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Characterization and identification of some Cyanophyta isolated from sandy soil

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ABSTRACT

Three cyanobacterial filamentous, heterocystous isolates (A, B, and C) were isolated from sandy soil collected from the Ismailia Agricultural Research Station (ARC), Ismailia Governorate, Egypt. A polygenic approach was used to characterize these isolates, which included morphology, ultrastructure, and molecular analyses. The morphological analyses for the three strains agree with the molecular data (16S, ITS, and 23S rRNA sequences and phylogeny); the order of our isolates is: Nostocales and family: Nostocaceae, they identified as the following: isolate A: *Trichormus variabilis*; isolate C: *Trichormus* sp.) which are filamentous and terminal heterocysts; and isolate B: *Nostoc* sp.) colonies formed of filamentous heterocysts enclosed in a membrane and gelatinous polysaccharide sheath. Dry weight, pigment content, phytohormones, total nitrogen, total protein, exopolysaccharides, and phosphate dissolving were determined for three strains. The results showed that *Trichormus variabilis* has the highest chlorophyll a content, *Nostoc* sp. has the highest carotenoid content, and three strains have almost similar phycocyanin content, while three strains can secrete phytohormones and nitrogen in their medium and dissolve phosphate, with near values in *Trichormus variabilis* and *Trichormus* sp. and a slight difference in *Nostoc* sp. According to the findings of this study, cyanobacterial strains isolated from Ismailia Agricultural Research Station can be used as biofertilizers and nitrogen-fixing fertilizers because of their ability to secrete phytohormones and bioactive compounds and fix air nitrogen in free nitrogen medium.

Keywords: Cyanobacteria, Phytohormones, Phosphate dissolving, Exopolysaccharides, Nitrogen fixing.

INTRODUCTION

Cyanobacteria are microorganisms that make their own food through photosynthesis. They are the first level of the soil's food chain, or the primary producers. They are frequently seen floating in wetland conditions. By releasing soluble organic compounds and bioactive metabolites, mobilizing nutrients, enhancing soil texture and carbon sequestration, and stimulating plant growth, they increase soil fertility and crop productivity (Rana *et al*.,2012). Currently, cyanobacteria are an essential part of many aquatic food webs and play a significant role in the global carbon and nitrogen cycles. They can, however, have significant negative effects on the environment, society, and the economy when they reach bloom proportions (Huisman *et al*., 2018). Cyanobacteria, despite their bacterial nature and gram-negative affiliation, can perform oxygenic photosynthesis, just like higher eukaryotic organisms (Rojas *et al*., 2020). They are aquatic organisms that have adapted to survive on bare rocks, arid deserts, high salinity waters, hot springs, and frozen Antarctica. They are also symbiotic with fungi (lichens), invertebrates (corals, sponges, and tunicates), legumes, ferns, cycads, and liverworts (Burford *et al*., 2020). Throughout Earth's history, cyanobacteria have evolved through periods of dramatic oxygen increases, CO_2 declines, and climatic variations, and have diversified into a wide range of natural habitats. Cyanobacteria have a strong allure as a natural source of bioactive molecules with a wide range of biological activities such as antimicrobial, antiviral, anticancer, antioxidant and anti-inflammatory properties (Pradhan *et al*.,2022). Cyanobacteria are well known for their role in the production of novel bioactive molecules and in recent years, research has focused on the hydrolytic enzymes and fungicidal compounds they can produce (Gupta *et al*.,2013; Prasanna *et al*., 2013). Cyanobacterial inoculation improves rice, wheat, legumes, and vegetable growth and yield significantly. (Nain *et al*., 2010 and Prasanna *et al*., 2015).

