عزل وتحديد بعض أنواع الرايزوبكتيريا المحفزة لنمو النبات(PGPR) من بعض المخصبات الحيوية

Isolation and Identification of some Species of Plant Growth Promoting Rhizobacteria (PGPR) from some Bio-fertilizers

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الملخّص

هدف البحث لعزل وتوصيف بعض أنواع البكتيريا المحفزة لنمو النبات (PGPR) من المستحضرات التجارية التي تحتويها، إذ أخذت ثلاث عينات من المستحضرات التجارية: رايزوباكتيرين وفوسفورين وبوتاسيوماج. استخدمت بيئات غذائية متخصصة لعزل وتفريق الأنواع البكتيرية، وعرفت العزلات على أساس الخصائص المزرعية للمستعمرات، والفحص المجهري، وصبغ غرام، واختبار الحركة، بالإضافة إلى الاختبارات الكيميائية الحيوية.

أظهرت النتائج أن العزلات البكتيرية المتحصل عليها من المستحضر ريزوباكتيرين جميعها تتبع للجنس *Azotobacter* مثبتة للآزوت الجوي، وللنوع (NFBac) *Azotobacter chroococcum*. كما تم الحصول على عزلة محلة للفوسفور تتبع النوع(PSBpf) *Pseudomonas fluorescens* من المستحضر التجاري فوسفورين، وأخرى محلة للبوتاس تتبع النوع (KSBbc) *Bacillus circulans* من المستحضر التجاري بوتاسيوماج.

الكلمات المفتاحية: البكتيريا المحفزة لنمو النبات (PGPR)، *Azotobacter chroococcum*، *Pseudomonas fluorescens*، *Bacillus circulans*، عزل، تعريف.

Abstract

This study was conducted to isolate and identify some species of Plant Growth Promoting Rhizobacteria (PGPR) form three biofertilizers; Rhizobacterin, Phosphorine and Potassiomag. Specific bacterial media were used for Isolation and identification of bacterial species. The isolates were identified according to its cultural and morphological characteristics, gram staining, microscopy examination, motility and biochemical characteristics. The results showed that the isolates which identified from Rhizobacterin were nitrogen-fixing bacteria *Azotobacter chroococcum* (NFBac). We also isolated and identified two other isolates; phosphate mobilizing bacteria *Pseudomonas fluorescens* (PSBpf) and Potas solubilizing bacteria *Bacillus circulans* (KSBbc) from commercial complex Phosphorine and Potassiomag, Respectively.

Key words: Plant Growth Promoting Rhizobacteria (PGPR), *Azotobacter chroococcum*, *Pseudomonas fluorescens*, *Bacillus circulans*, Isolation, Identification.

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Introduction

Plant Growth Promoting Rhizobacteria (PGPR) are a multivariate group of bacteria found in the plant's rhizosphere, that stimulate qualitative and quantitative growth by facilitating plant uptake soil materials, Change the concentration of growth hormones such indole acetic acid (IAA), Gibberellic acid, cytokinin, ethylene, nitrogen fixation, dissolving mineral phosphate, potassium and other nutrients (Saharan and Nehra, 2011; Singh, 2013).

PGPRs are used as bio-fertilizers products that containing bacterial cells of different species, which have the potential to convert the important nutrients from a non-available form to available nutrition for plants (Nalawde and Bhalerao, 2015). Bio-fertilizers have important place in the plant nutrition, because of they are the most environmentally friendly among other nutritional supplements, and improving plant growth and productivity (Sylvia, 1997; Chen *et al.*, 2007 ; Abdel Ghany *et al*., 2013), and inhibits the effect of pathogens on plant by contrasting with pathogens by producing siderophores, antibodies and cyanide gas (Bouizgarne, 2013; Jee, 2007; Sivasakthi *et al*., 2014).

Bin Ishak in (2008) isolated and characterised -described some of the bacteria found in the bio-fertilizers. Many researchers have isolated and characteristics PGPRs from the rhizospher of many plants, isolating several bacterial species that bio fixing atmosphere nitrogen gas, solubilization phosphorus or potassium, depending on cultural characteristics of bacteria, microbiological characteristics, movement and biochemical tests (Karpagam and Nagalakshmi, 2014; Tulajappa *et al*., 2008 ; Jiménez *et al*., 2011 ; Sachin and Misra, 2009 ; Raja, 2012 ; Sharma and Rai, 2013; Sharma *et al*., 2011; Slepecky and Hemphill, 2006; Aslim *et al*., 2002).

