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CHARACTERIZATION AND IDENTIFICATION OF NITROGEN FIXING CYANOBACTERIA IN EGYPTIAN SANDY AND ALLUVIAL (CLAY LOAM) SOILS

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ABSTRACT: *Samples of two different soils were collected from the surface layer (0 – 30 cm) of El- Ismailia Research Station (sandy soil) (El- Ismailia Governorate) (30°35' 41.901'' N, 32°16'45.834'' E) and Sids Res. Farm (alluvial clay loam soil) (Beni Swef Governorate) (29° 4' 54.349'' N, 31° 5' 25.775'' E), Agric. Res. Center, Giza Egypt, to isolate the prevailing cyanobacterial inhabitants. Characterization of the cyanobacterial biomass production and nitrogen fixing efficiency (nitrogenase activity) along 5 weeks incubation periods were also tested. Results revealed that, the six isolated cyanobacterial strains identified as Nostoc muscorum, Nostoc commune, Nostoc linckia, Nostoc ellipsosporum, Anabaena variabilis and Anabaena orientalis, and found to be commonly filamentous heterocystous nitrogen fixing agents. Nostoc muscorum recorded the highest biomass, followed by Anabaena variabilis, Anabaena orientalis, Nostoc linckia, Nostoc ellipsosporum and Nostoc commune. While, for nitrogenase activity, N. muscorum and A. variabilis gave the highest values against the others namely N. commune and N. linckia. Some of those cyanobacterial strains, i.e., N. muscorum and A. variabilis can be a promise to support and improve the fertility of soil, due to their high efficiency to fix the atmospheric nitrogen when inoculating the cropped soils.*

Key words: *Diazotrophy, autotrophic N2- fixers, nitrogenase activity, arid soils, biofertilizers.*

INTRODUCTION

Cyanobacteria formerly called blue-green algae (BGA) are the pioneer oxygenic, gram negative photosynthetic prokaryotes microorganisms distributed in almost all conceivable habitats. Some of which are capable of nitrogen fixation. Their main photosynthetic pigments are chlorophyll a, carotenes and xanthophylls together with phycobiliproteins, C-phycocyanin (blue) and C-phycoerythrin (red). Due to the presence of these latter pigments and mucilage, the color of cyanobacteria in nature may range from dirty yellow, through various shades of blue-green, to brown or black. Their range in vegetative form extends from simple unicells to multiseriate, true branching thalli (Ghadai *et al*., 2010). Cyanobacteria appeared to be a rich source for many useful products and are known to produce a number of bioactive compounds (Carmichael, 2001). During the last few decades, cyanobacteria have been described as potentially important source for vitamins, fuels, fine chemicals and many other pharmaceutical products (Chacon-de-Popioici, 1994, DeVries *et al*., 1993 and Miura *et al.,* 1993). Some of the species can fix dinitrogen and hence play a significant role in agriculture (Vaishampayan *et al*., 2001).

The rice paddy field ecosystem provides an environment favorable for the growth of cyanobacteria with respect to their requirements for light, water, high temperature and nutrient availability. This may account for the higher abundance of cyanobacteria in paddy soils than in other cultivated soils (Ahmed *et al*., 2010). Khadr (1975) studied the community of cyanobacteria in different rice soils in Governorates, i.e., Kafr EL-Sheikh, EL-Dakahlia and EL-Behaira and found that the genera of *Anabaena*, *Nostoc*, *Tolypothrix*, *Calothrix* and *Cylindrospermum* are prevailing. Ghazal (1987) and Adly (2011)

demonstrated that the genera of *Anabaena* and *Nostoc* are the most abundant and commonly inhabit the paddy rice soils. In the same concept, Hamed (2007) isolated an array of 15 filamentous nitrogen fixing cyanobacterial isolates from the Egyptian rice soils and found that they belong to the genera of *Anabaena*, *Nostoc*, *Tolypothrix*, *Calothrix* and *Oscillatoria.*

The aim of this work was to isolate, characterize and identify naturally occurring cyanobacteria from sandy and alluvial Egyptian soils. Their biomass production and efficiency for fixing atmospheric nitrogen (nitrogenase activity) were also evaluated.

