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Characterization of Soil-Indigenous Cyanobacterial Strains and Bioactivity Assessment

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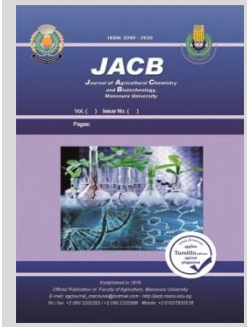

ABSTRACT

Some cyanobacteria isolates were collected from soil samples at various locations from governorates of Kafr El-Sheikh and El-Dakahlia. By using morphological characterizations to identify these isolates. Heterocyst-forming cyanobacteria were *Anabaena* sp., *Nostoc* sp., *Oscillatoria* sp. and *Chroococcus* sp. Only, *Nostoc* sp. and *Anabaena* sp. isolates were represented at high frequency in the two isolated areas. The less frequency of occurrence in this study were *Oscillatoria* sp., *Chroococcus* sp., *Phormidium* sp., *Pseudoanabaena* sp. *Nostoc calicola* D and *Anabaena cylindrica* D were recorded as the most active cyanobacteria according to nitrogen fixation, nitrogenase activity, Indole Acetic Acid(IAA) production and dry weight determination. Therefore, it would be recommended to apply these strains of cyanobacteria in bio-organic farming.

***Keywords*:** Cyanobacteria, nitrogen fixation, Nitrogenase activity, IAA

INTRODUCTION

The beneficial effects of cyanobacteria in paddy soils have recently received a lot of attention. The amount of nitrogen fixed by these blue-green algae may meet the nitrogen requirements of wheat plants. The release of growth-promoting substances during algal development should not be overlooked in this regard. Blue-green algae are a type of photosynthetic prokaryotes that can fix nitrogen from the atmosphere in submerged rice fields. The cyanobacterial process requires biological N2 fixation, which is dependent on a sufficient population. The diversity of cyanobacteria ranges from unicellular to multicellular, branched filamentous to pigmented, autotrophic to hetrotrophic, free-living to symbiotic, psychrophilic to thermophilic acidophilic to alkylotrophic, planktonic to epiphytic. Some workers have emphasized the importance of phytoplankton (Alam *et al*., 1989).

Cyanobacterial strains have the ability to fix nitrogen in the fields of culture, food, and fertilizer (Muthukumar *et al*., 2007). In Egypt, cyanobacterial strains were isolated, identified as *Anabaena* sp. and *Nostoc* sp. and used to provide rice crops with N_2 and made a high progress rice production (Afify *et al*., 2018)

The present research aims to study the diversity, isolations and identification of cyanobacteria from different locations in Egypt and study their capability for production PGPR as IAA and nitrogenase enzyme as evidence for producing nitrogen available for plants and to increase the crop.

MATERIALS AND METHODS

Soil samples collection

For cyanobacterial isolation, air-dried soil samples collected from different sites in Kafr El- Sheikh and El-Dakahlia governorates were used. The properties of the soil samples were determined according to Piper (1950) and Jackson (1973), as Mechanical analysis and obtained data are presented in Table (1).

Table 1. Physicochemical characteristics of the collected soil samples used in this stydy

Character	El-Dakahlia (Mansoura)	Kafr El-Sheikh (Sakha)
Physical analysis (%):		
Coarse Sand	2.24	1.55
Fine Sand	23.51	19.79
Silt	42.00	47.57
Clay	32.26	31.09
Soil Textural class	Clay loam	Clay loam
Chemical analysis:		
pH	7.97	7.9
EC (ds/m)	0.7	1.176
Cations (ppm)		
Ca^{++}	68.01	122.13
Mg++	34.50	65.41
Na+	397.17	646.85
K+	8.33	25.07
Anions (ppm)		
CO_3^{--}	0	0
HCO3-	725.17	792.17
Cl-	143.54	434.22
SO4--	0	18.69

Isolation of cyanobacteria

To obtain the cyanobacterial isolates, sterilized 0.7% agarized of modified Watanabe medium were poured into Petri dishes (10 cm in diameter) according to El-Ayouty and Ayyad, 1972.

Purification of cyanobacteria

The unialgal cultures were purified as described by Pringsheim (1949).

Maintenance of cyanobacterial cultures

Stock cultures were maintained in a refrigerator at 5ºC. on agar slants of Modified Watanabe Medium (El-Nawawy *et al.*, 1958).

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Identification of the isolated cyanobacteria

Characterization and identification of the purified cyanobacteria isolates were carried out according to Castenholz (2015).

