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Isolation and identification of *Bacillus subtilis* and testing its efficiency in the solubility of tri-calcium phosphate

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Abstract

A laboratory experiment was conducted to isolate and diagnose several isolates of Bacillus subtilis and test their efficiency in dissolving tri-calcium phosphate in solid cultures, So that ten samples were collected from the soil and rhizosphere of different crops such as (sweet, barley, radishes, alfalfa) for many regions, namely Thi-Qar, Al-Muthanna, Al-Qadisiyah and Najaf. The results indicated that bacterial isolates were obtained, which were diagnosed based on phenotypic characteristics and biochemical tests. The results also showed that the bacterial isolates differed in their ability to dissolve solid triphosphate in Pikovskay liquid medium. add unite of value and symbols of isolates, respectively, and the lowest values were represented by B9 isolate, which amounted to 1.09. The values of other isolates varied in their phosphate solubility values.

Keywords isolation, identification of bacteria, *Bacillus subtilis*

Introduction

Bacillus subtilis is one of the most important types of rhizosphere bacteria that possess advanced mechanisms to stimulate plant growth (Oteino et al. 2015). Research has shown its ability to produce many types of growth hormones, including auxins and gibberellins 3GA and indole acetic acid IAA, and reduce the harmful effects resulting from stress, including Salt stress (Mohamed and Gomaa, 2012, Siddikee et al. 2011). If the ability of some microorganisms, including Bacillus bacteria, was reached by reducing the pH of the soil, then about 20% of the Unavailable soil phosphorus turns into available phosphorus when adding the phosphorus-dissolving bacteria and reducing the soil reaction value from 7.14 to 5.75 compared to control, as well. It was found that the amount of phosphorus released increased when the acidity of the medium decreased (Portugal 2007, Hasan 2012). It was found that inoculation of barley seeds with six isolates of Bacillus bacteria led to an increase in plant growth and the amount of phosphorus absorbed in addition to an increase in microbial density with a significant increase in

the dry weight of the vegetative and root system (Timmusk et al., 2011). Phosphorus is one of the most important essential elements for plant growth after nitrogen (Timmusk et al., 2011). Sharma et al., 2013). The soil contains many beneficial microorganisms that are needed to increase soil fertility, whether they are bacterial or fungal capable of converting insoluble phosphate into soluble phosphate. These bacteria have the ability to secrete organic acids that lower the pH of the soil, some of which bind with calcium and iron and release phosphate (Talwar, 2018)

Given the importance of this topic, this study came to clarify the role of *B. subtilis* and its efficiency in converting insoluble phosphate into soluble phosphate in solid cultures.

MATERIALS AND METHODS

10samples were collected from the soil of the rhizosphere from fields cultivated with Delete replicate word, radish, basil, Zea mays and barley within the geographical area of the governorates of Dhi Qar, Muthanna, Qadisiyah and Najaf. The samples were collected from the fields and it were representative all area , all samples were placed in sterile plastic bags and kept in the refrigerator until use, as shown in Table No. (1)

Table (1): Numbers of soil samples and names of areas and fields from which they were collected

Form No.	Region Name	Field Type
1	Dhi Qar	alfalfa field
2	Dhi Qar	Jet field
3	Dhi Qar	alfalfa field
4	Dhi Qar	Basil field
5	Muthanna	radish field
6	Muthanna	Yellow corn field
7	Muthanna	Jet field
8	Muthanna	Jet field
9	Qadisiyah	radish field
10	Najaf	barley field