MATERIALS AND METHODS

Soil samples

Samples of two different soils were collected from the surface layer (0 - 30 cm) of El- Ismailia Research Station "sandy soil" (El- Ismailia Governorate, 30°35' 41.901'' N, 32°16'45.834'' E) and Sids Res. Farm "alluvial clay loam soil" (Beni Swef Governorate 29° 4' 54.349'' N, 31° 5' 25.775'' E), Agric. Res. Center, Giza, Egypt. Physical and chemical analytical data are presented in (Table 1) (Jackson, 1976). The soil samples were used for isolation of some cyanobacteria agents. The isolated cyanobacteria were purified, characterized and identified.

Table (1): Physical and chemical characteristics of the experimental soils used.

Properties			Sandy soil	Alluvial soil
Particle size distribution, %		Sand	91.4	25.5
		Silt	4.9	38.8
		Clay	3.7	25.5
Textural grade			Sandy	Clay loam
pH 1:2.5 (Soil: Water Susp.)			7.80	7.5
EC (dSm^{-1})			0.90	2.5
Organic matter (%)			0.27	1.07
Soluble ions (meq l^{-1})	Cations	Ca^{++}	2.00	6.30
		Mg^{++}	1.43	4.56
		Na^{+}	4.74	14.44
		K^{+}	0.83	0.56
	Anions	CO_3^{--}	--------	--------
		HCO_3^{-}	2.06	2.83
		Cl^{-}	1.83	4.09
		SO_4^{--}	5.11	18.08
Total N, %			0.9	1.04
Total P, %			0.04	0.17
Total K %			0.11	0.36
Soluble N, mg kg^{-1}			31.05	51.75
Soluble P, mg kg^{-1}			5.68	22.00
Soluble K, mg kg^{-1}			73.00	181.00

Plating Technique for Cyanobacteria Isolation

Nitrogen free culture "$BG11_0$" medium (Rippika *et al*., 1979) was used for isolation of N_2-fixing cyanobacteria. "$BG11_0$" medium is composed of (g l^{-1}): 0.04 K_2 $HPO_4.3H_2O$; 0.075 $MgSO_4.7H_2O$; 0.036 $CaCl_2.2H_2O$; 0.02 Na_2CO_3; 0.006 citric acid; 0.006 ferric ammonium citrate; 0.001 EDTA Na_2 (disodium – magnesium salt). One litre of "$BG11_0$" was mixed with 1 ml of trace metal mix., which was composed of (g l^{-1}): 2.86 H_3BO_3; 1.81$MnCl_2.4H_2O$; 0.222 $ZnSO_4.7H_2O$; 0.39 $NaMoO_4.2H_2O$; 0.079 $CuSO_4.5H_2O$; 0.0494 $CoCl_2.6H_2O$. The medium was autoclaved with 1% Bacto agar at 15 lbs/m^2 for 15 minutes, cooled down to 45^{o}C, and 30 ml volume each was poured into a sterile Petri dish. The culture plates were left to dry for 2 days before use. The soil samples were diluted with distilled water to a first 1/10 ratio. This first dilution was stirred for 30 minutes using a magnetic stirrer. Ten-fold dilutions were then furtherly made with distilled water. One ml aliquots each of the 10^{-2} to 10^{-6} dilutions were poured onto the surface of the agar medium and spread with an alcohol sterilized triangular glass rod whenever the plates were being rotated on a rotator. Triplicate plates were used for each soil dilution. The plates remained upright for 2-day to allow the agar to absorb the water before being incubated under continuous illumination in an inverted position at a room temperature for 3 weeks. Incident light of 500 Lux was provided by 20 watt cool-white fluorescent bulbs. Counting and isolation of the cyanobacteria were performed using a dissociating microscope and the cyanobacteria forming colonies were picked out using inoculation needle, for propagation, purification and identification.

