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Spatial distribution of nitrogen content and nitrogen fixing bacteria in soil of Damietta Governorate centers

El-Fadaly, H; El-Kadi, S. and Abd El-Twab, M.

Agricultural Biotechnology Department, Faculty of Agriculture, Damietta University, Damietta Egypt

ABSTRACT:

The present investigation deals with spatial distribution of nitrogen content and nitrogen fixing bacteria, such as *Azotobacter* sp., *Azospirillum* sp., *Clostridium* sp. and cyanobacteria from rhizosphere of *Triflium alexandrium* (Egyptian clover) from Damietta Governorate centers, Egypt. Twenty different sample were collected, five samples for each center (Damietta, Kafr Saad, Faraskour and El-Zarka). Nitrogen content was determined in examined comprised soil samples, N-fixing bacteria were counted and isolated in each center. All isolates were identified based on morphological and physiological characteristics and nitrogenase activity was also assayed. The highest value of nitrogen content was 448 mg/kg that found in El-Zarka center (S4). El-Zarka and Kafr Saad centers are considered the highest count with ratio of 35%, 32%, in case of *Azotobacter* sp. and cyanobacteria, respectively. El-Zarka center is considered the dominant center in terms of *Azospirillum* sp. (41%). While Damietta centers is considered the highest count with ratio 36% in case of *Clostridium* sp. The high N_2-ase activity value of *Azotobacter* spp. (M.A.M-5) was 130nmole C_2H_2/h/l while the high value of *Azospirillum* spp. (N.D.M-5) was 51nmole C_2H_2/h/l. The high N_2-ase activity value of *Clostridium* spp. (W.A.M-4) was 135 nmole C_2H_2/h/l. Based on this study the highest nitrogen fixing isolates of *Azotobacter* sp. , *Azospirillm* sp. and *Clostridium* sp. could be highly used in cultivation of soil in coastal environment such as Damietta Governorate soils as a bio fertilizer to reduce the chemical fertilizer and save the soil from chemical addition and keep the environmental clean.

Key words: Spatial distribution, *Azotobacter* sp., *Azospirillum* sp., *Clostridium* sp.

INTRODUCTION

Because of the increased population in the world, it had become necessary to increase the crop productivity by using different fertilizers, pesticides and insecticides. The soil has been badly affected because of enormous uses of these chemical fertilizers due to the depletion of the soil basic minerals **(Boraste *et al.*, 2009)**. Therefore, use of biofertilizers insteed of chemical fertilizers is getting significant today. Intensive that reach high yields require chemical fertilizers, which are not only costly but may also generate environmental problems. The intensive using of chemical fertilizers is currently subjected to discussion due to environmental concerns and consumer health concerns (**Rigby and Caceres, 2001 & Lee and Song, 2007**). Increasing and expanding the role use of biological fertilizers will reduce chemical fertilizers and reduce their harmful environmental effects. Bio-supply is of great importance in mitigating environmental pollution and the deterioration of nature (**Elkoca *et al.*, 2008**).

Nitrogen is primary limiting nutrient for growth of plant and all organisms need nitrogen for the synthesis of important

molecules including nucleic acids and proteins. Biological nitrogen fixation, which is the reduction of atmospheric nitrogen (N_2) to two molecules of ammonia (NH_3), nitrogen fixation is stimulate by the nitrogenase activity **(Michelsen *et al*., 2012).**

Biological nitrogen fixation (BNF) is an essential reaction in the universal nitrogen cycle in nature. BNF estimated as for roughly two-thirds of the fixing nitrogen produced on earth and is stimulated by the nitrogenase complex (**Rubio and Ludden, 2008**). The ability to fixing N_2 had been founded in most bacterial phylogenetic groups and is compatible with a wide range of physiologies **(Dixon and Kahn, 2004).** Non symbiotic nitrogen fixing has a great agronomic importance.