Filamentous nitrogen-fixing cyanobacteria of the Nostocales are ubiquitous in fresh and brackish water around the world. Cyanobacteria from the genera *Dolichospermum*, *Anabaena*, and *Aphanizomenon* are especially common and widespread in this group (Cir'es and Ballot, 2016; Li *et al.,* 2016), which grow as trichomes or vegetative cell filaments with a low proportion of two types of differentiated cells: heterocysts, the site of nitrogen fixation and akinetes, that also serve as robust over-wintering cells (Li *et al*., 2016).Therefore, the goal of this study is to isolate, identify and characterize some cyanobacteria isolates, which isolated from sandy soil in order to use them as biofertilizers and nitrogen fixing agents.

MATERIALS AND METHODS

Isolation of cyanobacteria

Soil samples were collected from the Ismailia Agricultural Research Station (ARC), Ismailia Governorate, Egypt (30° 35′ 41.901′′ N and 32° 16′ 45.843′′ E) and analyzed chemically and physically according to Jackson (1973) and Rasmussen et al. (2018), Table 1. Laboratory soil samples were cultured directly in nitrogen-free BG11 media (Browitzka and Browitzka, 1988). The culture was grown at 28–30 °C under continuous illumination of white continuous light at 3000 lux. Antibiotics such as ampicillin (25–50 mg/day) or trimethoprim (5–30 mg/day) were used to eliminate bacterial contamination. Single colonies appeared on fresh medium after 15 to 21 days.

Table 1. Physical and chemical properties of experimental soil**.**

Physical analysis		**Chemical analysis**	
Coarse sand%	69.58	**pH (1:2.5 suspension)**	7.8
Fine sand%	23.51	**EC dSm^{-1} in the soil water extract (1:5)**	0.137
Silt%	3.01	**O.M%**	0.461
Clay%	3.90	**Available N (ppm)**	18.198
Soil texture	sandy	**Available p (ppm)**	2 .189
		Available K (ppm)	71.897

Morphological Characterization and Molecular identification of Cyanobacterial Isolates

Morphological analysis was performed on the cell shape of different filaments from late exponentially growing fresh cultures of each strain. In N-free liquid (agar) BG_{11} media, regular visual examinations were performed. Olympus IX71 microscope with a digital camera attached Shishido et al. (2013) recommended that genetics be identified. BLAST (http://blast.ncbi.nlm.nih.gov) was used to align nucleotide sequences (Zhang et al., 2000). The phylogenetic tree was created using the phylogeny for programs.

Investigation of the Productivity of Isolated Cyanobacterial Strains on N-Free Medium

The isolated cyanobacteria grow in BG_{11} medium, which is nitrogen-free. The cyanobacteria biomass was separated from the culture at 7, 14, and 21 days, dried and the dry biomass yield was measured.

Exopolysaccharides determination

Exopolysaccharide cyanobacterial strains were grown in BG_{11} medium on an orbital shaker at 120 rpm at 25°C for 20 days under cool white fluorescent tubes (45 mol photons m^2 s^{-1}) of 12 h light per day. Supernatants were collected for released exopolysaccharide analysis by centrifugation at 25,155g for 15 minutes. Cyanobacterial cells were heated in boiling water for one hour before being centrifuged at 25,155g for 15 minutes to remove cell remains. A phenol sulfuric acid assay was used to determine the released and capsular polysaccharides from the total carbohydrate content (Dubois *et al*., 1956).

Phosphatase Enzyme

Tabatabai and Bremner's (1969) approach was used to assess phosphatase activity.

Pigments Content Estimation

To determine the quantitative composition of pigments, cyanobacterial isolates were cultured in BG11 media for 14 days, as well as in experimental variants with added metals, and then the pigment content was determined. 4 mL of a 14-day cyanobacterial culture suspension was taken from each variant studied, centrifuged at 15,000 g for 7 minutes, and the supernatant was discarded. 4 mL of pre-chilled (+4°C) methanol was then added, shaking (2000 g, 4 s), and pipetting were used to homogenize the sample. The samples were then wrapped in aluminum foil and incubated at +4°C for 20 minutes to extract pigments from the cells. The samples were then centrifuged for 7 minutes at 15,000 g, +4°C and the precipitate was discarded. A spectrophotometer was used to measure the pigments content (chlorophyll a, carotenoid and phycocyanin) at wavelengths (O.D) of 470, 615, 650, 652, 665, and 720 nm, with methanol as a control (Ritchie, 2006, Wellburn, 1994 and Bennett and Bogorad, 1973).

