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IDENTIFICATION AND CHARACTERIZATION OF *Azotobacter chroococcum* ISOLATED FROM SOME EGYPTIAN SOILS

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ABSTRACT

Bacteria with the ability to grow on nitrogen-free media and fixing atmospheric nitrogen were isolated from different locations in Egypt. Isolates were identified as *Azotobacter chroococcum* according to their morphological and physiological properties. Isolates were ovoid to rod shaped occurs in pairs, form cysts. Analysis with Random Amplified polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) to compare the similarity pattern of the selected *Azotobacter* isolates and reference strain was used as an identification tool. Physiological characteristics of *A. chroococcum* such as acetylene reduction activity (nitrogenase enzyme) and production of some plant growth promoting substances such as Indol acetic acid (IAA), Hydrogen cyanide (HCN) and Sidrophore production were also studied.

Keywords*: Azotobacter chroococcum*, Nitrogen Fixation, RAPD-PCR, Indol acetic acid (IAA), Hydrogen cyanide (HCN) and Sidrophore production.

INTRODUCTION

The use of free nitrogen fixing bacteria, i.e., *Azotobacter* sp. as a bioinoculant is widely applied for a wide variety of crops, such as rice, wheat, maize, sorghum, and sugarcane, due to its properties like nitrogen fixation, secretion of plant growth promoting substances, vitamins, antifungal metabolites, phosphate solubilization, soil aggregation and tolerance to pesticides (Inamdar *et al.,* 2000). The mentioned crops currently have much of their nitrogen needs supplied by costly mineral fertilizers (Döbereiner *et al.,* 1995 and Triplett, 1996). *Azotobacter* sp. is able to fix at least 10 mg N per gram of carbon (Becking, 1992). The ecological distribution of *Azotobacter* sp. is a complicated subject and is related with diverse factors, which determine the presence or absence of this bacterium in a specific soil. These bacteria have an advantage over root-associated diazotrophs, *Azotobacter* sp. and have better possibilities to exploit carbon substrates supplied by the plant (Boddey *et al.,* 1995; Sprent and James, 1995 and Triplett, 1996). More recently, molecular biology techniques have been utilized for refining or extending classifications, RAPD-PCR techniques for differentiating and tracking specific genetic elements within a complex genome or genomes. This method was originally developed to identify genetic polymorphism in plant, fungal and prokaryotic genomes (Mark and Don, 2000). Plant growth promoting rhizobacteria (PGPR) can trigger many benefits to plants through different mode of actions including the production of secondary metabolites

such as auxin mainly IAA, HCN and sidrophore, which may promote the plant growth indirectly *via* antagonism the soil borne pathogens by various mechanisms such as lytic enzymes, antibiotics production, competition and induction of systemic resistance, hence the main properties which appositionally mediated by the tested *A. chroococcum* isolates and strain cells.

This study aims to isolate and to identify the *Azotobacter* bacteria isolates isolated from some Egyptian soil samples using some biochemical tests and then to confirm the obtained data by the use of RAPD-PCR.

MATERIALS AND METHODS

Soil samples:

Four soil samples were collected from different locations in Egypt. The samples no. one and four from El-Behira Governorate, no. two and three from Assiut Governorate. Samples were picked from the upper 15 cm layer of soil and used to isolate the nitrogen fixing *A. chroococcum* bacteria. Some major properties of the experimental soil samples are presented in Table 1 according to Piper (1950).

Table (1): Some major properties of the experimental soil samples

No. of soil sample	pH (1:2.5 susp)	EC dSm^{-1}	$CaCO_3$ %	Organic Matter%	N-content mg Kg^{-1}	Texture class
1	8.5	0.13	9.70	1.00	15.0	Silty
2	7.8	0.19	9.90	0.60	80.0	Clay
3	7.6	3.40	17.2	0.60	60.0	Clay
4	7.4	2.00	9.40	0.70	70.0	Clay

Isolation and culturing of nitrogen-fixing bacteria:-

The specific *A. chroococcum* medium (Atlas, 1997), was used for the isolation of aerobic nitrogen-fixing bacteria *A. chroococcum*. Two grams of soil samples were added into 500 ml Erlenmeyer flasks containing 100 ml of *A. chroococcum* medium, then stirred on rotary shaker 180 rpm for 10 min, streaked out on agar *A. chroococcum* medium and incubated at 28 ± 2°C for 2-5 days to be checked for purity. Pure cultures were kept on agar slant at 4°C.