One main limitation that it faces is the availability of energy source and carbon for the energy intensive nitrogen fixation process. However, this limitation can be compensated by moveable nearer to or inside the plants, viz. in diazotrophs sitting in rhizosphere, rhizoplane or those increasing endophytically. Some important nonsymbiotic nitrogen-fixing bacteria as *Herbaspirillium* sp., *Azoarcus* sp., *Gluconacetobacter diazotrophicus* and *Azotobacter* sp. (**Vessey, 2003**).

Azotobacter is a genus consists of the members which are nitrogen-fixing, free-living, aerobic bacteria that are found over the world. These organisms are first isolated and descripted by Beijerinck in 1911 (**Patil *et al.*, 2014**). *Azospirillum* spp. are nitrogen fixers and IAA-producing bacteria that are well known to promot increase of plant growth and yield under normal as well as stressful conditions such as drought (**Barassi *et al.*, 2006**).

The genus *Clostridium* belongs to the obligate anaerobic, Gram-positive spore forming (drum stick shaped bacteria) and is composed of heterogeneous phylogenetic groups. Some species in this genus are known to be nitrogen-fixer **(Minamisawa *et al.*, 2004).** Recently, great attention has been paid to the beneficial effects of cyanobacteria in paddy soils. The amount of fixed nitrogen by these filamentous bacteria (blue- green algae) could supply rice plants with their needs of nitrogen **(Karthikeyan *et al*., 2007).**

Keeping the above facts, this research focused on: spatial distribution of nitrogen fixing bacterial content and its effect on nitrogen content in Damietta Governorate soils with determination the nitrogen content in Damietta 4 centers, counting and spatial distribution of nitrogen fixing bacteria on Damietta map, isolation, purification and identification of nitrogen fixing bacteria and select the highest nitrogen fixing bacterial isolates to use in cultivation of soil as a bio fertilizers individually or in mixed form.

MATERIALS AND METHODS:

3.1. Soil samples

The soil samples were collected at a depth of 5 to 30 cm from 20 different paddy fields Damietta **(Fig.1)**, Damietta governorate, Egypt. Five samples for each center (Damietta, Kafr Saad, Faraskour and El-Zarka). All samples were taken from rhizosphere of *Triflium alexandrium* (Egyptian clover) and the five samples of each center were mixed to obtain a representative and comprised sample for each center. The first center was Damietta (S1), the second was Kafr Saad (S2), the third one was Faraskour (S3) and the fourth was El-Zarka (S4) as can be seen on the map **(Fig. 1).**

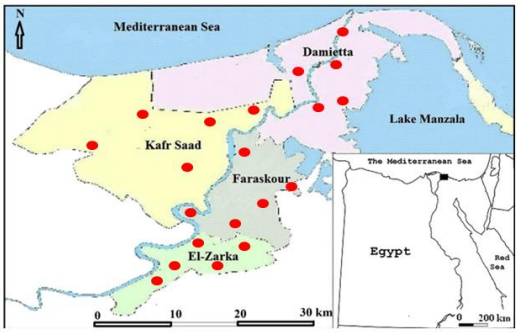


Fig.1. Locations of collected soil samples

3.2. Cultivation media

3.2.1. Modified Ashby's medium (MAM)

This cultivation medium was used for counting and isolating of *Azotobacter* spp. The chemical composition of this medium was (g/L), mannitol, 10.0; sucrose, 10.0; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; NaCl,0.2; $CaSO_4$, 0.1; $CaCO_3$, 5.0; $MnSO_4.4H_2O$, traces; $FeCl_3.6H_2O$, traces; $NaMoO_4.H_2O$, 1000 ml and pH was adjusted to 7.0± 0.2 at 25°C **(Akhter *et al.*, 2012).**

3.2.2. Nitrogen deficient medium (NDM)

This cultivation medium was used to count and to isolate *Azospirillum* spp. The chemical composition of this medium was (g/L), malic acid, 5.0; KOH, 4.0; K_2HPO_4, 0.5; $FeSO_4.7H_2O$, 0.5; $MnSO_4.H_2O$, 0.01; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.02; $Na_2MoO_4.2H_2O$, 0.002; bromothymol blue (0.5% alcoholic solution), 2.0 ml; agar agar, 1.75; distilled water, 1000 ml and pH was adjusted to 6.8 ± 0.2 at 25°C **(Alam *et al.*, 2002)**.