Selection of the most efficient N_2-fixing cyanobacteria strain

A growth curve experiment was conducted for 18 isolates to compare their growth activities and their capacities for N_2-fixation. Cyanobacteria isolates were cultivated for 30 days, to determine cyanobacteria dry weight, and the fixed nitrogen amount.

Preparation of standard cyanobacterial inoculum

The inoculum of the cyanobacterial identified strains was prepared by inoculating 500 ml Erlenmeyer flasks each containing 200 ml of Modified Watanabe liquid medium with a loopfull of 21 days old culture of each cyanobacteria strains. Inoculated flasks were incubated at 28-30ºC under continuous illumination (2500 lux) for 21 days.

Determination of total nitrogen content

Total nitrogen in the cyanobacterial culture for each isolates were determined using the micro-Kjeldahl method according to Jackson (1973).

Nitrogenase activity

The capacity of cyanobacteria isolates to fix nitrogen was assayed by acetylene reduction technique according to Hardy *et al* . (1973). Nitrogenase activity was then calculated by the following formula

µmole C_2H_4/ml culture =R x (container volume (test tube)/incubation time x Inj. vol. x D x 22.4) x 10^3

Where: Inj.= injecting volume – R = reading -D = The volume of the medium.

The results were presented as µ mole C_2H_4 / 100 ml culture /day.

Quantification of indole acetic acid (IAA) production

Each isolate was grown in its specific medium supplemented with 0.1% tryptophan according to Ahmad *et al*. (2005). Production of IAA in the supernatant was assayed using method described by Pilet and Chollet (1970). This method was shown to be most sensitive and most specific (Glickmann and Dessaux, 1995). The IAA concentrations were calculated from IAA standard curve (Salkowski Colorimetric Technique).

RESULTS AND DISCUSSION

Isolation, purification and identification of cyanobacteria

Out of the several isolates of cyanobacteria, from the rhizosphere soil of wheat plants grown in the different locations namely, kafr El-Sheikh, and El-Dakahlia governorates. Eighteen cyanobacterial isolates were obtained in pure cultures; bacteria free (El-Gamal *et al*., 2008). These isolates were examined for their morphological and cultural characteristics (Table 2), according to Venkataraman (1981) & Roger and Ardales (1991), in liquid and solid Watanabe medium (Staub, 1961).

Table (2) also showed the distribution of these isolates in the two Governorates. Eighteen cyanobacterial isolates were identified up to genera. Results showed that *Anabaena* sp. were the dominating organisms.

Anabaena sp. were isolated at high frequency; where, six cyanobacterial isolates, three of them representing in kafr El-Sheikh Governorate and three representing in Dakahlia Governorate . Less numbers of cyanobacteria belonging to different genera namely *Phormidium* sp. (3), *Oscillatoria* sp. (1), *Chroococcus* sp. (1) and *Nostoc* sp. (1) were also obtained in pure cultures from kafr El-Sheikh Governorate. But in El-Dakahlia Governorate *Nostoc* sp. and *Oscillatoria* sp. were isolated also at high frequency comparing to kafr El-Sheikh; where two isolates of each one of them were obtained. *Chroococcus* sp. isolates were equale where each governorate had one isolate. Zero numbers of cyanobacteria belonging to genus namely *Phormidium* sp. appeared in El-Dakahlia. On the other hand, appeared one isolate of *Pseudoanabaena* sp . which did not appear in kafr El-Sheikh.

Table 2. Occurrence of cyanobacterial genera in kafr El-Sheikh and El-Dakahlia soil samples.

Origen soil samples	Isolates Code	Cyanobacterial Genera	Isolates Frequency (%)
kafr El-Sheikh	N1	*Anabaena* sp.	16.67
	N3		
	K3		
	L3	*Nostoc* sp.	5.56
	H3	*Oscillatoria* sp.	5.56
	M1	*Chroococcus* sp.	5.56
	K5	*Phormidium* sp.	16.67
	O1		
	O3		
El-Dakahlia	B1	*Anabaena* sp.	16.67
	E2		
	E3		
	B2	*Nostoc* sp.	11.11
	F5		
	D2	*Oscillatoria* sp.	11.11
	D3		
	G3	*Chroococcus* sp.	5.56
	F2	*Pseudoanabaena* sp.	5.56
Total number of isolates		18	

Phenotypic properties of cyanobacterial genera and species

In the second edition of Bergey's Manual of Systematic Bacteriology (2001) cyanobacteria are subdivided into five quasi-taxonomic groups or subsections. Their diagnosis is based on the dichotomous key: the morphotype is unicellular or quasi-multicellular (trichome); division is binary or multiple (with formation of beocytes); presence or absence of differentiated cells (akinetes and heterocysts); and absence or presence of ramification (true or false) in the trichome (Pinevich, 2008 and Komárek *et al.*, 2014). All visible cultural characterizations and light microscopic preparations were observed and summarized in Table (3).

