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Effect of Nitrogen-Fixing Cyanobacteria on the Growth of Wheat Crop

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ABSTRACT

Isolation and purification of cyanobacteria from kafr El-Sheikh soil samples revealed that, two isolates (*Anabaena cylindrica* and *Nostoc clacicola*) were acquired as bacterial free cyanobacteria and nitrogen fixed. These cynobacteria isolates were produced auxin (Indole-3-acetic acid), gibberellins (Gibberellic acid) and cytokinin (Zeatin). Therefore, when inoculation with *Anabaena cylindrica*, *Nostoc clacicola* and their mixture were significantly increased the plant height, number of spikes/m^2, 1000-grain weight, grain yield, straw yield protein content% on wheat crop and total counts of cyanobacteria in soil.

Keywords: Cyanobacteria, Nitrogen fixers, Anabaena, Nostoc, Wheat crop.

INTRODUCTION

Cyanobacteria are an ancient diverse group of photosynthetic prokaryotes, which show morphological resemblances to Gram-negative bacteria but perform oxygenic photosynthesis like higher plants. Many of them also exhibit biological nitrogen fixation, and have been used as biofertilizers in agriculture, wherein they are known to contribute 20-25 kg N/ha/season and enhance soil fertility (Prasanna and Kaushik, 2006).

A variety of cyanobacterial strains colonize soil wherein heterocystous species are capable of fixing atmospheric nitrogen. However, several non-heterocystous cyanobacteria are able to fix atmospheric nitrogen under micro-aerophilic conditions. Some cyanobacteria are known to secrete growth promoters (auxin, gibberellins and cytokinin). The cyanobacterial isolates were purified, identified and evaluated for their nitrogen fixing efficiency, their production of growth regulators substances and their potential as a biofertilizer (Tantawy and Atef 2010). The present work aims to evaluate the potential of two cyanobacterial strains (*Nostoc clacicola* and *Anabaena cylindrica*) for developing association with wheat plant roots as well as their effect on wheat plant growth.

MATERIALS AND METHODS

Source of cyanobacterial isolates

The following methods were applied on air-dried soil samples collected from Kafr El- Sheikh using Z medium (Staub, 1961) and Modified Watanabe medium (El-Nawawy *et al.*, 1958) for isolation and culturing of cyanobacteria. Semi-solid medium as described by El- Ayouty and Ayyad (1972) were applied. For isolation the soil physical and chemical properties were determined in Soil and Water Department Al-azhar University according to Jackson (1973) and are presented in Table (1).

Seeds used

Wheat seeds variety sakha 94 were kindly provided by the Crops Res. Inst., Agric. Research Center, Giza, Egypt.

Purification of cyanobacteria

The unialgal cultures were purified according to Pringsheim (1949) any colored growth was selected, sub cultured and streaked several times in new agarized Watanabe medium plate. To get unialgal cultures, the previous technique was repeated many times.

Bacteria free cyanobacterial cultures

To get bacteria free cultures. Each culture was serially purified byWashing (Hoshaw and Rosowski 1973) and Ultra violet irradiation (Taha 1963)

Table 1. Physical and chemical analysis of soil samples

Characteristics	Clay loamy soil
Sand (%)	21.30
Silt (%)	33.00
Clay (%)	45.70
Chemical analysis meq-L	
$CaCo_3$	1.12
Ca++	1.90
Mg++	1.37
Na+	2.05
K+	0.65
$CO^{=}_3$	0.00
HCO^{-}_3	1.45
Cl^{-}	1.92
$SO^{=}_4$	2.60
E.C (dS m^{-1}, at 25oC)	0.59
pH	7.73
Organic matter	686.1 ppm
Total phosphorus	8.82 ppm
Total nitrogen	10 ppm

Identification of the isolated cyanobacteria.