3.2.3. Modified Winogradsky agar medium (WAM)

This cultivation medium was used to count and to isolate nitrogen fixing anaerobic bacteria. The chemical composition of this medium was (g/L), glucose, 20; K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; NaCl, 0.001; $MnSO_4.5H_2O$, 0.001; $FeSO_4.7H_2O$, 0.001; $CaCO_3$, 40; agar agar,15 and pH was adjusted to 7.8± 0.2 at 25°C (**Golas *et al.*, 2008)**.

3.2.4.Modified Watanabe medium (MWM)

This cultivation medium was used to count and to isolate nitrogen fixing cyanobacteria. The chemical composition of this medium was (g/L), K_2HPO_4, 0.3; $MgSO_4$, 0.2; K_2SO_4, 0.2; $CaCO_3$, 0.1; glucose, 2; $FeCl_3$ (13% freshly prepared) 0.2 ml, the group of minor salts 1 ml, and pH was adjusted to 7.5 **(EL-Zawawy, 2019).**

3.2.5. Milk agar medium (MA)

This cultivation medium was prepared and used for identifying the bacteria capable of casein hydrolyzing after adding milk as a protein source. The chemical composition of this medium was (g/L), agar, 20; peptone, 5; NaCl, 5; yeast extract, 2; beef extract, 1.0 and pH was adjusted to 7.4 ± 0.2 at 25°C **(Atlas, 2010)**.

3.2.6. Starch agar medium (S A)

This cultivation medium was used for identifying the bacteria capable of hydrolyzing starch **(Atlas, 2010)**.The chemical composition of this medium was (g/L), peptone, 10.0; beef extract, 3.0; starch, 2.0; agar, 20.0; distilled water, 1000ml; PH was adjusted to 7.4± 0.2 at 25°C and sterilized by an autoclave for 15 min at 121°C. This medium was used for detection of amylo-lytic bacteria **(Atlas, 2010)**.

3.3.Physical properties of examined soil

3.3.1.Measurement of pH

Twenty five g of each soil samples were taken in a clean dry conical flask (500 ml) and 225 ml of distilled water was added. The flasks were thoroughly stirred with mechanical shaker (Model, SHO-2D, votes 230 VAC, watts 45W, serial No. S/N 04005101200021) for 15 min. The supernatant was filtered through buchner funnel using a pump (A stage vacuum pump, model VG 215, Voltage 230V, 50/60HZ, power 1/4HP, oil capacity 160 ml). pH of the suspension was measured with a digital pH meter **(**Model SED-12500V, PRI 230-240V 50/60Hz, SEC 12V-500mA, Made in China**) (Akhter *et al.*, 2012).**

3.3.2. Measurement of electrical conductivity (EC)

The soil suspension was used for EC measurement. EC of the suspension was measured with a digital EC meter **(Akhter *et al*., 2012)**.

3.3.3. Measurement of moisture content

The gravity method was used to calculate the moisture content of the four comprised soil samples. In this method, the wet samples were put in a crucible with a cover and recorded the weight of the samples (wet weight) then, samples were dried at 105°C for constant weight. The dry weight was recorded by calculating the percent of moisture content by using the following equation **(Parsons *et al.*, 2001).**

$$\text{Moisture content} = \frac{\text{wet weight of soil} - \text{dry weight of soil}}{\text{dry weigh of soil}} \times 100$$

3.3.4. Mechanical analyses of soil samples

The Pipette method was used. Twenty grams of air dried soil samples that had passed through 2 mm sieve was placed in a beaker and digested using H_2O_2 to burn the organic matter. Fifty ml of 10% NaOH solution was added into the samples and shaken for 6 hours to facilitate the dispersion of individual particles. The suspension was then sieved through a 53µ sieve whereby the retained proportion was washed into a beaker, decanted and oven dried at 105ºC for weight constant then gravimetrically quantified as sand.