Identification and characterization of the *Azotobacter* spp:-

Pure isolates of the *Azotobacter* spp. from soil samples were characterized using the criteria of Bergey's Manual of Systematic Bacteriology (Brenner *et al*., 2005). The following morphological, physiological and biochemical tests were used: Colony morphology, Gram staining, productions of diffusible and non-diffusible pigments were determined on Modified Ashby's medium (Hegazi and Neimela, 1976) after 2-5 days of incubation at 28 ± 2°C. Motility was determined in wet mounts and flagella arrangement assessed by the technique of Rhodes (1958). Encystment was induced by the method of Socolofsky and Wyss (1961) and the cysts were stained by the method of Vela and Wyss (1964). Citrate utilization, H_2S production, catalase reaction acetone production (Margaret,

1989), utilization of some carbon sources, i.e., glucose, mannite, insoitol, sorbitol, rhamnose, sucrose, arabinose, ethanol, 0.2% butanol, isopropanol and methanol, which were assayed according to (Atlas, 1997). Starch hydrolysis was tested in cultures on starch agar medium containing 1% (w/v) potato starch by flooding with Lugol's iodine (Margaret, 1989). A reference strain of *A. chroococcum* (NNRL-B-14346) obtained from National Research Regional laboratory, USA was used for comparison.

Molecular studies using RAPD-PCR analysis:-

The molecular test of RAPD analysis by PCR was used to compare the similarity pattern of the selected isolates and the reference strain of *A. chroococcum* using it as identification tool. One hundred ml conical flasks containing 50 ml of liquid *A. chroococcum* medium (Atlas, 1997) were autoclaved (121°C for 20 min.), cooled down (45°C) and then inoculated with two ml of standard inocula of the tested isolates and for *A. chroococcum* strain. The flasks were incubated at 28±2°C in rotary shaker incubator (180 rpm) for 2-5 days. The cells were harvested by centrifugation at 10,000 rpm for 10 min and washed by distilled water. The collected cells were lysed by sonication for 10 Sec. under aseptic conditions. The lysed cells were stored at –20°C till used. A modified CTAB (hexadecyl trimethyl ammonium bromide) procedure based on the protocol of Porebski *et al.* (1997) was adopted for obtaining good quality total DNA. A set of five random primers; OP (A-11) (5´-CAATCGCCGT-3´), OP (B-10) (5´-CTGCTGGGAC-3´), OP (B-14) (5´-TCCGCTCTGG -3´), OP(C-02) (5´-GTGAGGCGTC-3´) and OP (C-14) (5´-TGCGTGCTTG-3´) were used in the detection of polymorphism among the four isolates and one strain of *A. chroococcum*. These primers synthesized on an ABI 392 DNA/RNA synthesizer (Applied Biosystems) were used for PCR amplification. All amplification products were separated electrophoretically in 1.5 % agarose gel.

Nitrogenase activity:-

Acetylene reduction activity (ARA) was measured according to the method of Stewart *et al.* (1967) for the isolates and the reference strain of *A. chroococcum*. Five ml of Modified Ashby's medium (Hegazi and Neimela, 1976) were added into 20 ml test tubes and inoculated with 50 µl of heavy inocula of 2-5 days old culture. Tubes were then incubated at 30°C for 2 hr. ARA was measured by replacing the cotton plugs of the tubes with rubber stopper and the head space (5 ml) was injected with 10% (v/v) acetylene. One ml of C_2H_2 was injected into tubes using disposables gas-tight siring and incubated at 30°C for 2 hr. C_2H_4 production was measured using gas chromatography (GC) DANI-1000.the ARA values were recorded as n mole C_2H_4/ ml / h.