Determination some biological activities of cyanobacteria *in vitro*

1. Cyanobacteria' growth

Results illustrated in Fig (1) indicate that a great variation in biomass production between the different cyanobacterial genera and in some cases ecological effects are appeared. In El-Dakahlia governorate isolates, ranges of cyanobacterial mass production (g gL^{-1} culture) during incubation periods (10, 20, 30 days) were found to be in the order *Nostoc* spp. (2 isolates) 0.4 to 3.0; *Anabaena* spp. (3 isolates) 0.2 to 3.0; *Oscillatoria* spp. (2 isolates) 0.3 to 2.1; *Chroococcus* sp. (1 isolate) 0.2 to 1.7, *Pseudoanabaena* sp. (1 isolate) 0.1 to 0.5 gL^{-1} culture. However, in kafr El-Sheikh governorate isolates, ranges of cyanobacterial mass production (g/100 ml-culture) also were found to be in the order *Anabaena* (3 isolates) spp. 0.6 to 3.2, *Chroococcus* sp. (1 isolate) 1.1 to 3.5, *Phormidium* spp. (3 isolates) 0.1 to 2.5, *Nostoc* sp. (1 isolate) 0.4 to 2.0, *Oscillatoria* sp. (1 isolate)

0.1 to 0.5 gL^{-1} culture. These results are in agreement with those obtained by (El-Zawawy, 2016 and Taha 2000) who found that cyanobacteria exhibited the highest dry weight of with increasing the incubation period.

Table 3. Summarized of cultural, morphological and microscopic characteristic of cyanobacteria isolated from soil revealed by microscopic observations

Isolates Code	Thallus color	Thallus morphology	Vegetative Cell			Heterocysts			Akinetes			Cyanobacteria Identified Name
			Shape	Width (µm)	Length (µm)	Site	Width (µm)	Length (µm)	Shape	Shape	Width (µm)	Length (µm)	
B2	Dark green	Gelatinous to rubbery	Cylindrical	2.3-2.7	2.7-5	Terminal and intercalary	3.2-4.1	3.8-5.9	Subspherical	Spherical or subspherical	3.6-5.4	2.7-6.3	***Nostoc calicola***
N3	Green	Filaments	Barrel	4	5	Terminal, intercalary	4-5 3-3.5	4-5 4-4.5	Subspherical conical	3-6 in series, sub-spherical	5-6	6-7	***Anabaena oryzae***
E2 & E3	Green	Filaments	Short angular	4-4.5	2-3.5	Intercalary	5-6	6.5-7.5	Barrel	-	-	-	***Anabaena cylindrica***
F5 & L3	Blue-green, green or brown; black and crusty when dry	Filaments	Subglobose to barrel	2–3	3–4	Intercalary or terminal	5–7	5–6	Globose or ellipsoid	Spherical to elliptical	10	10	***Nostoc pruniforme***
K3 & N1	Oliveceous green, blue green	Filaments	Cylindrical	3.6-4.1	4.1-5	Terminal or intercalary	4.2-5	4.2-5.4	Spherical	Ellipsoidal or oblong	5.0-6.3	5-8.1	***Anabaena variabilis***
B1	Blue green	Filaments	Sub-spherical	5.7-7.8	5.7-7.1	Intercalary	5.6- 7.1	6.5-7.1	Subspherical or nearly quadratic	Spherical	12.8-14.2	12.8-14.2	***Anabaena qelatinicola***
K5 & O1 & O3	Dark blue green	Filaments	-	1.6-1.8	2.6-3	-	-	-	-	-	-	-	***Phormidium foveolarum***
F2	Moving by gliding movement	Filaments	Cylindrical	1.5-1.8	3.9- 4.6	-	-	-	-	-	-	-	***Pseudanabaena qaleata***
G3 & M1	Dark blue green	Slimy-gelatinous	Spherical	3-4	3-4	-	-	-	-	-	-	-	***Chroococcus minor***
D2 & D3 & H3	Dark blue green	Trichome solitary and straight	Solitary	4-8.2	4-7.1	-	-	-	-	-	-	-	***Oscillatoria brevis***