Suspension that passed through the sieve and consisted of particles of silt and clay was transferred in a 1000 ml measuring cylinder. The sample was agitated by inverting 10 times. A pipette was used to extract 25 ml of the suspension in the upper 10 cm from the surface after 4.5 minutes, and 8 hours of settling at suspension temperature of 21ºC.The samples taken by the pipette were transferred to beakers of known weights and oven dried at 105º C for constant weight. The samples taken after 8 hours containing clay were calculated according to the following equation **(Mwendwa, 2020).**

$$\% \text{ Silt and clay} = \frac{\text{weight of silt and clay (after 4.5 minutes)} \times 1000}{25 \times \text{completely dry soil weight}} \times 100$$

$$\% \text{ Clay} = \frac{\text{weight of clay (after 8 hours)} \times 1000}{25 \times \text{completely dry soil weight}} \times 100$$

$$\% \text{ Silt} = \% \text{Silt and clay} - \% \text{ Clay}$$

$$\% \text{ Total sand} = 100 - \% \text{Silt and clay}$$

3.4. Counting, isolating and purification of nitrogen fixing bacteria

3.4.1. Count, isolation and purification of *Azotobacter* spp.

Count of *Azotobacter* spp. was done by Most Probable Number (MPN) method by the multiple tubes. Three decimal dilutions for each soil sample in three replicated tubes were employed using modified Ashby's medium. Tubes containing 5 ml of modified Ashby's medium were inoculated with 1 ml aliquot of prepared serial dilutions of the tested samples which obtained from plant rhizosphere. The inoculated tubes were incubated at 30°C for 7 days, and if *Azotobacter* sp. present at the surface of medium, the test was recorded as positive **(Kader *et al*., 2002).**

Isolation of *Azotobacter* sp. was done using modified Ashby's medium (MAM), inoculated and incubated at 30°C for 7days **(Akhter *et al*., 2012).** One hundred ml of modified Ashby's medium was taken in Erlenmyer flask (250ml) and 0.5g of glass beads was added. This medium was sterilized in autoclave at 121°C for 15 min and inoculated with *Azotobacter* spp. and incubated in shaking incubator (AC230V- 50/60HZ-900W-Model SKIR-602L- SERIAL NO.THK-KIR602L-0 2) at 30°C for 7 days. After incubation period, the isolates were purified through streaking plate technique **(El-Fadaly *et al*., 2019).**

3.4.2. Count, isolation and purification of *Azospirillum* spp.

Count of *Azospirillum* spp. was done by MPN by the multiple tubes. Three decimal dilutions for each sample in three replicated tubes were employed using nitrogen deficient medium. Tubes containing 5 ml of N-deficient semi-solid medium were inoculated with 1 ml of aliquot of serial dilutions of each soil sample. The inoculated tubes were incubated at 30°C for 7 days, and if *Azospirillum* sp. present at under surface of the medium, the test was recorded as positive **(Creus *et al*., 2005).**

Isolation of *Azospirillum* sp. was done using nitrogen deficient medium (NDM), inoculated and incubated at 30°C for 7days. The identification of obtained isolates was done using morphological and physiological characteristics**.** Obtained isolates were purified through streaking plate technique on a nitrogen deficient medium **(Usha and Kanimozhi, 2011).**

3.4. 3. Count, isolation and purification of anaerobic nitrogen fixig bacteria

Anaerobic nitrogen fixing bacteria of soil samples were counted by plate count method. Serial of decimal dilutions were prepared and shacked well. One ml of the last three dilutions was inoculated in Petri dishes in three replicates. After that, 15 ml of modified Winogradsky agar medium was added, and left to hardness and incubated at 30°C for 48 h in CO_2 incubator (Model: ICB-170, chamber volume(L) : 170, temperature range (℃) : RT+3-55, CO_2 range : 0-20%).