Physiological characteristics of *Azotobacter chroococcum*:-

a. Production of Indol Acetic Acid (IAA):-

Production of IAA by tested cells of *A. chroococcum* was estimated in Luria-Bertani agar medium (LB) amended with 5mM, L-tryptophan according to Bric *et al.* (1991). Bacterial cultures were spotted on the surface of the agarized Petri plates using micropipette (each drop equals 20µl). Plates were incubated at 28 ± 2°C for 2-5 days. Each inoculated plate was overlaid with sterilized Whatman paper No.2 that was treated with 2 % (0.5 M $FeCl_3$) and

35 % perchloric acid. Papers were saturated with the reagent, and then the reaction was allowed to proceed until adequate color developed. Bacteria producing IAA were identified by the formation of a characteristic red halo within the filter paper immediately surrounding the colony. The ability of producing IAA was recorded as positive or negative.

b. Production of cyanide (HCN):-

Cyanide production was detected by the method of Bakker and Schippers (1987). Petri plates containing Luria-Bertani agar medium (LB) supplemented with 4.4 g/L glycine was inoculated with tested isolates and reference strain of *A. chroococcum* using micropipettes (each drop equals 20µl). Plates were incubated at 28 ± 2°C for 2-4 days. Each inoculated plate was overlaid with Whatman paper No.2 that was impregnated with 0.5 % picric acid and 2 % sodium carbonate. Papers were saturated with the reagent the change in color from yellow to orange-brown on the filter paper is an indicator to the production of cyanide. The ability of producing HCN was recorded as positive or negative.

c. Sidrophore production:-

Chrome azurol-S (CAS) agar medium (Schwyn and Neilands, 1987) was used for detection of sidrophores production by *A. chroococcum*. For this purpose, the plates of Chrome azurol-S (CAS) agar medium (Schwyn and Neilands, 1987) were inoculated with a loop of an active culture of the tested isolates and strain. Inoculated plates were incubated at 28 ± 2°C for 2-5 days. Siderophore production was indicated by the formation of orange color around the bacterial growth against the blue background of the medium.

RESULTS AND DISCUSSION

The free nitrogen fixing bacteria *Azotobacter* contains several numbers of species within the order *Pseudomonadales*. Taxonomic status and phylogenetic analysis of *Azotobacter* have been based on a polyphasic approach including description and analysis of pigmentation, morphology, biochemical and physiological properties.

The colonies formed by *Azotobacter* spp. on nitrogen free *A.chroococcum* medium (Atlas, 1997) were slightly viscous, semi-transparent during the early growth and later changes to dark brown. Bacteria were Gram-negative with rounded ends, after 48 h growth in nitrogen free liquid culture. Biochemical and morphological characteristics of these bacteria included the following: motility with peritrichous flagella; ovoid to rod shaped occurs in pairs and form cysts Figure 1. Gram negative, aerobic, catalase positive, producing insoluble pigment creamy changes to brown. The isolates hydrolyze starch, utilize citrate, and produce H_2S and acetone. The ability of utilizing different carbon sources such as glucose, mannite, insoitol, rhamnose, arabinose, ethanol, sorbitol, butanol 0.2 %, trehalose and glutrate were estimated. The isolates were sensitive to Erythromycin 2 µg / ml, phenol, isopropanol and methanol. The isolates were classified according to Bergey's Manual of Determinative Bacteriology (Brenner *et al*., 2005) as *A. chroococcum*.

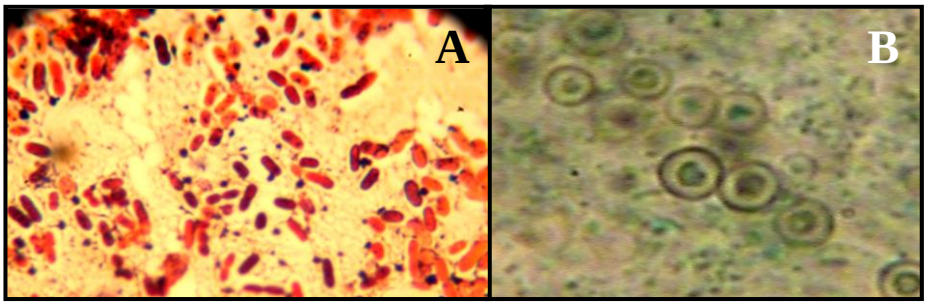

Figure (1): A- *Azotobacter* cells for isolate No.1 are Gram negative stain and straight rods or cocci single or in pair (diplococci) with (100 x). B- *Azotobacter* cyst with light microscope (staining by cyst stain) wet mount with (100 x).