Purification Techniques

The collected cyanobacterial isolates were exposed for purification using different techniques, i.e., phototactic technique (Stanier *et al.*, 1971), mercuric chloride technique (Gupta *et al*., l959), heat technique (Wierenga, 1968), antibiotic treatments technique (Felfoldy and Zsuzsa, 1959) and ultraviolet irradiation technique (Taha, 1963).

Identification of the Cyanobacteria isolates.

The developed colonies were exposed for identification through the examination of the cultural appearance on solid and liquid media as well as characteristics of trichomes, sheath vegetative cells and heterocyst produced by each isolate (Vennkataraman, 1981 and Roger and Ardales, 1991). The length and the width of the vegetative cells also the width of the sheath, type of spores, presence or absence of hormogonia, presence or absence of spores and its position, number of heterocyst and its repetition, presence of akinte and its type, the nature of cell wall, presence or absence gas vacuoles, as well as pigment color was taken in consideration according to Desikachary (1959).

Generally, diameters and lengths of 100 vegetative cells, 100 heterocysts and 100 akinetes were measured. Also, the type and the presence of branching were examined.

Growth Biomass and Nitrogen Fixing Efficiency of the Isolated Cyanobacterial Strains

The cyanobacterial strains were evaluated for their growth and nitrogen fixing ability through the determination of their growth biomass and nitrogenase activity. Conical flasks, each containing 100 ml of sterilized "$BG11_0$" medium was initially inoculated with 10 ml of an individual cyanobacteria strain (10^9 cfu ml^{-1}) and incubated under continuous white light exposure (3000 Lux) at 28-32^{o}C for periods of 1, 2, 3, 4 and 5 weeks to determine both the cyanobacterial growth biomass and nitrogenase activity (Hardy *et al*., 1973).

RESULTS AND DISCUSSION

The six cyanobacterial isolates collected from either the sandy or alluvial (clay loam) were identified according the description of Desikachary (1959). All the cyanobacterial isolates were belonged to the order Nostocales and family Nostocaceae and the

gen*era Anabaena and Nostoc.* The description of those six cyanobacterial isolates includes six filamentous heterocystous species. Their characteristic features are given below.

a) *Nostoc muscorum* (plate 1)

It was characterized by:

Gelatinous-membranous thullus, attached by the lower surface, tuberculate, dull olive or brown, 2-5cm diam.; filaments densely entangled; sheath distinct only at periphery of the thallus, yellowish brown, trichome 3-4 µ broad; cells short barrel-shaped to cylindrical, up to twice as long as broad; heterocysts nearly spherical, 6-7 µ broad; spores oblong, many in series, 4-8 µ broad, 8-12 µ long, epispore smooth and yellowish. The isolate was identified as *Nostoc muscorum* Ag. ex Born et Flah.

b) *Nostoc commune* (Plate 2)

This species was described as firm thullus, gelatinous, at first globose, later flattened, expanding, undulated, membranous or leathery, sometimes irregularly torn, often perforated, many centimeters diam., blue-green, olivaccous or brown; filaments flexuous, entangled; sheath mostly distinct only at the periphery, thick, yellowish brown, often lamellated inside the thullus more or less distinct, but hyaline; trichome 4.5- 6 µ broad, cells short barrel-shaped or nearly spherical, mostly shorter or a little longer than broad, 5 µ long; heterocysts nearly spherical, about 7 µ broad; spore only once observed, as big as vegetative cells epispore smooth colorless. Such isolate was identified as *Nostoc commune* Vaucher ex Born et Falah.

Plate (1): *Nostoc muscorum* Ag Born et Flah.