The sub surface colonies were counted as positive results. Isolation of anaerobic nitrogen fixating bacteria was done using the above modified Winogradsky agar medium (MW), inoculated and incubated at 30°C for 24 hour. The sub surface colonies were isolated and maintained on MW. Purification of anaerobic nitrogen fixing bacteria was done using the nutrient broth medium. The sub surface colonies taken from MW were inoculated on nutrient broth medium, covered with 2m fasbar layer to make sure it is anaerobic colonies and subcultured 3 times on nutrient broth medium and microscopic examination until pure culture was appeared (**Golas *et al*., 2008)**.

3.4. 4. Count, isolation and purification of Cyanobacteria

Count of cyanobacteria was done by MPN method. Three decimal dilutions for each sample in three replicated conical flask were employed using modified Watanabe medium. Flask containing 100 ml of modified Watanabe medium were inoculated with 1 ml of aliquot of serial dilutions of each soil sample.

The inoculated flask were incubated in the window at room temperature for 12 h in sun light for 14 days, and the color of the media was greenish and bubbles appear on the surface of the culture, the test was recorded as positive**.** Isolation of cyanobacteria was done using modified Watanabe medium (MWM), inoculated and incubated in the window at room temperature for 12 h in sun light for 14 day.

The identification of obtained isolates was done using morphological characteristics**.** Purification of cyanobacteria was done using modified Watanabe medium. The growth on positive Erlenmyer flask was subcultured 4 times on the same medium and microscopically examined until pure culture was appeared **(EL-Zawawy, 2019).**

3. 5. Nitrogenase activity of the obtained isolates

Nitrogenase activities (N_2ase) of the obtained isolates, as acetylene reduction activity (ARA). Five ml of modified Ashby's medium, nitrogen deficient medium and modified Winogradsky broth medium were added into 20 ml test tubes and inoculated with 50 µl of heavy inocula of 2-5 days old cultures. 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 of C_2H_4/ ml / h **(Abdel-Hamid *et al.*, 2010).**

3.6. The identification of obtained isolates

3.6.1. Morphological characteristics of bacterial isolates

Simple staining, Gram and spore staining were done according to Akhter et al. (2012). Capsule staining **(Bhaduri *et al.*, 2016)** and motility (**Bisen, 2014**) were microscopically examined studied.

3.6. 2. Biochemicals characteristics of bacterial isolates

Catalase test and hydrolysis of starch **(Akhter *et al.*, 2012),** hydrolysis of casein and Voges-Proskauer Test (**Atlas, 2010)** were carried out.

2.7.Statistical analyses

Data obtained throughout this study were analyzed by computer-assisted one-way ANOVA, using the software package statgraphics version 5.0 (costat). Least significant differences (LSDs) were calculated at 99% level of significance $P < 0.05$ **(Murica *et al.*, 1997)**.

RESULTS AND DISCUSSION:

4.1.Physical properties

4.1.1. Soil pH

The pH value of comprised soil samples ranged between 8.09 to 8.46 **(Table 1)**. There were significant differences (LSD 0.05 = 0.10) among pH values in different locations of the same examined soil. Similar results were also obtained by **El-Gammal *et al.* (2014)** who studied the soil pH in Damietta, Egypt, and they found that, the highest value of pH was 8.85 while the lowest one was 7.1.