RAPD-PCR analysis:-

Five random amplified polymorphic DNA (RAPD) primers against the reference strain *A. chroococcum* (S) (NNRL-B-14346), and four confirmed strains (isolates No. one, two, three and four) were studied. Figure 2 presented that the RAPD pattern of primer OP (A-11) were detected 35 fragments that ranged from 1200 to 190 bp, all fragments were polymorphic. Fragments were distributed as follows, 13 fragments for isolate number one and seven fragments for isolate number two, five fragments for isolate number three and three fragments for isolate number four and three fragments for *A. chroococcum* strain (S). Furthermore, nine specific fragments were detected for isolate number one, and four for isolate number two, while one specific fragment was detected for isolate number three, two fragments for isolate number four and three specific fragments for *Azotobacter chroococcum* strain (S). Figure 2 showed that the RAPD pattern of primer OP (B-10) has detected 33 fragments that ranged from 1200 to 300 bp, 12 are polymorphism. Fragments were distributed as follows, six fragments for both isolates number one and two, five fragments for isolate number three and nine fragments for isolate number four, while seven fragments for *A. chroococcum* strain were detected.

Furthermore, one specific fragment was detected for isolate number three, and three for isolate number four, while one specific fragment was detected for *A. chroococcum* strain (S). No specific fragments were detected for isolates number one and two. On the other hand both isolates number one and two have the same DNA pattern. Furthermore, Figure 2 showed that the RAPD pattern of primer OP(C-14) have detected 26 fragments ranged from 1000 to 250 bp, where 18 are polymorphic. Fragments were distributed as follows, six fragments for isolates number one, two and three. Four fragments for isolate number four and for *A. chroococcum* strain (S). Furthermore, one specific fragment was detected for both isolates number four and *A. chroococcum* strain (S). No specific fragment was detected for isolates number one, two and three. On the other hand both isolates number one and three have the same DNA pattern Table 2. Results in Figure 2 refer to that the RAPD pattern of primer OP (B-14) have detected 34 fragments that ranged from 1600 to 200 bp, all were polymorphic.

Figure (2): RAPD pattern of local *Azotobacter* isolates 1, 2, 3, 4 and a reference strain of *Azotobacter chroococcum* using primers OP (A-11), OP (B-10), OP(C-14), OP (B-14) and OP (C-02).

Fragments were distributed as follows; seven fragments for both isolates number one and number three, six fragments for both isolate number two and *A. chroococcum* strain (S). Eight fragments were found for isolate number four. Furthermore, five specific fragments were detected for isolate number one and three fragments for isolates number two, three, four and for *A. chroococcum* strain (S). Data in Figure 2 revealed that the RAPD pattern of primer OP(C-02) have detected 41 fragments that ranged from 1600 to 440 bp. Twenty one are polymorphic. Fragments were distributed as follows; eleven fragments for isolate number one and nine fragments for isolate number two, six fragments for isolate number four, where ten fragments for isolate number four and only five fragments for *A. chroococcum* strain (S).

Furthermore one specific fragment was detected for both isolates number two and three. No specific fragments were detected for the isolate number four and *A. chroococcum* strain (S). Results revealed that isolates number one and two have the same amplified DNA pattern when primer OP (B-10) was used and also isolates number one and three have the same amplified DNA pattern when primer OP(C-14) was used, while their pattern differed in one or two fragment when primers OP (A-11), OP (B-10) and OP(C-02) were used.