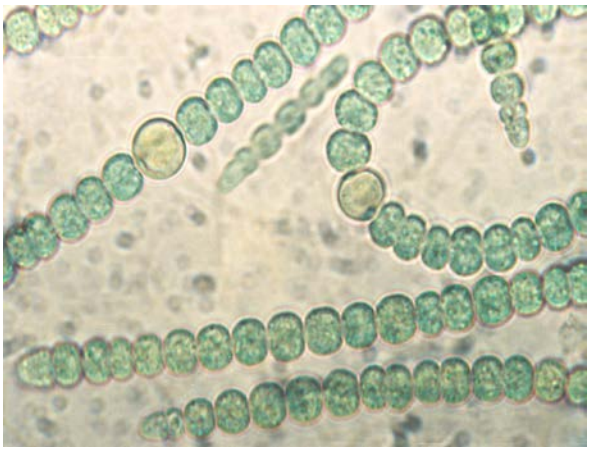

Plate (2): *Nostoc commune* Vaucher ex Born .et Flah.

a) *Nostoc linckia* (Plate 3)

This species was characterized by densely arranged trichomes coiled, thallus varying in size, sometimes tuberculate, at first glopose later irregularly expanding, torn, gelatinous, blue green to violet, or blackish green or brown; filaments densely entangled, flexuous or highly coiled; sheath diffluent and colorless inside, distinct only in the peripheral protein; heterocysts subspherical; spores subspherical, 6-7 µ broad, 7-8 µ long, epispore smooth. The isolate was identified as *Nostoc linckia* (Roth) Bornet ex Born et Flah.

b) Nostoc ellipsosporum (Plate 4)

Its thallus was gelatinous irrigulary expanded, attached by the lower surface, reddish browen; filaments flexuous, loosely entangled; trichome about 4 µ broad, light blue-green or olivceous; cells cylindrical, 6-14 µ long, heterocysts subspherical or oblong 6-7 µ broad, 6-14 µ long, spores ellipsoidal to oblong cylindrical, 6-8 µ broad, 14-19 µ long, epispore smooth, hyaline or browenish. The isolate was identified as *Nostoc ellipsosporum* (Desm) Rabenh. Ex Born. Et Flah.

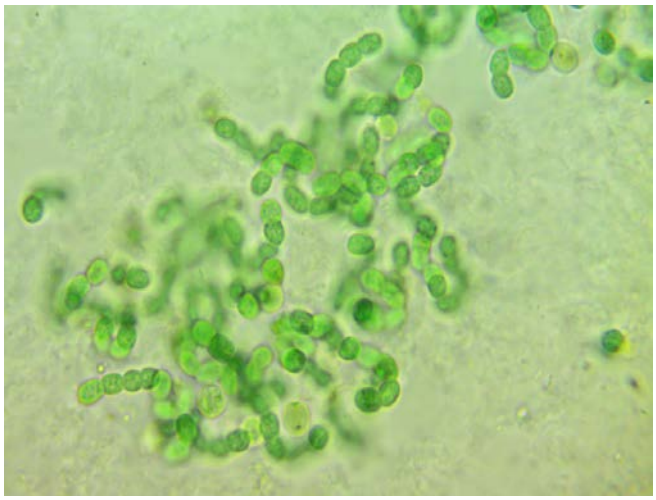

Plate (3): *Nostoc linckia* (Roth)Bornet ex Born. et Flah.

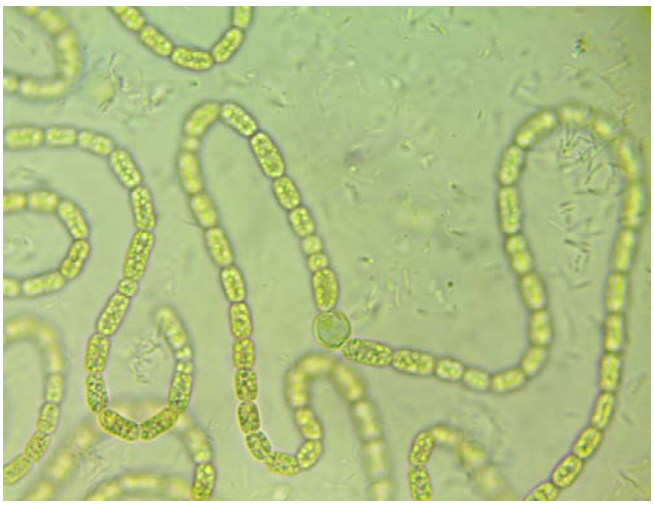

Plate (4): *Nostoc ellipsosporum* (Desm).Rabenh. ex Born. et Flah.