Table 1. Physical properties of examined soil samples

Samples No.	pH values	EC, dSm^{-1}	MC, (%)
S1(Damietta**)**	8.46^{a}	5.00^{a}	9.95^{d}
S2(Kafr Saad**)**	8.16^{bc}	3.90^{b}	23.06^{c}
S3(Faraskour**)**	8.22^{b}	3.20^{c}	25.27^{b}
S4(El-Zarka**)**	8.09^{bc}	2.10^{d}	30.23^{a}
LSD 0.05	0.10	0.64	1.72

n=3, EC: Electrical conductivity, MC: Moisture content, dSm^{-1}=The unit used to estimate salinity in the form of electrical conductivity

Unsimilar results were obtained by **Akhter *et al.*, (2012)** who isolated and characterized salinity tolerance *Azotobacter* sp. and they found that, the highest value of pH was 7.21 while the lowest one was 4.95. The pH is important in nitrogen fixation ability. It

remained almost unchanged within the pH range of 6.24 to 8.54, decreased at more acidic or alkaline pH and complete inhibition was detected above 8.75 and below 5.00 **(El-Gammal *et al*., 2014)**.

4.1.2. Soil electrical conductivity EC(dSm^{-1})

Results in **Table 1 and** showing electrical conductivity values which determined in examined soil samples S1, S2, S3and S4 being 5.00, 3.90, 3.20 and 2.10, respectively. There were significant differences (LSD 0.05= 0.64) among EC values in different locations of the same examined soil.

These results are not accordance with those obtained by **El-Gammal *et al*. (2014)** who studied the soil EC in Damietta, Egypt and they found that, the highest value of EC was 29 while the lowest one was 1.13. EC is important in soil salinity and it is considered one of the main factors contributing to crop loss and plant instability. In saline soils, osmotic potential is low and minerals cycles can be reduced. Hydraulic conductivity is also lowered with increasing salt concentration and loss of water can cause stunted growth and consequent low plant production **(El-Gammal *et al*., 2014)**.

4.1.3. Moisture content

Moisture content (MC%) was in the range between 9.95 to 30.23 **(Table 1**). The differences in moisture content (%) values are most probably due to collection of comprised samples from different locations and there were an inverse relationship between moisture and salinity in soil samples. Moisture content (MC%) guide to quantity of organic matter in soil and soil texture **(El-Fadaly *et al*., 2019).**

4.1.4. Soil structure

The examined comprised soil samples were clay soil **(Nath, 2014).** The texture of the soil samples are given in **Table 2** The results show that clay (%) dominates over sand (%) and silt(%), and the values could be arranged in the ranges of clay: 71.26 to 74.93%, silt: 5.9 to 7.6 % and sand: 18.42 to 22.84 %.

As a soil textural class, clay refers to soil material when that is 40 percent or more of clay, less than 40 percent of silt and less than 45 percent of sand as detected by **Peterson (2012).**

Table 2. Soil texture of examined comprised soil samples

Sample No.	Clay (%)	Silt (%)	Sand (%)	Soil texture
S1	71.26	5.9	22.84	Clay Soil
S2	72.93	6.4	20.67	Clay Soil
S3	73.98	7.6	18.42	Clay Soil
S4	74.93	6.6	18.47	Clay Soil

4.2. Total nitrogen

All organisms need nitrogen for the synthesis of important molecules including nucleic acids and proteins. Results in **Table 3** showing total nitrogen (TN) in comprised soil sample. The highest value was 448 mg/kg in S4 and the lowest value was 308 mg/kg in S1 and there were significant deferentce amongst samples. El-Zarka center is considered to have the highest nitrogen content of the soil, due to low salinity content. On the other hand, the Damietta center is considered the lowest in its nitrogen content, due to high salinity content.