For identification of the purified isolated cyanobacteria, 500 ml Erlenmeyer flasks each containing 250 ml of Modified Watanabe liquid medium and plates of agarized Modified Watanabe medium were inoculated with a loop full of 10 days old culture of each cyanobacterial isolates. For 10 days, Inoculated plates and flasks were incubated at 28-30ºC under continuous illumination (2500 lux). The identification of cyanobacteria was done as follows: Thallus morphology and dimension, thallus color, vegetative and reproductive cells, size of heterocyst. Heterocyst-forming cyanobacteria were also cultured in nitrogen-free Z-medium according to Venkataraman (1981)

Plating technique of cyanobacteria enumeration

N-free culture medium Watanabe medium Allen and Stanier (1968) was used for culturing N_2-fixing cyanobacteria plates

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Determination of phytohormones:

Separation and determination of phytohormones (auxin, geberillin and cytokinin) were carried out by gas liquid chromatography in Al-Azhar university (the regional center for mycology and biotechnology). HPLC analysis was performed on GBC- germey by winChrome Chromatography Ver. 1.3 which equipped a GBC U.V/vis Detector and Hypercarb (C18,Sum 100x4.6 cm) the detective wavelength was 254nm flow rate of mobilephse was 7 ml/min which 85% Acent: 15% water. method according to Van Staden *et al.*, (1973)

Total nitrogen:

Total nitrogen in the cyanobacteria were measured using the micro-kjeldahl method according to Jackson (1973). Results were expressed as mg nitrogen/100 ml culture.

Field experiment:-

A field experiment was carried out in the Agricultural Farm at Sakha Research Station, Kafr EL-Shiekh, Egypt during 2018/2019 wheat growing season. The sowing date for the variety Sakha 94 was 1/11/2017 and harvest dot was 25/5/2018. The design of the experiment as following

1- Main plot cyanobacteria isolates(*Anabaena cylindrical*, *Nostoc clacicola* and mixture)
2- Sub plot nitrogen level (0,20,40 and 60) Nitrogen level /Fed.

Wheat seeds were wetted with mixture of biofertilizers with types of cyanobacteria strains and starch mixed carefully and spread on plastic sheet far from the direct sun effect for a short time before sowing and the soil was irrigated immediately after the sowing. This experiment was performed for one season.

Wheat plant parameters.

Plant height (cm), Number of spikelets/m^2,1000-Grain weight, Wheat yield (tons/fed.) and Protein (%)

Cyanobacterial count determination

The count of cyanobacteria in soil-based inoculants were determined using the colony forming unit/g soil (cfu dry soil).

Statistical analysis:

The collected data were analyzed according to the procedure outlined by Steel and Torrie (1980). Spilt spilt plot design with three replications was used. Differences among treatment means were compared using the Revised L.S.D. at 5% and 1% levels of significant adoption.

RESULTS AND DISCUSSION

Isolation and purification of cyanobacteria from kafr El-Sheikh soil samples revealed that two isolates were obtained as bacterial free cyanobacteria. They were identified according to Geitler (1932) as *Anabaena cylindrica* and *Nostoc clacicola*, as the most of cyanobacteria they were associated with other microorganisms, hence, these must be purified from any contaminants, they exposed to different trials of purification. Roger and Ardales (1991), in liquid and solid Modified Watanabe medium.

Results indicated that on solid medium the 21 day-old culture of the firsetisolate gave a localized growth with fibrous appearance on the agar surface. Growth was opaque and green in color without coloration of the medium. The 18 day-old culture, in liquid medium, showed green homogenous growth.

Microscopic examination revealed that trichomes were not-ramified, uniseriate, singly arranged wavy without tapering and were not enveloped within a sheath. Vegetative cells were short angular (4-4.3 x 2-3.9 µm). Most apical cells were pointed and heterocysts were of single occurrence and mostly produced intercalary. They were of barrel-shape (5-5.8 x 6.3-7.2µm). No spores were observed. Hormogonia have the same width as the filaments. Therefore, these cyanobacteria isolate is belonging to Anabaena cylindrical plate No.1.