c) *Anabaena variabilis* (Plate 5)

Thallus was dense, soft, mucilaginous, deep green; trichomes 3.1 -4.2 µ broad, blue green, often irregularly curved and more or less entangled with each other, slightly constricted at joints, attenuated at the ends, the terminal cell being conical with a sharp or rounded apex, without mucilaginous sheath, cells cylindrical, up to twice as long as broad, rarely barrel-shaped, and almost as long as broad; heterocysts single intercalary, and distributed at regular intervals throughout the length of the trichome, cylindrical, 4.2-5.2 µ broad and 8.4 to 12.6 µ long; spores in short or long chains, ellipsoidal or barrel-shaped, remote from heterocyst, 4.2-6.3 µ broad and 6.3-10.5 µ long, with a thick, smooth and colorless outer wall. The strain was identified as *Anabaena variabilis* v. Kashiensis. The isolate was identified as *Anabaena variabilis*.

d) *Anabaena orientalis*

This species was described as the trichome is single, straight or slightly curved, 2.5-4 µ broad, cells quadrate or cylindrical, rarely slightly barrel-shaped, up to twice (or even thrice) as long as broad, 3.7- 4.8 µ long, end cell conical with rounded apex; heterocysts single, intercalary, cylindrical or slightly ellipsoidal with rounded end-walls, 4.8 - 5.5µ broad and 7.4-9.2µ long; spores one on each side of a heterocyst, ellipsoidal 7.4-9.2µ broad and 14.8-16.6 µ long. The isolate was identified as *Anabaena orientalis* Dixit.

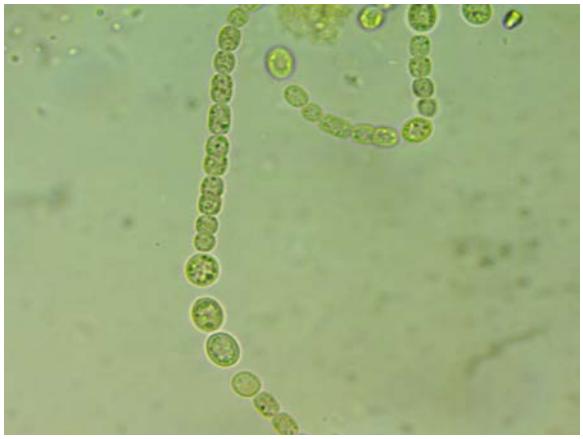

Plate (5): *Anabaena variabilis* v. Kashiensis.

Plate (6): *Anabaena orientalis* Dixit.

Generally, these results are in confirmation with those reported by Ghazal (1992) who examined some Egyptian rice soils for inhabiting cyanobacteria, and found that the dominancy was for the genera *Anabaena* and *Nostoc*. He also owed the abundance of cyanobacteria to that the rice soils can save the favorable condition, (water, light and temperature for the growth of cyanobacteria. Kim and Lee (2010) reported that a total of 142 isolates of heterocystous filamentous cyanobacteria were screened from 100 soil samples taken from rice paddy fields in 10 different locations across Korea, classified according to their morphological characteristics under light microscopy. The collected cyanobacteria were classified into a total of 14 genera, including seven genera of filamentous cyanobacteria and seven genera of nonfilamentous cyanobacteria. In particular, 142 heterocystous filamentous cyanobacteria were isolated and classified into six genera, including *Anabaena*, *Nostoc*, *Calothrix*, *Cylindrospermum*, *Nodularia*, *Scytomena*, and *Tolypothrix*. They added that yet, over 90% of the heterocystous filamentous cyanobacteria isolated from the rice paddy fields belonged to two genera: *Anabaena* and *Nostoc*. In the same line and in parallel to the results of the present study, Adly (2011) found that the genera *Anabaena* and *Nostoc* are commonly prevailed in the rice soils of EL-Dakahlia, EL-Sharkia and Damietta Governorates and explained that this was due to that the ecology of rice soils are very convenient habitat for the proliferation of cyanobacteria.