Nitrogen is primary limiting nutrient for plant growth, consequently, the lack of nitrogen causes a decrease in plant growth and yield. The deficiency in the nitrogen content is due to the lack of soil fertility, organic matter, and nitrogen-fixing microbes. This problem can be solved by nitrogen fertilization or biofertilization with nitrogen-fixing bacteria **(Michelsen *et al*., 2012).**

Table 3. Values (mg/kg) of total nitrogen in examined composite soil samples

Samples No.	TN (mg/kg)
S1	**308^{d}**
S2	**425^{b}**
S3	**406^{c}**
S4	**448^{a}**
LSD, 0.05	**11.302**

4.3. Count of nitrogen fixing bacteria

4.3.1. Aerobic N-fixing bacteria

Results in **Table 4 and Fig. 2 (Damietta map)** showing the aerobic free N-

fixing bacteria expressed in *Azotobacter* sp. count (cfu $\times 10^5$/g) using Ashby·s medium with MPN technique in examined four composite soil samples. *Azotobacter sp.* counted on Ashby,s medium ranged from 110 $\times 10^5$ cfu /g soil to 46 $\times 10^5$ cfu/g. Based on the spatial distribution of *Azotobacter* sp. count in Damietta Governorate soil, El-Zarka and Kafr Saad centers are considered the highest count with ratio of 35%, Damietta and Faraskour centers are considered the lowest in ratio of 15%.

Unsimilar results were obtained by **Kumar *et al*. (2016)** who studied the microbiological properties of soil influenced by weed control methods in maize-pea cropping system under mid hill conditions of Himachal Pradesh, India. They found that the highest value of *Azotobacter* spp. was 30.3 $\times 10^4$ cfu/g soil while the lowest one was 6.06 $\times 10^4$ cfu/g soil. **Akhter *et al*. (2012)** isolated and characterized salinity tolerant *Azotobacter* sp. They found that the highest count of *Azotobacter* sp. was 7×10^5 cfu/g soil while the lowest one was 1×10^5 cfu/g soil.

4.3.2. Microaerophilic N-fixing bacteria

Results of the MPN technique using Dobriner medium showing the microaerophilic N-fixing bacterial count (cfu/g $\times 10^5$) by means of *Azospirillum* sp. in composite soil samples are displayed in **Table 4 and Fig. 3.** (Damietta map).The values of *Azospirillum* spp. varied between 15 $\times 10^5$ and 46 $\times 10^5$ cfu/g soil. Looking at the map **(Fig. 3)**, El-Zarka center is considered the dominant center in terms of *Azospirillum* sp. because it contains nearly half of the content of 41%, and in contrast, the remaining three center contain 59% of the obtained count.

Similar results were obtained by **Kumar *et al*. (2016)** who examined the microbiological properties of soil influenced by weed control methods in maize-pea cropping system under mid hill conditions of Himachal Pradesh, India. They found that the highest count of *Azospirillum* sp. was 62.8 $\times 10^4$ cfu/g while the lowest one was 23.1 $\times 10^4$ cfu/g soil.

4.3.3. Anaerobic N-fixing bacteria

The genus *Clostridium* belongs to the obligate anaerobic, Gram-positive spore forming (drum stick shaped bacteria). Some species in this genus are known to be nitrogen-fixer, **(Minamisawa *et al*., 2004).** Results in **Table 4 and Fig. 4** (Damietta map) showing the count of *Clostridium* sp. using plate count technique in examined four composite soil samples.

Clostridium spp. ranged from 10.3 $\times 10^4$ cfu/g soil to 19.9 $\times 10^4$ cfu/g. According to the relative distribution of *Clostridium* sp. count, Damietta centers can be arranged from highest to lowest on the map **(Fig.4).** Damietta, Kafr Saad, Faraskour and El-Zarka were 36%, 25%, 20% and 19%, respectively.

The results are in disagreement with those obtained by **Bin *et al*. (2005)** who studied the effect of inoculation with anaerobic nitrogen-fixing *Clostridium* spp. on salt tolerance of *Miscanthus sinensis*. They found that the highest count of *Clostridium* sp. was 4.1 X 10^4 cfu/g soil while the lowest one was

1.4 X 10^4 cfu/g soil. Similar results were obtained by **Minamisawa *et al.*, (2004)** who studied anaerobic nitrogen-fixing Clostridia isolated from gramineous plants. They found that the highest count of *Clostridium* sp. was 15 X 10^4 cfu/g soil while the lowest one was 0.74 X 10^4 cfu/g soil.