As for second isolate the 20 day-old culture of this isolate on solid medium showed colonial growth. Colonies were 1-1.3 mm in diameter and had a low convex at its side with rough margin. The colonies were opaque and dark green coloured. A16 day-old culture, in liquid medium, had an aggregative sedimentary type of growth and an in cohesive patchy growth at the bottom of the flask. The culture showed green medium color and the patchy growth was medium yellowish green. Microscopic examination revealed that trichomes had no ramifications. They were uniseriate, single, aggregated, and showed neither polarity nor tapering. No sheath was formed. Trichomes composed of three sizes and shapes of cells; a barrel cells (5-5.8 x 5.5-6.8µm);b) granular, ellipsoidal cells (4.9 x 5.7-7µm); c) yellowish- brown rounded cells of 8.6µm in diameter. Few heterocysts were observed. They were of single occurrence with 2 position, intercalary and terminal, (Plates 2). And namely Nostoc clacicola.

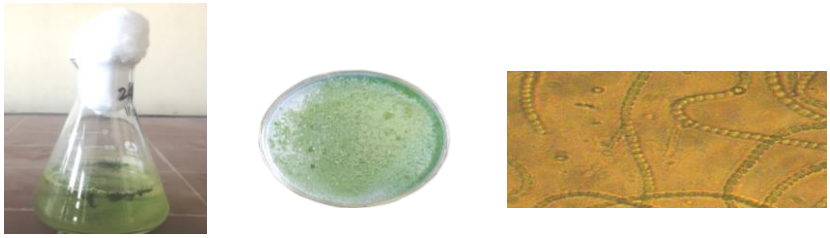

Plate No 1. ***Anabaena cylindrica***

Plate No 2. ***Nostoc clacicola***

Results in (Table 2), showed a gradual increase in fixed-nitrogen using both cyanobacterial isolate. The highest value of fixed-nitrogen(Intracellular, Extracellular and Total) (8.53, 1.67, 10.30 and 11.76, 2.83,14.59 mg N/100 ml- cu)

was recorded with the both isolates at the end of the experimental period (after 35 days) *Anabaena cylindrica* and *Nostoc clacicola* respectively.

The efficiency of cyanobacterial isolates to fix the atmospheric nitrogen is one of the most important parameters used for selection of cyanobacterial isolates for preparing cyanobacteria inoculants

Table 2. Amounts of fixed-nitrogen by cyanobacterial isolates (mg N/100 ml-culture)

	Anabaena cylindrica			*Nostoc clacicola*		
Cyanobacterial isolates / **Culture age days**	**Intracellular**	**Extracellular**	**Total**	**Intracellular**	**Extracellular**	**Total**
7	2.75	0.42	3.17	4.76	0.75	5.21
14	4.16	0.68	4.84	6.25	1.16	7.41
21	6.13	1.06	7.19	8.69	1.76	10.45
28	7.55	1.43	8.98	10.51	2.37	12.88
35	8.53	1.67	10.30	11.76	2.83	14.59

Phytohormoes production by cyanobacterial isolates

Data in Table (3) showed that, isolates *Nostoc calcicola* and *Anabaena cylindrica* were produced auxin (Indole 3 acetic acid (IAA)) (7.15 and 6.55 ug/100 ml), gibberellins (Gibberellic acid (GA_3)) (9.92 and 6.12 ug/100 ml), and cytokinin (Zeatin)(4.00 and 3.88 ug/100 ml) respectively. Tantawy and Atef (2010) revealed that, cyanabacterial filtrates in suspensions significantly increased the IAA, GA_3 and cytokinin.

Table 3. Composition and analysis of phytohormones of *Anabaena cylindrica* and *Nostoc clacicola* in filtrates (µg/100 ml).

	Anabaena cylindrica	*Nostoc calcicola*
Indole 3 acetic acid (IAA)	6.55	7.15
Gibberellic acid (GA_3)	6.12	9.92
cytokinin (Zeatin)	3.88	4.00

Growth parameters of wheat

Plant height (cm)

Data in Table (4) indicate the effect of inoculation under different nitrogen levels on wheat plant height (cm).