The importance and aims of the research:

The importance of this research comes from the emergence of the concept of clean agriculture that free of chemicals and organic agriculture, the consumer's need for food free of chemical compounds and the ability of PGPRs bacteria to stimulate plant growth and increase its productivity and reduce the use of chemical fertilizers to convert nutrients that are not available to plants by biological processes of the form available for absorption, Therefore, the objective of the research isolated these bacteria from some commercial preparations containing them and identification, for using later as bio fertilizers and improving the efficiency of the bacteria found in the bio fertilizers in the optimal supply of the plant major elements N.P.K

Material and Methods

The research was carried out within microbiological laboratory and soil and water sciences research laboratory at the Faculty of Agriculture, Tishreen University (Lattakia, Syria).The following medium, reagents and diagnostic methods were used:

1- Sampling:

The study samples were taken from the following commercial complexes:

1-1 Rezobacetrin: A bio-fertilizer used on field crops vegetables and fruits. Its effectiveness was because of containing high numbers of fixed nitrogen bacteria loaded on pitmos, that were found on the roots plant and surrounding soil area with high efficiency during plant life. 1g was taken from the commercial product and underwent several dilutions and then inoculated on the bacterial SMSA medium and the Ashbys Mannitol Agar medium within the Petri dishes and incubated at 28 °C for three days. We are performed isolation and purification until we obtained pure bacterial colonies and then conducted the tests, and left bacterial dishes for a week until the distinctive color of the bacterial isolates studied (Abdullah and Abis, 2015; Al-Moussawi and Jabr, 2012).

1-2. Phosphorin: An Egyptian commercial complex which is containing a very active bacteria in the solubilization unavailable tri- calcium phosphate into available mono-phosphate of the plant. These bacteria soon multiply and spread in the root area of the plant and supply it with phosphorus necessary during different plant growth stages. Pikoviskaya's Agar medium was prepared, and then taken (1 g) of the commercial complex. It was underwent to several dilutions and inoculated on the Pikoviskayas Agar medium within Petri dishes, then

incubated the dishes at a temperature of 33°c for three days, and repeated the isolation until Single and pure bacterial colonies for characterization (Sharma *et al*., 2011).

1-3- Potassiumag: An Egyptian commercial complex, a bio-fertilizer containing a solubilization potassium bacteria that used to fertilize a wide spectrum of field crops, vegetables and fruits. We prepared the specialized medium of the bacteria Glocuse- Yeast extract-$CaCO_3$, then was taken (1 g) of The commercial product and given several dilutions and inoculation on the Glocuse-Yeast extract-$CaCO_3$ medium within Petri dishes. The dishes were incubated at 28 ° C for three days. We repeated the isolation and purification process until the pure bacterial colonies (Lisdiyanti *et al*., 2003).

2- Isolation and identification of the bacteria:

2.1 Bacterial media used in morphological diagnosis and movement test:

The following media were prepared:

- Sucrose Mineral Salts Agar, a general medium for the isolation of nitrogen-stabilizing bacteria (Krueger *et al*., 1970). The medium consisted of the following chemicals, per liter: sucrose, 1g ; NaCl, 1g ; $MgSO_4$'$7H_2O$, 0.2g ; KH_2PO_4, 1.2g ; $(NH_4)2HPO_4$, 5.5g ; $FeSO_4$-$7H_2O$, 10mg ; $ZnSO_4$-$7H_2O$, 10mg ; $MnSO_4$-H_2O, 7.8mg ; $CaCl_2$-$2H_2O$, 5.6 mg ; and $CuSO_4$-$5H_2O$, 0.10mg ; in demineralized distilled water, final pH 7.2.
- Ashbys Mannitol Agar, an medium that specializes in the isolation of the *Azotobacter chroococcum*, an atmospheric stabilizer (Technical Data, 2011).
- Glucose-Yeast extract- $CaCO_3$ a specialized medium for the isolation of *Fraturia aurantia bacterium* solubilization potassium (Lisdiyanti *et al*., 2003).
- Pikoviskayas Agar, a specialized medium for the isolation of *Bacillus megaterium* bacterium solubilization phosphorus (Technical Data, 2011).
- Motility Test Medium used for motion test (Green *et al.*, 1951).
- Pseudomonas F Agar was used to classify and differentiate bacterial species of the Pseudomonas species.