Equations were used to calculate the pigment concentration (1–3):

1- Chlorophyll a (µg ml^{-1}) = 12.9447 (A665 - A720)
2- Carotenoid (µg ml^{-1}) = 1000 (A470 - A720) - 2.86
3- Phycocyanin (µg ml^{-1}) = (A615 - 0.474A652)/5.34

Screening of Phytohormones

IAA screening, in summary, revealed that 21-day-old cyanobacterial cultures (grown in BG11 medium supplemented with tryptophan at 0.5 g/l) were harvested by centrifugation (11,000 rpm). 1 ml of Salkowski reagent was added to the supernatant and incubated at room temperature for 30 minutes, according to Tsavkelova *et al.* (2007). The color developed was measured using a spectrophotometer at 540 nm.

Estimation of Total Nitrogen and Protein Content

Jackson (1973), micro-kjeldahl method was used to determine total nitrogen in cyanobacteria. The results were expressed in milligrams of nitrogen per 100 ml culture.

The protein content of culture filtrate was calculated as follows. Herbert *et al*. (1971) determined total soluble protein.

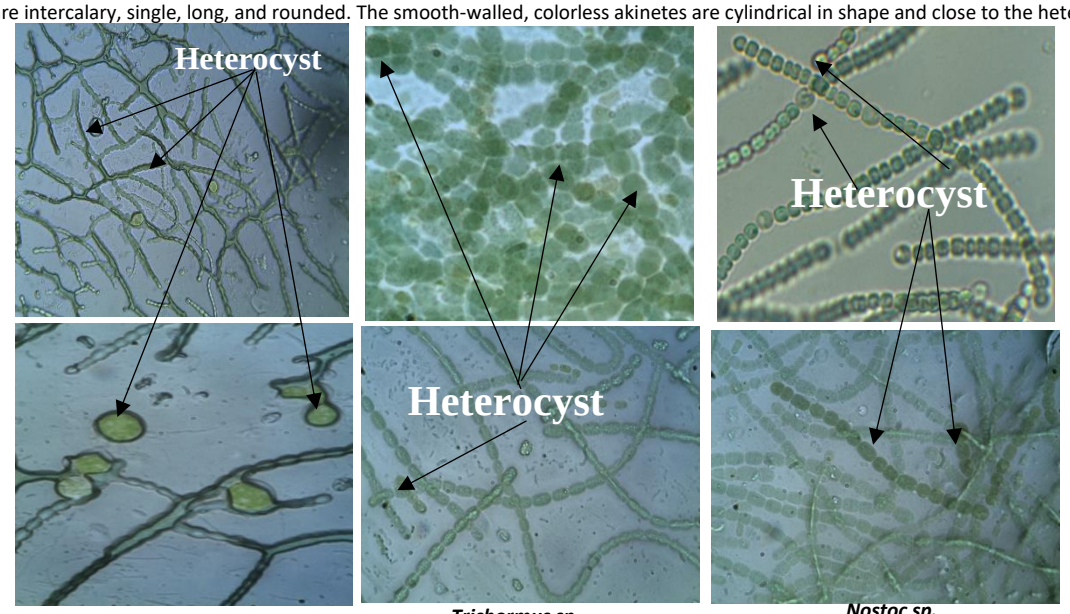
RESULTS

More than ten isolates were displayed on Petri dishes, cyanobacterial single colonies were collected from BG_{11} medium and transferred into free nitrogen liquid BG_{11} medium, to select the most biomass producing isolates. A, B, and C isolates were assigned to selected isolates. They were subjected to various tests to determine their suitability as biofertilizers and nitrogen fixers.