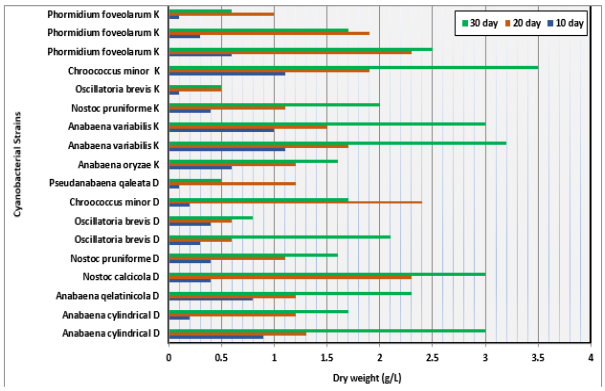


Fig. 1. Growth (g dry weight L^{-1}) of identified cyanobacterial strains isolated from El-Dakahlia (D) and kafr El-Sheikh (K) governorates

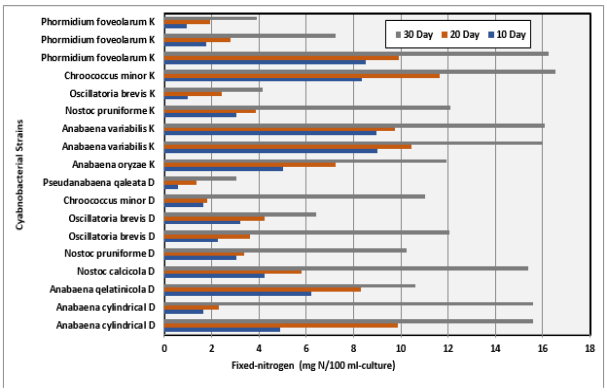


Fig. 2. Fixed nitrogen (mg N/100 ml culture) by cyanobacterial strains isolated from El-Dakahlia (D) and kafr El-Sheikh (K) governorates

2. Nitrogen fixation by cyanobacteria

It is important to determine the efficiency of cyanobacterial isolates to fix the atmospheric nitrogen as one of the most important parameters used for selection of cyanobacterial isolates in the further experiments.

Results graphically in Fig. (2) show that amounts of fixed nitrogen (mg N/100 ml culture) gradual increased by all tested cyanobacterial strains with incubation period increment, where higher values of the fixed nitrogen amounts were recorded with all strains at 30 days of growth. *Anabaena cylindrica* D and *Nostoc calcicola* D were recorded 15.60 and 15.37 mg N/100 ml liquid culture, respectively. On the other hand, only 3.06 mg N/100ml culture was recorded by the lowest one, *Pseudanabaena qaleata* D at 30 days incubation.

Firstly, in (1995) Stal stated that in nature cyanobacterial nitrogen fixation appears different patterns depending on the type of strain and environmental conditions. Boussiba *et al.*, (1984) found out that the use of cyanobacteria as a biofertilizer for rice fields is very promising but limited due to fluctuation in quality and quantity of inoculum and its physiological attributes in varied agroecological regions. Utilization efficiency of fixed nitrogen by rice plants is often low and efforts are therefore being extended to isolate suitable strains of cyanobacteria that would be prolific not only in fixing atmospheric nitrogen but also in excreting it continuously, thus making it available to the growing rice plants.

3. Cyanobacteria nitrogenase activity

The eighteen strains of cyanobacteria had various efficiency levels of the N2-fixation as previously mentioned (Fig. 3). Thus, it is necessary to detect nitrogenase activity of these cyano-strains. Data illustrated in Fig (4) indicate that cyanobacterial strains had ability to reduced acetylene at varied rate from 20 to 540 n moles C_2H_4/ml/day. In addition, heterocystous cyanobacteria *Nostoc calcicola* and *Anabaena cylindrical* proved to be the most active strains for nitrogenase activity which recorded 540 and 360 n moles C_2H_4/ml/day, respectively. These findings in agreement with Singh *et al.*, (2011) who reported that filamentous cyanobacteria have a specialized structure for nitrogen fixation called heterocysts which containing a key enzyme "nitrogenase" that is responsible and involved in the nitrogen fixation process.

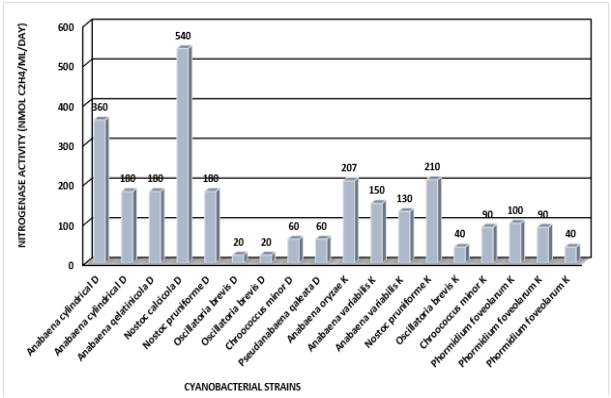


Fig. 3. Nitrogenase activity (n moles C_2H_4/ml/day) of cyanobacterial strains isolated from El-Dakahlia (D) and kafr El-Sheikh (K) governorates.