Results revealed that inoculation of wheat with both cyanobacteria strains, i.e., *Anabaena cylindrica*, *Nostoc calcicola* and their mixture recorded wheat plant height (cm) ranged from 88.25 to 110.88 cm in the effect of zero and 60 kg N/fed., respectively. However, in both treatments with the mixture of cyanobacteria inoculation combined with 60 kg N/fed. gave the highly significant different of wheat plant height compared to the control.

Table 4. Effect of cyanobacteria inoculation on wheat plant height(cm) under different nitrogen levels.

Nitrogen level /Fed.	**Without inoculation (control)**	***Anabaena cylindrica***	***Nostoc clacicola***	**Mixture of cyanobacteria**
0	88.25	91.15	92.20	93.80
20	92.30	101.15	102.55	103.65
40	102.15	102.95	103.30	106.55
60	106.12	107.12	108.70	110.88
LSD =		0.05		0.01
Nitrogen		4.01		10.52
cyanobacteria		13.21619		18.68745

This result is similar to those reported by Ahmed *et al.* (2010), Nishar and Kaushik (2007) and El-Zemrany (2017) who noted that Plant growth and yield of pearl millet-wheat sequence in the soil increased in response to cyanobacterial biofertilizer due to the increase in water holding capacity, hydraulic conductivity and mean weight diameter at the end of both pearl millet and wheat crop.

Number of spikes/m^2

Results in Table (5) show the effect of cyanobacterial isolates and nitrogen leveles on number of spikes/m^2 in wheat which showed highly significant different values. Therefore, the highest value obtained from the mixture of cyanobacteria with 60 kg N/fed. (110.88) and the lowest value (88.25) produced from without inoculation of cyanobacteria in control of nitrogen. The best combinations of mixture of cyanobacteria and 60 kg N/fed. on this trait. This result is similar to those reported by Ahmed *et al*., (2010) and El-Zemrany (2017) who isolated several non-heterocystous cyanobacteria and tested for their capacity to produce the plant hormone indole-3- acetic acid (IAA), and the possible role of IAA in the association of cyanobacteria with seedling roots was evaluated. Strains producing IAA were more efficient in the colonization of the roots than those lacked this ability. Also they conclude that nonheterocystous cyanobacteria also have the potential for use in agriculture to improve the growth and yield of crop plants that do not naturally form associations with cyanobacteria.

Table 5. Effect of cyanobacteria inoculation on number of spikes/m^2 in wheat under different nitrogen levels.

Nitrogen level /Fed.	**Without inoculation (control)**	***Anabaena cylindrica***	***Nostoc clacicola***	**Mixture of cyanobacteria**
0	285.83	304.22	315.56	337.54
20	305.18	314.33	329.22	353.58
40	340.52	349.51	371.85	397.77
60	376.05	379.72	390.98	415.97
LSD =			0.05	0.01
Nitrogen			22.29	33.22
cyanobacteria			58.12	82.19

Weight of 1000-grain

Results in Table (6) indicate the effect of cyanobacteria strains and nitrogen leveles on 1000-grain weight in wheat .

Results reveled that inoculation with two locally species and their of *Anabaena cylindrica*, *Nostoc calcicola* and mixture cyanobacteria were affected significantly on 1000-grain weight therefore, the range of 1000-grain weight in wheat was from 10.95 to 35.80 under the effects of both various cyanobacteria as well as the nitrogen levels. The best combinations of treatments with mixture of cyanobacteria and 40 kg N/fed. on this trait. This result is similar to those repotted by Ahmed *et al*., (2010) and Nishar and Kaushik (2007) El-Zemrany (2017) who reported the effects of a commercial inoculant of cyanobacteria on wheat cv. Sakha 69.

Table 6. Effect of cyanobacteria inoculation on 1000-grain weight under different nitrogen levels in wheat.