Morphological Characterization and molecular identification of cyanobacterial isolates

Figure 1 shows the morphological properties of the three strains. Three isolates are filamentous cyanobacteria, which are made up of blue-green spherical vegetative cells that are single, smooth, and not closed at the ends or constricted noticeably at the stigma sites. The heterocysts are typically light brown, solitary, intercalary, and intercalary. Akinetes are uncommon, barely discernibly oval, identical in size to surrounding cells, and have a granular consistency. Filaments Cyanobacteria are primarily composed of chains of barrel-shaped gram-negative vegetative cells encased in a thin gelatinous polysaccharide sheath. The mucilaginous sheath encasing the filaments is formed by polysaccharide secretions by the cells. Isolate A was identified as OP730954. *Trichormus variabilis* FMG, while isolate B was identified as OQ207016. *Trichormus* sp. FMG and isolate C were defined as OP730945. 1 *Nostoc* sp. FMG by using universal forward and reverse primers, the PCR amplicon was further

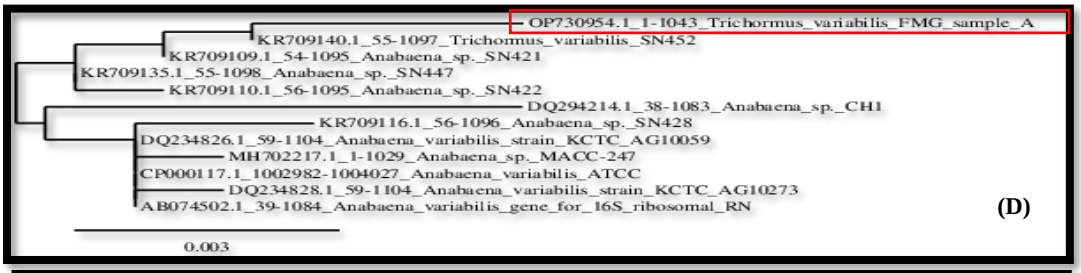
amplified. Using the online BLAST program, the consensus sequences were compared to a database of the NCBI GenBank that was easily accessible. Using phylogeny and the neighbor-joining method, a phylogenetic tree was created through comparison with the literature that was available (Figs. 1a and b). The vegetative cells of that kind of species are circular, lack aerotopes, and have spherical apical cells. The heterocysts are intercalary, single, long, and rounded. The smooth-walled, colorless akinetes are cylindrical in shape and close to the heterocyst.