2.2 Bacterial media used in Biochemical Tests:

The following media were prepared:

Nutrient Gelatin was used to test gelatin liquefaction (Difco and BBL Manual, 2009) .Starch Agar (Technical Data, 2011) was used to test for starch degradation. Kligler Iron Agar (Technical Data, 2011) used to test amino acids decomposition. Tryptophan Culture Broth was used for the test of indol (Conda, 1960). Phenol Red Broth Base (Technical Data, 2011) was used to test the digestion of sugars. Glucose Agar (Technical Data, 2015) was used to test oxidase. Methyl Red Voges Proskauer Broth (Technical Documentation, 2001) was used to test Voges Proskauer. All the media were sterilized at 121° C for 20 minutes.

2-3- Reagents used for biochemical tests:

Iodine solution was used for test starch degradation, Dimethyl phenylene diamine hydrochloride for test oxidase, oxygen water 3 to test catalase, Methyl Red, And Alpha-naphthol and potassium hydroxide regents (Voges-Proskauer-regents) VPA and VP B (Technical Documentation, 2001)

2-4- Classification of bacteria:

Bacterial isolates were classified according to:

-Agricultural characteristics of general and specialized media (colony shape, color, texture, height, edges, size).

-Gram stain for differentiation between gram-negative bacteria and gram-positive bacteria and their microscopic properties.

-Movement test to diagnose bacteria whether they are moving or not moving.

-Biochemical tests (catalase - oxidase - sugar fermentation: glucose, sucrose, lactose, mannitol - gelatin liquefaction test - indol test - starch test - Voges Proskauer test - amino acid decomposition test).

Results and Discussion

1 Identification using the cultural characteristics:

The isolates obtained from commercial rhizobacterin showed distinct and similar growths in the general medium Sucrose Mineral Salts Agar (SMSA). They gave small, smooth, sticky, dark-colored circular colonies that were transformed into a brown color over time, as shown in Table.1 and Figure.1, Otherwise, the colonies on Ashbys Mannitol Agar of all the isolates colonies were bright, convex, sticky, medium to large in size, transparent, dense and covered the surface of the dish, and isolated these bacteria, the medium on which they grow and gave brown to the medium over time. As shown in (Table.1 and Figure.1). These results correspond to (Benson, 2001; Bergey's, 2004), which showed that the coloration of colonies of brown color on the specialized medium of nitrogen-based bacteria is due to the formation of water-soluble pigments to protect the nitrogenase enzyme of atmospheric oxygen oxidation because Azotobacter bacteria are mandatory, and the appearance of brown is considered a taxonomic characterization of the species *Azotobacter chroococcum* for bacterial species of the same genus.

Whenever isolating and purifying the bacterial isolates from the commercial complex Phosphorin to the specialized medium Pikovaskaya's Agar, all the isolates studied showed small to medium sized circular colonies with regular edges, white turned dark gray with time, soft cotton appearance, and formed a transparent ring around the bacterial colonies, Whereas, the isolates on medium Pseudomonas F Agar have yellow colonies that are characteristic of the *Pseudomonas flourescens*, as shown in (Table.1 and Fig.1). These results correspond to (Rhodes 1959; Mayz *et al*., 2013; Suman *et al*., 2016 ; Kipgen and Bora, 2017). They pointed out that the hallo zone of solubilization of tricalcium phosphate on Pikovskayas agar medium formed around the bacterial colony and that the yellowish yellow color is the result of the production of bacteria to the yellow color flourescein green, a classification characteristic of the species *Pseudomonas fluorescens*, and the more the diameter of this transparent ring, the more efficient bacteria to dissolve the phosphorus and convert it to the form available plant (Karpagam and Nagalakshmi, 2014).