Nitrogen level /Fed.	**Without inoculation (control)**	***Anabaena cylindrica***	***Nostoc clacicola***	**Mixture of cyanobacteria**
0	10.95	30.46	30.4	33.00
20	27.50	30.29	31.31	35.75
40	30.15	31.62	31.40	35.80
60	29.35	30.58	29.33	35.25
LSD =			0.05	0.01
Nitrogen			3.63	5.42
cyanobacteria			0.48	0.69

Grain yield (ton/fed.)

Resultes in Table (7) illustrated the effect of cyanobacteria taypes and nitrogen leveles on grain yield (ton/fed.) in wheat .

The data in Table (7) indicated that inoculation with various two locally species of *Anabaena cylindrica*, *Nostoc calcicola* and their mixture cyanobacteria concerning the range of grain yield in wheat ranged from 1.48 to 3.21 (ton/fed.) under the effects of two various of cyanobacteria as well as the nitrogen levels. The best combinations of treatments was cyanobacteria mixture with 60 kg N/fed.

This result is similar to those repotted by Karthikeyan (2006) and El-Zemrany (2017) who reported the effects of inoculant cyanobacterial strains on wheat involving (single or in combination) showed visible differences in terms of the appearance of plants. This was accompanied by enhancement in plant height, dry weight and grain yield of wheat crop. Therefore, plant growth stimulation, in terms of plant height, dry weight and grain yield in pot culture experiment can be attributed to IAA-like compounds and photo-heterotrophic/heterotrophic abilities of the cyanobacterial strains, as observed in our earlier studies.

Table 7. Effect of cyanobacteria inoculation on the grain yield (ton/fed.) under different nitrogen levels in wheat.

Nitrogen level /Fed.	Without inoculation (control)	*Anabaena cylindrica*	*Nostoc clacicola*	*Mixture of cyanobacteria*
0	1.48	1.612	1.66	1.86
20	1.71	2.42	2.42	2.43
40	1.99	2.76	2.77	2.64
60	2.16	2.90	2.90	3.21
LSD =			0.05	0.01
Nitrogen			1.03	1.53
cyanobacteria			1.19	1.68

Straw yield (ton/fed.)

Data in Table (8) revealed that the highest straw yield (5.76 ton/fed.) was due to the mixture of cyanobacteria with 60kg N/fed. While, the lowest straw (3.69 ton/fed.) produced from non-inoculated soils and without nitrogen.

This result is similar to those repotted by Obreht *et al.* (1993) and El-Zemrany (2017)

Table 8. Effect of cyanobacteria inoculation on the straw yield (ton/fed.) under different nitrogen levels in wheat.

Nitrogen level /Fed.	Without inoculation (control)	*Anabaena cylindrica*	*Nostoc clacicola*	Mixture of cyanobacteria
0	3.69	3.74	3.96	4.57
20	4.31	4.33	4.39	4.59
40	4.39	4.47	5.04	5.47
60	5.53	5.57	5.65	5.76
LSD =			0.05	0.01
Nitrogen			2.68	4.00
cyanobacteria			1.73	2.45

Biological yield (ton/fed.)

Results in Table (9) show the effect of cyanobacteria types and nitrogen levels on biological yield (ton/fed.) in wheat.

Results revealed that both tested inoculation with various two locally species of *Anabaena cylindrica*, *Nostoc calcicola* and their mixture gave a wheat biological yield ranged from 5.17 to 8.97 (ton/fed.) due to the effects of two various inoculated cyanobacteria as well as the nitrogen levels. The best combinations between two treatments were cyanobacteria mixture and 60 kg N/fed.

Table 9. Effect of cyanobacteria inoculation on the biological yield (ton/fed.) under different nitrogen levels in wheat.

Nitrogen level /Fed.	Without inoculation (control)	*Anabaena cylindrica*	*Nostoc clacicola*	Mixture of cyanobacteria
0	5.17	5.35	5.62	6.43
20	6.02	6.75	6.81	7.03
40	6.38	7.23	7.81	8.11
60	7.69	8.47	8.55	8.97
LSD =			0.05	0.01
Nitrogen			3.60	5.37
cyanobacteria			2.54	3.59

Protein content on wheat

Results in Table (10) show the effect of cyanobacteria types and nitrogen levels on wheat protein content.