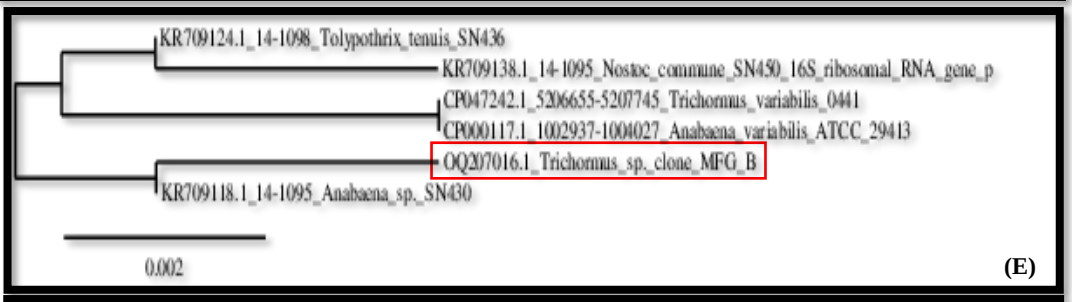


Trichormus variabilis **(isolate A)**

Trichormus sp. **(isolate B)**

Nostoc sp. **(isolate C)**





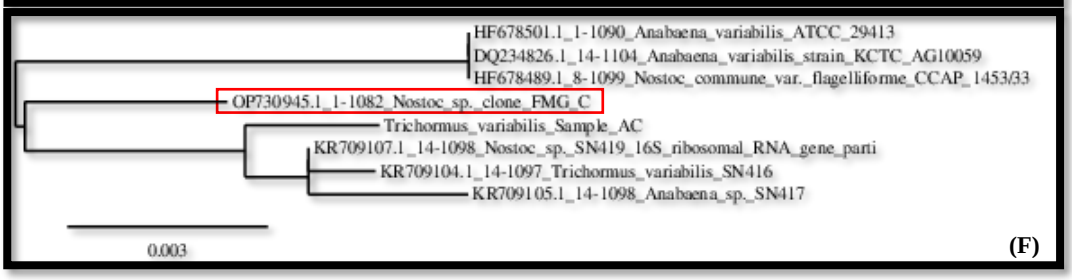


Fig. 1:(A , B and C) Microscopic appearance of cyanobacterial strains at 40 X magnification under and Nikon digital microscope (Eclipse80i), (D) phylogenetic tree of neighboring species of isolate A (*Trichormus variabilis*) , (E) phylogenetic tree of neighboring species of isolate B (*Trichormus* sp.), (F) phylogenetic tree of neighboring species of isolate C (*Nostoc* sp.), depicting the relationships among partial 16S rDNA sequences of isolates, all of the sequences and their most closely related matches from the NCBI BLAST.

Dry weight of biomass, total nitrogen, and total soluble protein content

Table (2) shows that the cyanobacteria biomass dry weight gradually increased as the experiment progressed in 100 ml culture, with the highest cyanobacteria dry weights recorded with all strains, which occurred from 7 to 21 days of incubation. After 21 days, *Trichormus variabilis* had the highest dry weight (16.121 g), followed by *Trichormus* sp. (13.8428 g), and Nostoc sp. (10.3517 g). The ability of cyanobacterial strains to fix nitrogen from the air was one of the criteria used in the selection of cyanobacterial isolates for preparing cyanobacterial inoculum. Data revealed that all strains differed in their ability to secrete extracellular nitrogen after 21 days of incubation. Nitrogen content increased in cyanobacterial fixed-nitrogen strains during the test, with high fixed-nitrogen values recorded for all tested cyanobacteria strains. *Trichormus variabilis* strain had the highest nitrogen content (4.10mg /100ml culture), followed by *Trichormus* sp. (3.55mg /100ml culture) and *Nostoc* sp. (3.12mg /100ml culture). The nitrogen-to-protein conversion factor was strongly influenced by total nitrogen, which was used to calculate protein content. As a result, the highest protein content was (25.625 mg of 100 ml culture), followed by the highest nitrogen content, which was reported by the *Trichormus variabilis* strain then (22.188 mg of 100 ml culture), and *Trichormus* sp. and *Nostoc* sp. (19.50 mg of 100 ml culture).

Table 2. Biomass dry weight and total nitrogen fixed by cyanobacterial strains (mg/100ml-culture)

Cyanobacterial strains	Dry Weight (g/100ml culture)			Total Nitrogen (mg/100ml culture)	Total Soluble Proteins (mg of 100 ml culture)
	7days	14 days	21 days		
Trichormus variabilis	3.264	9.831	16.121	4.10	25.625
***Trichormus* sp.**	2.4568	8.4302	13.8428	3.55	22.188
***Nostoc* sp.**	2.1673	7.6530	10.3517	3.12	19.50

Pigment and Phytohormone Contents

Data in table (3) demonstrated that all cyanobacterial strains could produce pigments and phytohormone (IAA). *Trichormus variabilis* strain had the highest chlorophyll a content (28.04 µg ml^{-1}), while *Nostoc* sp. had the highest carotenoid content (15.88 µg ml^{-1}) and all strains produced phycocyanin pigment in nearly equal proportions. Furthermore, all cyanobacterial strains can secrete indole acetic acid at slightly different rates; the highest value was 29.86 g ml^{-1}, which strain *Nostoc* sp. recorded, followed by *Trichormus variabilis* and *Trichormus* sp.

Table 3. Pigments and IAA content of cyanobacterial strains.