4. Production of indole acetic acid (IAA):

Results represent in Fig. (4) indicate that indole acetic acid production by tested cyanobacterial strains through experiment age, 30 days. Most cyanobacterial strains showed positive results for IAA production in the media supplemented with tryptophan. It was also noted that through the first 10 days cyanobacterial strains had a high variable amounts then gradually decreased through next 10 days and some cyanobacterial strains became (Nil) IAA undetected at the end of incubation time (30 days). These results clearly demonstrated IAA production varied according to isolates that obtained from different environments. *Nostoc calcicola* D, *Anabaena cylindrica* D showed the superior significant production of IAA in first 10 days which were (59.85 mg /ml), (39.26 mg /ml) respectively.

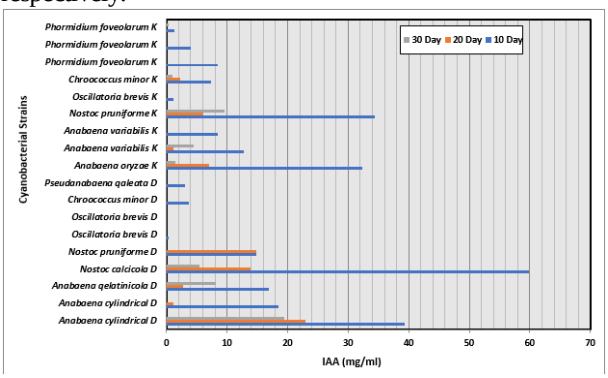
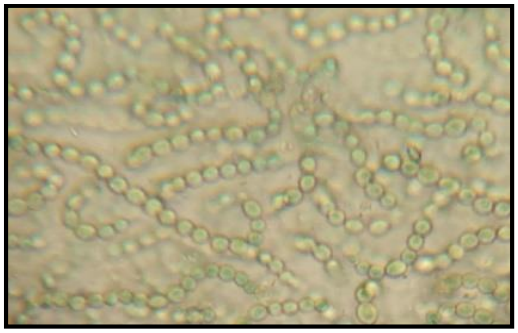


Fig. 4. IAA – production (mg /ml) of identified cyanobacterial strains isolated from El-Dakahlia (D) and kafr El-Sheikh (K) governorates.

It could be concluded that the cyanobacteria strains of *Anabaena cylindrica* D and *Nostoc calcicola* D (Fig. 5) were of the most efficient strains in biomass, IAA productions and nitrogen fixation capacity and could be used as bioagents for most economic plants.

Anabaena cylindrica



Nostoc calcicole

Fig. 5. Photographs of microscopic preparations of superior cyanobacterial strains.

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توصيف سلالات السيانوبكتيريا المتوطنة في التربة وتقييم النشاط الحيوي

راندا محمد زكي السعداوى[1]، أحلام علي مصطفي محيسن[2]، ايمان حسين عاشور[1] وعايدة حافظ عفيفى عامر[1]*

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تم جمع عينات تربه من مواقع مختلفه في محافظتى كفر الشيخ والدقهليه وذلك لعزل السيانوبكتيريا حيث تم الحصول على عزلات السيانوبكتيريا نقيه ثم تعريفها وذلك طبقا شكل و لون الثالوس وحجم الهتيروسست بالإضافه إلى الخلايا الخضريه و التكاثريه ، وعند تنميتها اتضح أن اجناس السيانوبكتيريا لها القدرة على تكوين الهتيروسست مثل أنابينا و نوستوك و أوسيلاتوريا وكذلك كرووكوكس والفورميديم وسيدوانابينا. وكانت معظم الأنواع تتبع جنسى النوستوك و الأنابينا و ذلك في محافظتى كفر الشيخ والدقهليه بينما كانت أقل الأنواع تنتمى إلى جنسى الأسيلاتوريا و الكرووكوكس اما الفورميديم ينعدم وجودها في الدقهلية بينما السيدوأنابينا لم تتواجد في أراضي كفر الشيخ وطبقا لتقديرات النيتروجين المثبت و الوزن الجاف و الاندول اسيتيك اسيد وانزيم النيتروجينيز. يتضح من النتائج المتحصل عليها ان النوستوك كاليكولا والانابينا سيلاندريكا هما أفضل العزلات التي يمكن أن يوصى باستخدامهما وذلك بالتطبيق في التجارب الزراعية لدعم الاتجاه الى الزراعة الحيوية والعضوية النظيفة.