Table (2): Total amplified fragments in RAPD-PCR analysis

Primers	*Azotobacter* spp.						
	CF		1	2	3	4	S
OP(A-11)	2	PF	13	7	5	3	7
		SF	9	4	1	2	3
OP(B-10)	4	PF	6	6	5	9	7
		SF	0	0	1	3	1
OP(C-14)	2	PF	6	6	6	4	4
		SF	0	0	0	1	1
OP(B-14)	0	PF	7	6	7	8	6
		SF	5	3	3	3	3
OP(C-2)	4	PF	11	9	10	6	5
		SF	2	1	1	0	0
	12	TPF	32	34	33	32	25
		TSF	16	8	6	9	8
		TAF	48	42	39	41	33

S: Reference strain *Azotobacter chroococcum.*
CF: Number of common fragments.
PF: Number of polymorphic fragments.
SF: Number of specific fragments. (Considered only one or two fragments detected under PCR conditions).
TPF: Total number of polymorphic fragments.
TSF: Total number of specific fragments.
TAF: Total amplification fragments.

Table 3 illustrates the similarity matrix of the four isolates and *A.chroococcum* strain (S) and the highest similarity of 81.6 % was detected between isolates number one and two, while 71 % similarity was found between isolate number four and *A. chroococcum* strain (S). In addition the lowest similarity was detected between isolates number 2 and 4, which was 51.4 %. Also isolate number one and *A. chroococcum* strain (S) have recorded 53.1 % similarity. However, both isolates number one and number

two showed relative similarity ratio when compared with the other tested isolates as well as to isolate number four and *A .chroococcum* strain (S). Also Figure 3 showed that the dendrogram contains two clusters. The first cluster has one branch for isolate number four and *A. chroococcum* strain (S) with 71 % similarity. While, the second cluster has two branches one for isolates number one and number two with 82 % similarity in addition to 69 % similarity for isolate number one, two and three. On the other hand RAPD technique can be used as identification tool (Mark and Don, 2000 and Abbas, 2006). These obtained results are confirmed by Azza (2002) who had used same biochemical, morphological and molecular tests applied in the present study in characterization and identification of *A. chroococcum* isolated from soil samples. So, in conclusion the present local isolates are identified almost probably as *A. chroococcum*.

Table (3): Similarity matrix (%) calculated by NTSYS program among five *Azotobacter* spp. based on RAPD-PCR analysis

***Azotobacter* isolates No.**	**1**	**2**	**3**	**4**	**S**
1	100				
2	81.6	100			
3	69.7	67.6	100		
4	60.6	51.4	68.8	100	
S	53.1	61.1	58.1	71	100

Figure (3): Dandrogram based on the RAPD-PCR showing the similarity among five different *Azotobacter* spp. isolates No. 1, 2, 3, 4 and *Azotobacter chroococcum* strain (S).

Production of some PGPR of *Azotobacter chroococcum*:-

Inoculation of plants with plant growth promoting rhizobacteria has proposed as a useful agricultural tool to enhance crop yield (Baldani *et al.*, 1997). PGPR stimulate plant growth by mechanisms such as nitrogen fixation, phosphate solubilization, phytohormones production, sidrophore synthesis or biocontrol of phytopathology (Bashan and Holguin, 1997).

Nitrogenase activity:-

The acetylene reduction assays (nitrogenase activity) was used as an index of the rate of nitrogen fixation (Hitchins and Sadoff, 1973). The isolates were screened *in vitro* for their N_2- fixing ability, data in Table 4 recorded the amounts of acetylene reduced by *A. chroococcum* isolates and the reference strain, and the amounts were quite different. Rates obtained in isolates were in the range from 76.79 to 189.6 n mole C_2H_4/ ml/ h. It has been shown that the soil characteristics; organic matter content; moisture; C/N ratio; pH and host plant can affect on the nitrogenase activity (Döbereiner and Pedrosa, 1987, González-López, 1992 and Tejera *et al.,* 2006). Many rhizobacteria can produce phytohormones that believed to be related to their ability to stimulate plant growth. Among these phytohormones auxin may play a major role in the promoting ability. Data in Table 5 which are derived from the qualitative screening showed that *A. chroococcum* isolates and the reference strain cells can produce auxin but they greatly fluctuated in their color appearance on the tested plates, where all isolates and the reference strain produce IAA in the same level. In accordance with our results, many investigators showed that PGPR, i.e., can produce *in vitro* IAA and other phytohormones such as gibberellins and cytokinins. (Malik *et al*., 1992; Rademacher, 1994; Iosipenko and Ignatov 1995; Radwan *et al*., 2000).