Cyanobacterial Strain	Pigment Content (µg ml^{-1})			IAA (µg ml^{-1})
	Chlorophyll a	Carotenoid	Phycocyanin	
Trichormus variabilis	28.04	12.51	8.00	25.60
***Trichormus* sp.**	25.200	14.65	7.80	27.04
***Nostoc* sp.**	23.86	15.88	7.70	29.86

Exopolysaccharides and Phosphate dissolving

Exopolysaccharides and phosphatase enzyme were secreted by cyanobacterial strains, as shown in Table (4) and Fig (2). The data followed the same pattern as the previous parameters, revealing that the *Trichormus variabilis* strain had higher activities than the other two strains. The largest amount of exopolysaccharides was (1.02 mg ml^{-1}) while phosphatase was (11.99µg inorganic phosphorus/ml culture /day), followed by *Trichormus* sp. exopolysaccharides was (0.895mg ml^{-1}) while phosphatase was (9.99µg inorganic phosphorus/ml culture /day) and *Nostoc* sp. exopolysaccharides was (0.665 mg l^{-1}) while phosphatase was (9.44µg inorganic phosphorus/ml culture /day).

Table 4. Exopolysaccharides and phosphatase activity of cyanobacterial strains.

Cyanobacterial Strains	EPS (mg ml^{-1})	phosphatase activity (µg inorganic phosphorus/ml culture/day)
Trichormus variabilis	1.02	11.99
***Trichormus* sp**	0.895	9.99
***Nostoc* sp.**	0.665	9.44

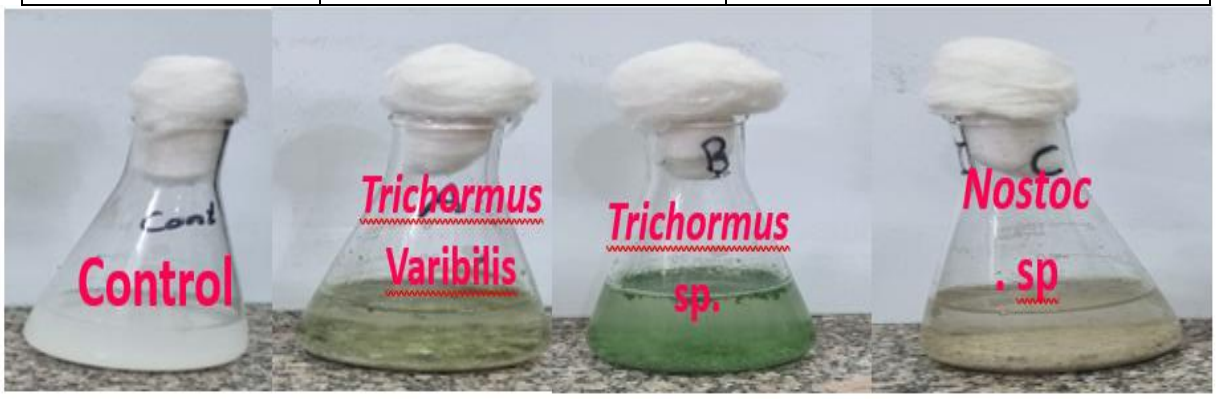


Fig. 2. Phosphatase activity of cyanobacterial strains grown in BG_{11} medium in the presence of tricalcium phosphate.

DISCUSSION

Cyanobacteria taxonomy has historically been based on morphological, physiological, and ecological properties, but more recently, biochemical, and molecular approaches have been used (Li *et al*., 2008; Zapomlová *et al*., 2010). This means that, apart from microscopic analysis, multiple disciplines' approaches to cyanobacteria characterization and categorization are now recognized (Hayes *et al*., 2007; Koma´rek, 2010). Many cyanobacteriologists worldwide are increasingly employing this so-called morphogenetic methodology (which incorporates traits, chemotaxonomic, and genomic data) (Schleifer, 2009). Advanced cyanobacterial classification has evolved because of molecular methods based on PCR amplification that target essential genes within the 16S rRNA gene, which allowed genomic links among cyanobacteria to be determined (Koma'rek, 2006). The analysis of phylogenetically similar species as well as the growth parameters of the isolates A, B, and C enabled us to identify their relation to the order Nostocales. The formation of heterocysts, as is well known, begins after up to 20 hours of nitrogen poverty. This is because nitrogen fixation genes (nif) are demonstrated between 18 and 24 hours after nitrogen deprivation (Walsby, 2007). An investigation of the isolated heterocystous cyanobacteria's ability to grow on a nitrogen-free medium revealed that the formation of heterocysts on a filament under these conditions indicated that all of the isolates examined could grow on a nitrogen-free medium.