Growth Biomass and Nitrogen Fixing Efficiency of the Isolated Cyanobacteria Strains

Biomass Production:

The isolated cyanobacterial strains were left to grow under continuous illumination (3000 lux) and ambient temperature (28-32 ^{o}C) for different growth periods (1, 2, 3, 4 and 5 weeks) in the laboratory. Data in Table (2) reveal the patterns growth of such cyanobacterial strains. The highest biomass, i.e., 117 mg dry weight l^{-1} medium was recorded by *Nostoc muscorum* after 5 weeks and followed by 109, 105, 102, 99 and 89 mg dry weight 100 ml^{-1} medium for *Anabaena variabilis*, *Anabaena orientalis*, *Nostoc linckia*, *Nostoc ellipsosporum* and *Nostoc commune*, respectively, after the same incubation period. All tested cyanobacteria strains increased by elevating the incubation period. These results are in harmony with those of Adly (2011) who found that the biomass production of the cyanobacterial strains isolated from Egyptian rice fields, i. e., *Anabaena flos aquae*, *Nostoc muscorum*, *Nostoc maculiforme*, *Noctoc calcicola*, *Microchate tenra*, *Anabaena laxa*, *Nostoc humifusum* and *Wollea* sp. had progressively increased with increasing the incubation period from one to 4 weeks.

Table (2): Biomass growth (mg dry weight 100ml^{-1} medium) of the cyanobacterial strains cultured on "BG 11_0" medium at different incubation periods.

Cyanobacteria strains	Incubation period (weeks)				
	1	2	3	4	5
Nostoc muscorum	33	43	75	100	117
Nostoc commune	35	48	72	85	89
Nostoc linckia	21	37	69	87	102
Nostoc ellipsosporum	29	51	69	87	99
Anabaena variabils	30	44	82	97	109
Anabaena orientalis	22	45	62	93	105

*Initial inoculum was 10 ml filtrate cyanobacterial culture containing 10^{8}cfu hormogonia cyanobacterial cells ml^{-1}.

Nitrogenase Activity

Data listed in Table (3) indicate that, the measured nitrogenase activity of the isolated cyanobacterial strains proved their ability to fix atmospheric nitrogen. Increasing the incubation period from 1 to 5 weeks increased the activity of such enzyme to show the highest values,.i.e., 60.45 and 50.12 mmol C_2H_4 g dry algae^{-1} hr^{-1}, to be given by *N. muscorum,* and *A. variabilis,* respectively. Whereas the lowest values of such enzyme were recorded by *N. commune* followed by *N. linckia* having values of 35.18 and 38.14 mmol C_2H_4 g dry algae^{-1} hr^{-1}, in order.

In this regard, El-Gaml (2006) noted that the cyanobacterial strains of *Nostoc moculforum*, *Nostoc calcicola*, *Micrichate tenra*, *Anabaena laxa* and *Nostoc humifusum*, which were previously isolated from the Egyptian rice fields exhibited high nitrogenase activity. She added that those strains were applied to prepare a cyanobacterial inoculum to be used as N_2-fixing biofertilizer.

Some cyanobacteria are able to reduce atmospheric nitrogen to ammonia inside their cells. Diazotrophic cyanobacteria require sunlight as a sole energy source for fixation of both CO_2 and N_2. Thus, they have a great potential as biofertilizers, for environmental and economic benefits.

In this study, six local cyanobacterial strains, namely *Nostoc muscorum, Nostoc commune, Nostoc linckia, Nostoc ellipsosporum, Anabaena variabilis* and *Anabaena orientalis* were tested for their efficiency in both biomass production and fixation of the atmospheric nitrogen in order to be used as biofertilizers for assumed cereal crops cultivated in both poor sandy and alluvial clay loam soils. Thus, recommendations of El -Ayouty (1998), Hamed (2007), Adly (2011) Shatta *et al*. (2011), Ghazal *et al*. (2011) and Zein El-Abdeen (2013) are, confirmed by the present results.