Table (4): The amount of nitrogenase activity from the tested *Azotobacter* isolates and reference strain of *Azotobacter chroococcum*

***Azotobacter* isolates No.**	**Nitrogenase activity n mole C_2H_4 / ml / h**
1	**189.6**
2	**183.59**
3	**91.14**
4	**169.17**
S	**76.79**

Cyanide production (HCN):-

The ability of *A. chroococcum* isolates and the reference strain for cyanide production was examined in culture Table (5). Both isolates and the reference strain were varied in their ability to produce (HCN) where all isolates appeared the most active producers in comparison with the reference strain. Results also revealed that *A. chroococcum* isolates can produce the cyanide *in vitro* (Bashan and Levanony, 1990; Radwan *et al*., 2002). Cyanide is a secondary metabolite of several microorganisms, it can be produced directly from glycine and form cyanogenic glycosides (Knowles, 1976). The visual inspection of the tested plates revealed that *A. chroococcum* isolates and the reference strain have a cyanogenic potential changing due to the

color of indicator paper. This ability of the *A. chroococcum* isolates and strain to produce reasonable quantities may be useful to imply such rhizobacteria as suppressive bioagents soil borne phytopathogens.

Sidrophores production:-

Iron is an essential element for the growth and function of most living cells and it is vital component in a wide variety of biochemical reactions in plants and microorganisms. Data in Table (5) elicited for excreting sidrophore compounds where isolate No. two showed the superior production of sidrophores than all other isolates and the reference strain. The ability of producing sidrophores is important to the vital role of organic compounds to improve the iron nutrition (Lesueur *et al*., 1993). Additionally, microbial sidrophores play a prominent role in the biocontrol of some soil borne plant disease *via* sequestering available iron consequently deprive pathogenic fungi from this an essential element (Stephane *et al.*, 2005).

Table (5): Production of some PGPR of *Azotobacter chroococcum*

***Azotobacter* isolates No.**	**PGPR**		
	IAA	**HCN**	**Sidrophores**
1	**+**	**++**	**+**
2	**+**	**++**	**++**
3	**+**	**++**	**+**
4	**+**	**++**	**+**
***Azotobacter chroococcum* (S)**	**+**	**+**	**+**

CONCLUSION

The present results concluded that the successful isolation of local isolates of *Azotobacter chroococcum* from some Egyptian soils have an important role to enhance the soil fertility (nitrogen fixers). Identification of these isolates showed genetically different properties; adapted; compatible with the Egyptian conditions and the ability for production of important plant growth promoting substances.

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تعريف وخصائص الأزوتوبكتر كروكوكم المعزولة من بعض الأراضي المصرية

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البكتريا ذات القدرة على النمو في البيئات الخالية من النيتروجين والتى يمكنها تثبيت النيترجين الجوي تم عزلها من أماكن مختلفة في مصر. العزلات تحت الدراسة سالبة لجرام وعصوية في أزواج وتكَون حويصلات وتم تعريفها بأستخدام التجارب المورفولوجية والفسيولوجية وإستخدام تفاعل البلمرة المتسلسل لمقارنة أنماط التشابه بين العزلات تحت الدراسة وبين السلالة المرجعية للأزتوبكتر كروكوكم. بعض الخصائص الفسيولوجية للأزوتوبكتر مثل إختبارإختزال الأستيلين و إنتاج المواد المحفزة لنمو النباتات مثل الإندول أستيك أسد وسياند الهيدروجين وأيضا إنتاج المواد المخلبية (السيدروفورز) تمت دراستها.

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