It was discovered that the capacity of all strains for secreted biomass varied. These findings are consistent with those of El-Zawawy *et al.* (2021) and Afify *et al*. (2018), who discovered that cyanobacteria had the highest dry weight as incubation time increased. The ability of all strains studied to develop on a nitrogen-free medium indicates that there is nitrogen-fixing activity in isolated cyanobacteria cultures (Gladkikh *et al*., 2008). Furthermore, the quantity of heterocysts in a nitrogen-free medium increased. These strains were chosen for the preparation of cyanobacterial inoculants as biofertilizers due to their high biomass production and nitrogen fixation capacity, These findings were accepted by Sadvakasova *et al*. (2022).

Pigments are chemical compounds that absorb light in the visible wavelength range as well as cyanobacteria are photosynthetic organisms. The ability of all strains studied to develop on a nitrogen-free medium indicates that there is nitrogen-fixing activity in isolated cyanobacteria cultures. that can synthesis chlorophyll and variety of valuable compounds such as carotenoids and phycocyanin (which are used as antioxidants) as accessory pigments to absorb light energy. The phycocyanin, water-soluble pigments that transfer captured energy to chlorophyll a, are found in cyanobacteria but not in plants (Tiwari *et al*., 2021). The findings of this study agreed with those of Jaiswal *et al*. (2018), who found that cyanobacteria are the photosynthetic organisms capable of synthesizing pigments. Cyanobacterial species from the *Anabaena, Trichormus*, *Nostoc* and *Spirulina* genera can secrete pigments in their culture media. In addition to chlorophyll, filamentous cyanobacteria are thought to be desirable organisms for the production of carotenoids, phycocyanin and other significant chemicals that serve as accessory pigments in photosynthesis (Deepika *et al*., 2022). Auxins, gibberellins and cytokinins are just a few of the growth-promoting compounds that cyanobacteria have been found to synthesize and secrete (Kaushik, 2014). These bioactive substances play important roles in plant development, and metabolism (Hashtroudi *et al*., 2013). IAA and cytokinin are thought to be development compounds that increase grain weight, stem length, root growth, root hair number, and seed germination (Mazhar *et al*., 2013).

Cyanobacterial extracellular polysaccharide formed a biofilm layer. Moreover, the capability of these strains to secrete exopolysaccharide gives to their promising biofertilizer properties (Rossi *et al*.,2015). Direct application of *Nostoc muscorum*, crude polysaccharide to soil increased soil aggregate stability more than cyanobacterial biomass inoculation only. Cyanobacterial exopolysaccharide has been proposed to reduce heavy metal effect *via* biosorption processes (Chittapun *et al*., 2018). Furthermore, interest in the exploitation of valuable exopolysaccharides for various industrial applications has risen in recent years, as has interest in polysaccharide producing bacteria and cyanobacteria. High flocculation activity observed implies the possibility of industrial applications. However, more research on process conditions is needed until considering large-scale production (Khangembam *et al*., 2016). Microalgae and cyanobacteria are known to moderate exopolysaccharide and endopolysaccharide creation in response to environmental conditions such as salinity stress, high illuminance, and nitrogen shortage (Sadjeu Tchakouteu , 2015).