It was also observed that the colony form of the isolated bacterial isolated from the commercial Potassium complex and inoculated with the specialized medium Glucose- Yeast extract- $CaCO_3$ was convex, medium, sticky, transparent ring in the insulation medium, and gave light orange color to this medium. As shown in (Table. 1 and Figure.1). These results are consistent with similar studies (Nakamura and Swezey, 1983; UK Standards, 2015) and are representative of *Bacillus circulans*.

Tabel 1. Cultural characteristics' for colonies of bacterial isolates.

bacterial isolates	Medium	Shape of Bacterial isolate	Size	Height and edge	color
Rhizobacterin isolates	AMA	bright, circular, sticky	Mediate	Convex height	Non transparent takes brown over time
	SMSA	small, smooth, sticky, circular	Smal	Little height	Non transparent takes brown over time
Phosphorin isolates	Pikovaskaya,s Agar	Circular	Small	Little height	muddy gray
Potassiumag isolates	GY- $CaCO_3$	Sticky, circular	Mediate	medium height	muddy orange

*** Mediate: diameter of colony between 2- 3 mm, Small: diametr of colony less than-2 mm.**

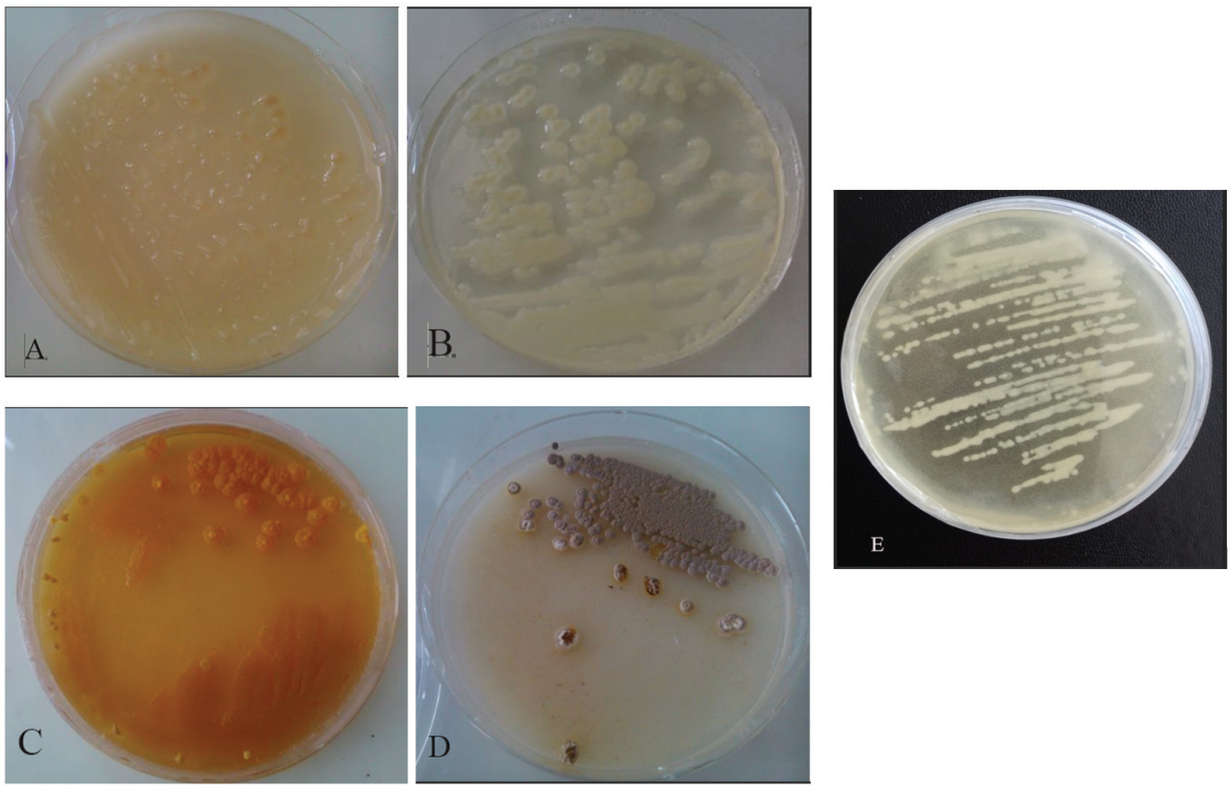

Fig 1. Cultural characteristics of isolates in nutrient media: A: Rhizobacterin isolate colonies on AMA medium were bright, convex, sticky, medium to large in size, transparent, dense and brown. B: Rhizobacterin isolate colonies on SMSA medium small, smooth, sticky, dark-colored circular colonies. C: Rhizobacterin isolate colonies on Pseudomonas F Agar medium have yellow colonies that are characteristic of the Pseudomonas flourescens. D: Phosphorin isolate colonies on Pikovaskaya,s Agar medium were small to medium sized circular colonies with regular edges, white turned dark gray with time, soft cotton appearance, and formed a transparent ring. E: Potassiumag isolate colonies on specialized medium Glucose- Yeast extract- $CaCO_3$ were convex, medium, sticky, transparent ring in the insulation medium, and gave light orange color to this medium.

3- Identification by pigmentation, microscopy and Motility test:

It was found that the bacterial cell form of the bacterial isolates from the commercial rhizobacterin comlex was short to oval, gram-negative, often in the form of pairs, a real movement in the Motility Test Medium, as shown in Table.2, Figure.2, and these results are consistent with *Azotobacter chroococcum* in similar studies (Abdullah and Abis, 2015 ; Tejera *et al*., 2005 ; Tulajappa *et al*., 2008; Jiménez *et al*., 2011 ; Sachin and Misra, 2009 ; Sharma and Rai, 2013 ; Dadook *et al*., 2014).

Tabel 2. Identification bacterial isolates according to pigments, microscopy and Motility test.

Bacterial isolates	Gram pigment	Bacterial shape	Motility
Rhizobacterin	-	Short rod, oval	+
Phosphorin	-	Short rod in pairs	+
Potassiumag	+	Rod in chain	+

