculums
Maize Seeds were coated with dry inoculums of BS12 which prepared by added 10 gm of sucrose to 10 ml of bacterial suspension with concentration 10^8 cell/ml, then incubated in 28 °C for 4 days, and dried at room temperature. Then 50% of gum was added to the seeds and rolled into the dry inoculums [13, 14].

Bacterial addition to soil
100 ml of bacterial culture with 10^8cell/ml was mixed well with 3 kilogram of soil.
25 seeds were sowing in every pot. The pots were watered with tap water every two days. After 15 days the germination percent was calculated. The germination plants were reduced to 7 in every pot. These tests were performed at the plant field of Baghdad University/ college of science.
The shoot height was measured in 15, 30, 45 days after planting. The root height was measured after covering the pots with water and randomly harvested plant. Then the dry mass of these plants was measured by putting them in paper sack, next put in oven at 60°C for 24 hours.
Calcium and Manganese were measured by using the atomic absorption spectrophotometer of department of biology/college of science/ Baghdad University.
After the plants were dried at 60°C for 24 hours then, samples were smashed and 0.25 gm of the plant sample were digested by 10 ml of H_2So_4 and heated till boiling for 2 hours until the color of sample turned black. The solution was cooled, and 1.5 ml of $HCLO_4$ was added, reheated until the solution became clear. The volume was completed to 50 ml with distilled water [15].

Statistical analysis

The SAS program [16] was used in Statistical analysis to distinguish if there any significant differences between different parameters include germination percent, shoot high, root high and dry biomass. The degree for significant variation used in statistical analysis was $P<0.05$.

Results and discussion

20 *Bacillus* isolates were obtained from rhizospheric soils of different plants. Many studies confirmed the existence of many types of bacteria in this region benefiting from the root exudation. *Bacillus* is considered one of the most important of microorganism that exist in rhizosphere for its ability to form spores, so it has the ability to resist many hard environmental conditions like drought [10, 17].

IAA production
It's found that all *Bacillus* strains used, have the ability to produced the phytonormones IAA with different concentration. The most efficient strain in IAA production was BS12 with 8.4 µg/ml concentration as shown in figure (1). While the BS16 is the least with 0.7 µg/ml. the other strains have different ability in the production of IAA.

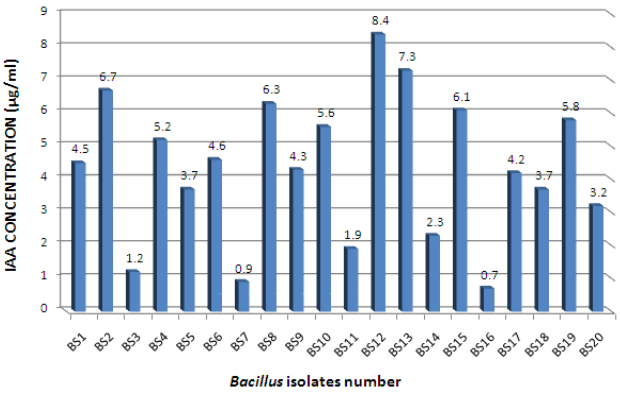

Figure 1: Indole acetic acid production by locally isolated *Bcillus* spp. after 72 of incubation in nutrient broth with 0.1% Tryptophan. Each value represents average of three replicates

Many researches referred to the ability of different types of bacteria to produce IAA, like many isolates of *Pseudomonas* and *Azotobacter* species [18]. *Bacillus* is also able to produce different types of phytoarmones like gibbrellines and IAA with best concentration after 72 hours of incubation [19, 20]. The high level of IAA can produced by modifying the medium with the addition of glycerol or different concentration of tryptophan [21, 22].

Phosphate soluoblizing

BS12 strain has the ability to solubilizing phosphate by forming halo zone around their growth in the medium containing the tricalcium phosphate as phosphate source.

The phosphate solublizing by bacteria was studied widely by many researchers for its plant growth promoting advantage. Many researchers mentioned the ability of different types of bacteria especially *Bacillus* in phosphate solublizing by using different liquid and solid medium [2, 11, 23].

Nitrogen fixation

The BS12 strain show ability to fix nitrogen by developing a heavy growth on N_2-free medium. Because of the ability of the nitrogenase enzyme to fix free nitrogen. There are many species of *Bacillus* isolated from soil able to fix nitrogen even in anaerobic condition [9].

Ammonia production

The BS12 show ability to produce ammonia by developing a yellow color after incubation for 72 hours on peptone water. Mostly isolated strain of *Bacillus*, *Pseudomonas* and less frequently *Azotobacter* are able to produce ammonia which one of the important trait of PGPR [4].

Bacterial identification

The BS12 is identified depending on microscopical, cultural and biochemical tests as shown in table (1).

Table 1: BS12 identification tests

Test	Result
Gram stain	+
Spore forming	+
Egg yolk lecithinase	-
Voges-proskauer	+
Utilization of:	
Citrate	+
Glucose	+
Arabinose	+
Anaerobic growth	+
Hydrolysis of:	
Gelatin	+
Starch	+
Growth in 7% NaCl	+

+ Positive result

- Negative result

According to this results, it's proved that strain belong to *Bacillus licheniformis*. Results showed that the BS12 is able to produced IAA, phosphate solubilizing, ammonia and nitrogen fixation. So, the BS12 has many traits of plant growth promoting rhizobacteria.

The pot experiments

The results showed that the BS12 has ability to improve the maize growth by enhancement the germination shoot high, root high, dry biomass and nutrient concentration. As shown in table (2) the germination percent when the bacterial used (liquid inoculums, dry inoculums or bacterial added to soil) was higher than the control.

Table 2: Effect of different treatment on germination, shoot high, root high and dry biomass after 15 days.