Thus, cyanobacteria play a role in phosphate decomposition in agriculture by converting undissolved phosphate compounds into simpler compounds that can be easily absorbed by crops (Kaushik, 2014 and Singh *et al*., 2014). Additionally, it has been demonstrated that certain herbicides and an organo-phosphorus insecticide are degraded by cyanobacteria from the genera *Nostoc*, *Phormidium*, and *Oscillatoria*, improving the soil's suitability for agriculture (Singh *et al*., 2014). Additionally, supplying organic matter, nitrogen and phosphorus to the soil causes the decomposition of cyanobacteria. Furthermore, *Anabaena* spp. have been shown to have effective biocontrol potential against phytopathogenic fungi (Chaudhary *et al*., 2013). Besides, by increasing soil bacterial populations, CO_2 evolution, enzyme dehydrogenase and nitrogenase activities, cyanobacteria improve soil quality and plant growth (Hegazi *et al*., 2010). N_2-fixing Cyanobacteria is one of the most promising microorganisms for use as a biofertilizer. They can fix atmospheric nitrogen, improve physical and chemical soil properties, and produce active compounds that promote plant growth (Ghazal *et al*.,2022). Cyanobacteria are currently being proposed as eco- and agrofriendly candidate organisms for restoring, maintaining, and improving soil and land stability, fertility, quality, and productivity in degraded ecosystems (Kaushik 2014; Singh *et al*., 2014, 2016).

CONCLUSION

Cyanobacteria are eco-friendly microorganisms that are photosynthetic and can fix atmospheric nitrogen as well as produce plant-enhancing bioactive compounds. This study's isolated cyanobacterial strains, *Trichomus variabilis* , *Thrichormus* sp. and *Nostoc* sp can be used as biofertilizers and nitrogen fixing agents.

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توصيف و تعريف بعض أنواع السيانوبكتريا المعزولة من التربة الرملية

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تم عزل ثلاث عزلات (A,B,C) من السيانوبكتريا (الطحالب الخضراء المزرقة) من منطقة محطة بحوث الاسماعيلية – مركز البحوث الزراعية – بالإسماعيلية – مصر. تم تعريف العزلات مورفولوجيا بواسطة الميكروسكوب الضوئي، ووراثيا بواسطة (16S-ITS-23S rRNA sequences and phylogeny) و قد توافق التعريف المورفولوجي مع التعريف الجيني ووجد أن الثلاث سلالات تنتمى إلى order: Nostocales and family: Nostocaceae ، و تم تعريف العزلات كالآتى (السلالة A: *Trichormus variabilis*) و (السلالة C:*Trichormus sp.*) وهى طحالب خيطية تحتوي على heterocyst طرفية بينما تم تعريف (السلالة.*Nostoc* sp) B : وتكون عبارة عن طحلب خيطى ولكن Heterocyst مغلفة بغشاء جيلاتينى داخل غلاف من السكريات العديدة . وقد تم تقدير كل من محتوى الوزن الجاف والصبغات والهرمونات النباتية والنيتروجين وقدرة السلالات على إذابة الفوسفات للثلاث سلالات. وأوضحت النتائج أن السلالة *Trichormus variabilis* كانت أكثر السلالات الثلاثة في الوزن الجاف و افراز صبغة الكلوروفيل أ ، بينما كانت السلالة .*Nostoc* sp أكثر السلالات إفراز لصبغة الكاروتين وكانت الثلاث سلالات متساوية تقريبا في إفراز صبغة الفيكوسيانين ، في حين أن السلالتين *Trichormus variabilis* و .*Trichormus* sp لها القدرة على افراز الهرمونات النباتية والنيتروجين و لها القدرة على إذابة الفوسفور بقيم متقاربة و باختلاف طفيف عن السلالة .*Nostoc* sp . ووفقا للنتائج المتحصل عليها من هذه الدراسة يمكن استخدام سلالات السيانوبكتريا المعزولة من محطة بحوث الاسماعيلية كمخصبات حيوية لقدرتها على افراز الهرمونات النباتية أيضاً كمثبتات نيتروجين لقدرتها على تثبيت النيتروجين الجوي في البيئات الخالية من النيتروجين.

الكلمات المفتاحية : سيانوبكتريا ، الهرمونات النباتية ، مذيبات الفوسفات ،السكريات العديدة الخارجية ، مثبتات النيتروجين