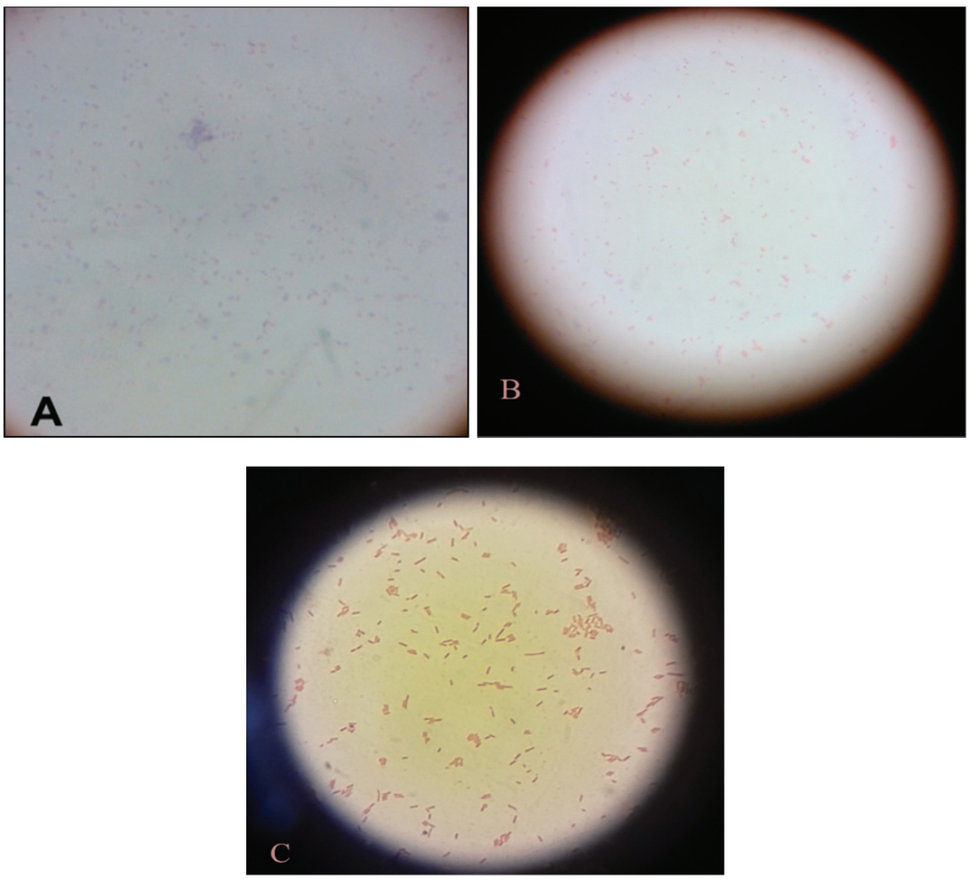

Fig 2. Bacterial cells for the bacterial isolates under microscope (100X): A: Rhizobacterin isolate is short rod, gram negative. B: Phosphorin isolate isolate is short rod, gram negative. C: Potassiumag is short rod, gram negative.

All bacterial isolates of commercial phosphorin were Gram-negative, short-rod in pairs and mobile when tested in the Motility Test Medium, as shown in Table 2 and Fig. 2, corresponding to the *Pseudomonas fluorescens* Similar studies have isolated and characterized them (Rhodes, 1959; Bergey's, 2004; Mayz *et al*., 2013 ; Suman *et al*., 2016 ; Kipgen and Bora, 2017).

For all bacterial isolates of commercial potassiumag were Gram- negative and the bacterial cells were shown to be very short-rod in chain, a real active movement when tested in the Motility Test Medium, as shown in Table 2 and Figure 2, which agree with characteristics of bacterial specie *Bacillus ciculans* in similar studies (Burdon *et al*.,1955 ; Nakamura and Swenzy, 1983; Bergey's, 2004; UK Standards, 2015)

4- Identification by biochemical characteristics:

Table 3 shows the characteristics of the bacterial isolates studied according to the biochemical tests (catalase test - oxidase test - sugary fermentation: glucose, sucrose, lactose, mannitol - gelatin liquefaction - Ethanol test, Indole test - starch test - Voges Proskauer test).

It was found in Table (3) that the results of biochemical tests of all isolates of the commercial rhizobacterin are consistent with the biochemical tests of the bacterial species *Azotobacter chroococcum* (Abdullah and Abis, 2015; Bergey's, 2004; Tulajappa *et al*., 2008; Jiménez *et al*. 2011); this indicates that the tested isolates are traceable to the bacterial specie *Azotobacter chroococcum*.