Serial dilutions were prepared for each soil sample by adding 10 g of each soil sample to 90 ml of sterile distilled water in 250 ml beakers, mixed well, and a series of serial dilutions (10-1-10-7) were carried out by transferring 1 ml of soil suspension into test tubes containing 9 ml of sterile distilled water, and for each soil sample, medium (King B) was used to grow soil dilutions on it. Taking 1 ml of soil dilutions prepared above to inoculate test tubes containing 9 ml of King B sterile medium above, in duplicate for each

dilution. The tubes were incubated at a temperature of 28 °C for 3 days. The tubes were examined to note the formation of a white thin film on the surface, which is a preliminary indicator of the growth of Bacillus spp. 0.1 ml was taken from the tubes that gave a growth indicator and spread on the surface of a Petri dish containing King A solid medium. The dishes were incubated at a temperature of 28 °C for a period of (2-3) days. The dishes were re-planed to obtain pure colonies of bacteria. After that, greenish-yellow colonies were obtained, which were isolated purely on solid environments, and they were given symbols and numbers according to the region from which they were isolated. For the purpose of obtaining pure colonies of Bacillus subtilis, a number of single colonies were selected that were given phenotypic characteristics identical to Bacillus subtilis, as the method was used Streaking for the purpose of re-seeding these bacteria on the surface of King B medium using the loop carrier and under sterilization conditions, the dishes were incubated for a period of (24 - 48 hours) until colonies appeared distant or close to each other, for the purpose of conducting microscopic examination and biochemical tests. Phenotypic, microscopic and biochemical tests were carried out for the colonies. growing in terms of their shapes, colors, surface and edges of the colonies, the presence of distinctive odors, transparency and texture on the surface of the nutrient agar (Nutrient agar, Black 1965), and the bacterial isolates were examined microscopically by taking a swab from the colony Bacterial cultures growing on the culture media were fixed and stained with a creamy stain to observe the shapes and arrangement of the bacterial cells and their interaction with the dye (positive or negative). The catalase enzyme production test, the oxidase enzyme production test, the motility test, and the nitrate reduction test were practically conducted. Nitrate, urease enzyme production test, methyl red test (MR), Fox Proskauer VP test, test Indol test, gelatin hydrolysis test test, and test Vtilization Citrate consumption test, then a bacterial diagnosis was carried out based on the private keys and according to what was stated in (Bergey, 1974)

Solubility coefficient (SI) estimation experiment

The dissolution coefficient of triple calcium phosphate (which is the ratio of the total diameter of the colony + the transparent corona to the diameter of the colony) was estimated by bacterial isolates, where Pikovskaye nutrient medium was used, as this medium was prepared by dissolving 10 g of Glucose, 5.0 g of Cas3(PO4)2 and 0.1 g of SO4 2(4NH) and 0.1 g MgSO4. $7H_2O$, 0.2g KCL, 0.01g MnSO4, 0.001g FeSO4, 0.5g Yeast extract, 15.1g Agar and 1000ml Distilled water. At a temperature of 121 °C and a pressure of 15 pounds / inch2 for 20 minutes, then the medium was poured into Petri dishes and left to solidify, then 0.1 ml was transferred by a sterile mechanical pipette from the vaccine isolates and spread on the surface of the medium by means of an L-shape spreader, using three replicates, and the dishes were incubated at a temperature of 28 ° C After three days of incubation, the diameter of the colony and the diameter of the transparent corona were measured using the following equation to estimate the dissolution coefficient (Edi-Premono et al., 1996):-

SI = D/C

whereas-:

SI = solubility coefficient

D = total diameter of the colony + areola pellucida

C = colony diameter only

Results and discussion

Table (2) shows the morphology of the bacterial isolates of *Bacillus subtilis*, isolated from the soil and rhizosphere of some crops, some of the agronomic and microscopic characteristics of the bacterial isolates, as it was found that most of the colonies of these bacterial isolates are circular, smooth, with a convex surface (convex), yellowish-white in color (white yellow), medium to large in size (mucous), the edge is lobed (lobate), while the results of examining slides stained with cream color showed that these cells have shaped Bacillus straight or oblong rod, Gram-positive. They are often in the form of pairs or chains. Based on these microscopic characteristics, it was found to be compatible with the characteristics of Saha bacteria (Bacillus et al., 2012)