Data revealed that, the effect of both different soil types, and inoculation with various locally cyanobacteria species of *Anabaena cylindrica*, *Nostoc calcicola* and mixture on protein content arranged from 8.76 to 11.45%. The highest protein content among the treatments was due to mixture of cyanobacteria along with 60 kg N/fed. Mfundo *et al*., (2010) stated that the ability of cyanobacteria to fix N_2 and produce exopolysacharides varies widely among different cyanobacteria strains. Rogers and Ardales (1991) showed that, total protein content and microbial numbers were significant in the inoculated soil.

Table 10. Effect of cyanobacteria inoculation on the protein content% under different nitrogen levels in wheat.

Nitrogen level /Fed.	Without inoculation (control)	*Anabaena cylindrica*	*Nostoc clacicola*	Mixture of cyanobacteria
0	8.76	9.44	9.90	10.25
20	9.12	9.51	10.01	10.40
40	9.47	9.97	10.68	11.31
60	9.71	10.14	10.74	11.45
LSD =			0.05	0.01
Nitrogen			0.85	1.26
cyanobacteria			0.88	1.24

Cynobacteria propagation:

Microbial count in soil was varied according to the inoculated cyanobacterial species (Table 11) .The count was the highest followed. Inoculation with mixture with 60 nitrogen level/fed. and 60 dayes attained the highest number for viable counts compared to those recorded by the other tested cyanobacterial species. However, the inoculation of by cyanobacterial species mixture gave higher microbial counts rather than any of them inoculated alone. On the other hand, viable count was increased with increasing the nitrogen dose. At the 60 nitrogen level/fed. and 60 dayes , the viable count was the highest. (Rogers and Ardales., 1991). The survival of the inoculated species for at least 300 days is in contrast to results from other studies using phototrophic inocula (Rao and Burns, 1990)

Table 11. Effect of nitrogen level and wheat plant on cyanobacterial species cfu (x10^4g dry $soil^{-1}$)

Nitrogen level /Fed.	Without inoculation (control))			*Anabaena cylindrica*			*Nostoc clacicola*			Mixture of cyanobacteria		
	Zero time	15	30	Zero time	15	30	Zero time	15	30	Zero time	15	30
0	0.001	0.38	0.40	3.8	4.1	52	3.9	4.7	0.6.2	4.5	59	64
20	0.001	0.39	0.41	3.8	4.2	058	3.9	4.8	0.6.4	4.5	62	7.1
40	0.001	0.44	0.47	3.8	5.9	18	3.9	12	21	4.5	15	76
60	0.001	0.45	0.48	3.8	4.7	62	3.9	14	22	4.5	28	110

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تاثيرالسيانوبكتيريا المثبتة للنيتروجين على نموحصول القمح

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في هذه الدراسة تم عزل وتنقية بعض أنواع من السينوبكتيريا السائدة في عينات التربة التي تم جمعها من محافظة كفر الشيخ وقد تبين من الدراسة أنه تم الحصول على عزلتين في صورة نقية وعرفت العزلتين على انهما Anabaena cylindrica وNostoc clacicola وبقياس كفائة العزلتين وجد ان السيانوبكتيريا Nostoc calcicola, Anabaena cylindrica تثبت النيتروجين وتنتج الاوكسين (اندول 3 حامض الخليك) والجبريلين وكذلك السيتوكينين (الزيتين). وقد أدى تلقيح Anabaena cylindrica و Nostoc clacicola في صورة منفردة او خليط على القمح إلى زيادة كبيرة في طول النبات وعدد الافرع / م 2 ووزن 1000 حبة ومحصول الحبوب وإنتاجية القش ومحتوى البروتين وكذلك زيادة فى عدد السيانوبكتيريا فى التربه وذلك على نبات القمح.