Treatment	Average ± Standard error			
	Growth plant No.	Shoot high (cm)	Root high (cm)	Dry biomass (gm)
Control	18.00 ± 0.75 d	4.68 ±0.08 c	0.43 ±0.014 d	0.046 ±0.001 c
Liquid Inoculums	21.66 ±0.33 a	8.42±0.13 b	0.51 ±0.005 b	0.068 ±0.001 b
Dry inoculums	20.10 ±0.57 b	9.25 ±0.24 a	0.52 ±0.003 ab	0.060 ±0.001 b
Bacteria + soil	19.66 ±0.33 bc	9.01 ±0.22 a	0.55 ±0.015 a	0.077 ±0.001 a
LSD		0.505 *	0.31 *	0.37 *

***(P<0.05)**

Different letters indicate significant differences (P<0.05)

This enhancement may be the result of IAA produced by the BS12 strain. Hormones like Indole acitic Acid and gibberellins, which would have triggered the activity of specific enzymes that promoted early germination, such as α-amylase, brought an increase in availability of starch assimilation. Beside, significant increase in seedling vigor would have occurred by better synthesis of auxins [24].

The germination percent with liquid inoculums was higher than other treatment and this is because that maize seeds maybe imbibitions with different secretion of bacterial culture which make seeds benefiting directly from the different material especially Indole acitic Acid that produced by BS12.

While the seeds with dry inoculums was benefiting after a period of time as shown in

shoot high for this treatment, table (2, 3, 4).The dry inoculums maybe enhance the adhesion of the bacteria to seeds and consequently the seeds got benefit from the bacteria promoting trait slower than the liquid inoculums. The Sucrose enhances the adhesion of the inoculums to the seed and increase the survival rate of bacteria by protecting it from desiccation and death of cells [13, 25], hence the bacteria begin produce the IAA which enhances the plant growth by stimulating cell elongation or cell division [26]. The first sign of the adaptability of these PGPR strains to the root system of maize and wheat was the proliferation and clumping of bacteria around the root tips followed by entry into cortical cells of the root. In the case of maize, the PGPRs, particularly *Azospirillum* and the species of *Bacillus* were able to enter into vascular tissues of the root system [27].

Table 3: Effect of different treatment on germination, shoot high, root high and dry biomass after 30 days.

Treatment	Average ± Standard error		
	Shoot high (cm)	Root high (cm)	Dry biomass (gm)
Control	19.00 ±0.24 c	8.43 ±0.08 c	0.166 ±0.008 b
Liquid Inoculums	20.55 ±0.27 a	10.36 ±0.09 b	0.203 ±0.014 ab
Dry inoculums	22.53 ±0.40 b	10.66 ±0.09 b	0.246±0.023 a
a Bacteria + soil	20.99 ±0.37 a	10.53 ±0.63 b	0.233 ±0.088
LSD	0.908 *	0.943 *	0.044 *

***(P<0.05)**
Different letters indicate significant differences (P<0.05)

The root high, dry biomass and nutrient concentration were higher than the other treatments when bacteria added to soil as shown table (3, 4 ,5). The soil contains the high number of the BS12 strain which has may have traits that promote the maize growth like IAA and Ammonia production, Phosphate solublizing and nitrogen fixation, all these traits maybe the reasons that made this treatment standing in root high dry biomass and nutrient concentration.

Table 4: Effect of different treatment on germination, shoot high, root high and dry biomass after 45 days.

Treatment	Average ± Standard error		
	Shoot high (cm)	Root high (cm)	Dry biomass (gm)
Control	20.91 ±0.43 c	9.50 ±0.35 c	0.276 ±0.020 c
Liquid Inoculums	24.22 ±0.22 b	13.06 ±0.10 b	0.350 ±0.017 b
Dry inoculums	26.11 ±0.17 a	13.20 ±0.15 b	0.386 ±0.013 b
a Bacteria + soil	24.86 ±0.42 b	15.10 ±0.10 a	0.410 ±0.015 a
LSD	0.992*	0.943*	0.048 *

***(P<0.05), Different letters indicate significant differences (P<0.05)**

Table 5: Effects of different treatments on nutrient Concentration

Treatment	Mn (p.p.m)	Calcium %
Control	24.3	0.15
Liquid inoculums	37.1	0.17
Dry inoculums	25.7	0.19
Bacteria + soil	44.3	0.27

Each number represents average of three replicates

Phytohormone producing bacteria encouraged adventitious root formation. Rapid establishment of roots, whether by elongation of primary roots or by proliferation of lateral and adventitious roots. PGPR inoculation may effectively increase the surface area of roots and root weight. the inoculation of the N_2-fixing and Phosphate solubilising bacteria helped in maintaining a higher number of total bacteria for up to 45 days. The beneficial effect of inoculation on microbial population may have been due to an increase in the supply of available P and N, directly and indirectly through changing the growth rate and metabolic activities of the crop, which resulted in more root exudates and thereby created a favorable

habitat for the growth and development of microorganisms [22].

These results were agreement with many results of other studies which using the PGPR in improving the plant growth and increasing the plant yield, Freitas [28] used *Bacillus polymyxa* to increase the Wheat yield, while Anjum *et. al.* [29] used *Azotobacter* and *Azospirllum* to improving the cotton growth as a substitution to N.P.K. fertilizer. Adesemoye and Ugoji [30] used *Pseudomonas aeruginosa* to increase Okra, tomato and African spinach growth.

The present study suggests substituting the chemical fertilizers with biofertilizer such as *Bacillus licheniformis* BS12 strain according to the ability of the last one to stimulate plant growth as well as it is non environmental hazardous, also it suggests screening for bacterial strains that have other growth promoting traits and achieve more studies that explain the specific role of different traits in plant growth promotion.

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