Table (2) Biochemical tests for *Bacillus subtilis*

Iso.no. / Bio.che. / Test	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Gram stain	+	+	-	+	+	+	-	-	+	-
Motility t.	+	+	+	-	-	+	+	+	-	-
Vo . Pro.t	+	+	+	-	+	+	-	+	+	+
Starch hydrolysis	+	-	+	+	+	+	+	+	-	-
Nitrate reduction	+	+	+	+	+	+	+	+	+	+
Indole t.	-	+	+	+	-	-	-	+	-	+
Catalase test	-	-	+	+	+	+	-	+	+	+
Oxidase test	+	+	+	+	+	+	+	+	+	+
Urease test	-	-	-	-	-	-	-	-	-	-
MethylRed T.	-	-	-	+	-	-	+	-	-	-
Gelatin hydrolysis test	-	-	-	+	+	+	-	-	-	-
Citrate Vtilization test	-	-	-	+	+	+	+	+	+	-

Table (3) the results of the biochemical tests that were adopted in the diagnosis of bacterial isolates, as most of the isolates gave a positive result for the catalase enzyme test and positive for the citrate test, while these isolates gave a negative test for urease secretion and positive for the nitrate reduction test. The isolates showed a positive result for the Fuchs-

Proscauer test and negative for methyl red. They were able to analyze the starch as a result of their ability to secrete the amylase enzyme. This bacteria showed its ability to move. It also gave a negative result for the test for the gelatinase enzyme and positive for the oxidase test. These bacterial isolates bear characteristics of Bacillus subtilis, and this is consistent with what was mentioned by Starr et al. (1981) and Collee et al. (1996)

Table (3) Morphology of bacterial isolates of Bacillus subtilis

Isolate No	Soil isolated from it	Description
1	field persimmon	, cream-coloured, brittle, lobed, convex, round, short rod, mobile, gram-positive
2	Jet field	colony yellow, opaque, convex-lobed, globular, mobile, gram-positive
3	field alfalfa	colony creamy, gelatinous, flat, motile, rod, gram-negative
4	field basil	colony yellow, round, scalloped edge, mobile, short rods, gram-positive
5	field radish	brown colony, round, opaque, mobile, sticky, gram-positive
6	Yellow **Corn**	Colonial yellow cornfield, creamy, irregular edge, slightly raised, short sticks, gram-positive, mobile.
7	field jet	colony white, flat, serrated edge, short rod, motile, gram negative
8	Jet field	white, opaque, lobed-edged, domed, short rod, motile gram-negative colonies.
9	field radish	white colonies, scalloped edge, sticky, gram-positive, mobile, flat
10	Barley field	yellow colonies, edge-lobed, domed, short sticky, mobile, gram-negative.

Efficiency Testing of bacterial isolates in dissolving tri-calcium phosphate in solid media

The results shown in Table No. (4) indicate the effect of bacterial isolates on the solubility values, as isolate No. 3 outperformed the rest of the isolates and amounted to 3.50. p registered.

The results also indicated the superiority of isolate No. 2 over the other isolates, which amounted to 2.60, which did not differ significantly from isolate No. 1, which amounted to 2.28. Isolate No. 9, which amounted to 1.09, recorded the lowest values of the solubility coefficient. The superiority of the isolates above in the solubility of phosphate may be due to some genetic and environmental characteristics. Which is

characterized by these isolates as a result of their secretion of some organic acids and a decrease in PH and thus an increase in the solubility of phosphates (Sabri et al., 2017)

The study recommends the use of bacterial isolates of *Bacillus subtilis* that are efficient in dissolving tri-calcium phosphate, due to its effectiveness in increasing phosphorus availability.

Table (4) shows the effect of bacterial isolates on the solubility values

isolation number	**Dissolving factor values of B. subtilis**
1	**2.28**
2	**2.60**
3	**3.50**
4	**1.33**
5	**1.73**
6	**1.90**
7	**1.50**
8	**1.30**
9	**1.09**
10	**1.57**
Lsd	**0.47**

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