On conclusion, examining the naturally occurring community of cyanobacteria in both the Egyptian sandy and alluvial (clay loam) soils proved the dominance of the filamentous heterocystous nitrogen fixing genera of "*Nostoc*" and "*Anabaena* "that are derived from the family Nostocaceae.

Table (3): Nitrogenase activity of the isolated cyanobacterial strains cultured on "BG 11$_0$" medium at different incubation periods (mmol C_2H_4 g dry algae^{-1} hr^{-1}).

Cyanobacterial strains	Incubation period (weeks)				
	1	2	3	4	5
Nostoc muscorum	9.45	18.9	29.65	31.12	60.45
Nostoc commune	6.64	14.96	20.81	23.16	35.18
Nostoc linckia	7.49	15.03	22.97	26.12	38.14
Nostoc ellipsosporum	8.16	16.21	25.63	30.12	45.12
Anabaena variabils	9.78	20.94	32.55	36.15	50.12
Anabaena orientalis	6.69	16.78	28.00	22.00	45.00

* Initial inoculum was 10 ml filtrate cyanobacterial culture containing 10^8cfu hormogonia cyanobacterial cells ml^{-1}.

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وصف وتعريف السيانوبكتيريا المثبتة للنيتروجين الجوى فى ألأراضى الرملية والرسوبية (الطينية طمييية) المصرية

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[٣] قسم علوم الأراضى – كلية الزراعة – جامعة المنوفية

الملخص العربى

تم جمع عينتين من التربة احداهما رملية (من محطة البحوث الزراعية – مركز البحوث الزراعية – محافظة الاسماعيلية) والأخرى رسوبية (طينية طمييية) (من محطة بحوث سدس – مركز البحوث الزراعية – محافظة بنى سويف) وذلك بغرض وصف وتعريف تجمعات السيانوبكتريا الشائعة بهذه الأراضى. حيث تم عزل السيانوبكتريا من التربة وتنقيتها ثم وصفها و تعريفها. هذا بالإضافة الى دراسة كفاءتها فى النمو و فى تثبيت النيتروجين الجوى عن طريق تقدير نشاط انزيم النيتروجينيز بعد تحضين السلالات المتحصل عليها لمدة خمسة أسابيع تحت الاضاءة المستمرة.

وكانت أهم النتائج المتحصل عليها هى مايلى:

١-أمكن عزل ستة سلالات من السيانوبكتيريا من نوعى التربة وهى:

Nostoc muscorum, *Nostoc commune*, *Nostoc linckia, Nostoc ellipsosporum*, *Anabaena variabilis* and *Anabaena orientalis*.

٢-تبين أن هذه السلالات خيطية ومحتوية على خلايا الهيتيروسيست ومثبتة لنيتروجين الهواء الجوى وأيضا تابعة لجنسى النوستوك والآنابينا والتابعين لعائلة Nostocaceae.

٣-أوضحت النتائج أن سلالة الـ *Nostoc muscorum* **أعطت أعلى كتلة حيوية (نمو) ، تليها سلالات الـــ** *Anabaena variabilis*, *Anabaena orientalis*, *Nostoc linckia*, *Nostoc ellipsosporum* and *Nostoc commune* **،على التوالى.**

٤-أظهرت سلالتى الـــ *N. muscorum and A. variabilis* **أعلى نشاط لانزيم النيتروجينيز (كفاءة تثبيت النيتروجين) بينما أعطت سلالتى الـــ** *N. linckia* and *N. commune* **أقل نشاط لانزيم النيتروجينيز.**

٥-تعتبر سلالتى الـــ *N. muscorum and A. variabilis* **من سلالات السيانوبكتيريا الواعدة فى تحسين وزيادة خصوبة التربة لما لها من كفاءة عالية فى النمو و تثبيت النيتروجين اذا ماستخدمت فى التلقيح لمثل هذه الأراضى.**