Table 3. Biochemical characteristics for bacterial isolates.

Biochemical tests	Bacterial Isolates		
	Rhizobacterin	Phosphorin	Potassiumag
Sucrose	+	-	+
Glucose	++	+	+
Mannitol	++	+	+
Lactose	+	-	+
Ethanol	-	+	-
starch test	+	-	+
catalase test	+	+	+
oxidase test	+	+	+
Gelatin hydrolyse	+	+	+
indol test	-	-	-
Amino Acids Hydrolysis test	-	-	+ -
NaCl 7%		+	+
Voges Proskauer test	-	+	-

+ : positive . - : negative

The results of the biochemical tests of isolated bacterial isolates of commercial phosphorin with *Pseudomonas fluorescens* are similar with studies (Rhodes, 1959 ; Bergey's, 2004 ; Mayz *et al*., 2013 ; Suman *et al*., 2016 ; Kipgen and Bora, 2017) (And thus the experimental isolation follows the bacterial specie *Pseudomonas fluorescens*. Biochemical tests for bacterial isolates isolated from the commercial potassiumag complex have been followed by *Bacillus circulans* and are consistent with the results of similar studies (Burdon *et al*., 1955; Nakamura and Swenzy, 1983 ; Bergeys, 2004 ; UK Standards, 2015) The tested isolates are traceable to bacterial species *Bacillus circulans*.

Conclusion and recommendations:

Azotobacter chroococcum isolates were obtained from rhizobacirin complex, *Pseudomonas fluorescens* from commercial phosphorin, and pure isolates of *Bacillus circulans* from Potassiumag. The isolates of studied bacterial could be used in plant bio-fertilization and integrated pest management programs.

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