4.3.4. Count of Cyanobacteria

Recently, great attention has been paid to the beneficial effects of cyanobacteria in paddy soils. The amount of fixed nitrogen by these bacteria (blue- green algae) could supply rice plants with their needs of nitrogen. Results in **Table 4** and **Fig. 5** (Damietta map) showing the count ($\times 10^2$ cfu /g soil) of cyanobacteria using MPN technique in examined four composite soil samples on watanab medium.

Cyanobacteria ranged from 20 $\times 10^2$ cfu/g soil to 7.4 $\times 10^2$ cfu/g soil. El-Zarka and Faraskour centers are thought to have the highest count of 32%, while Damietta center was have the lowest ratio with of 12%, based on the spatial distribution of cyanobacterial count in Damietta Governor soil.

Similar results were obtained by **Karthikeyan *et al*. (2007)** who studied the potential of plant growth promoting cyanobacteria as inoculants for wheat. They found that the highest count of cyanobacteria was 9.2 X 10^2 cfu/g while the lowest one was 5.4 X 10^2 cfu/g.

4.4.Morphological and physiological characteristics of obtained bacterial isolates

All obtained bacterial isolates appeared on Modified Ashby's medium are coccoid shaped cells and they arranged in diplococcoid, Gram negative, none spore formers, capsule positive, none motile cells. Some isolates produced brown pigment while other produced yellow pigment, Besides, catalase test, starch hydrolysis, and protein hydrolysis were positive result and negative of Voges-Proskauer Test (V.P) (**Table 5 and Fig.6)**.

From these characteristics, all isolates were classically identified as *Azotobacter* sp**. Tejera *et al.*, (2005)& Young and Park 92007)** reported that *Azotobacter* is a genus usually non motile, oval or spherical shaped bacteria that form thick-walled cysts (has hard crust) and may produce large quantities of capsular slime. *Azotobacter* species are Gram-negative bacteria.

Cells of the genus *Azotobacter* are relatively large for bacteria (2–4 μm in diameter). *Azotobacter* aerobically, receives energy from redox reactions, using organic compounds as electron donors, and can use a variety of carbohydrates, alcohols, and salts of organic acids as sources of carbon. *Azotobacter* produces pigments. For example, *Azotobacter chroococcum* forms a dark-brown water-soluble melanin pigment**.**

All isolates obtained on NDM are short rods and arranged in single form. Gram negative, none spore formers and capsule

negative. The results of motilty, catalase test, starch hydrolysis, and protein hydrolysis were positive and negative of Voges-Proskauer Test (**Table 6 and Fig. 7)**. From these characteristics, all isolates were classically identified as *Azospirillum* sp. **Rosenberg *et al*., (2014 and Madigan *et al*., (2003)** *Azospirillum* are Gram-negative, do not form spores, and have a slightly twisted rod shaped cells. *Azospirillum* have at least one flagellum and sometimes multiple flagella, which they use to move rapidly.

Azospirillum are aerobic, but many can also function as microaerobic diazotrophs, under low oxygen conditions, they can change inert nitrogen from the air (N_2) into biologically useable forms (NH_4)**.**

All isolates obtained on WAM are long rods and arranged in single form. Gram positive, spore formers, capsule negative, motile cells. They showed negative results with catalase test, (**Table 7 and Fig. 8)**. From these characteristics, all isolates are classically identified as *Clostridium* sp. **Dieterle *et al*., (2019)***Clostridium* is a genus of anaerobic, Gram-positive bacteria and rod-shaped in drum stick shaped cells, species of *Clostridium* inhabit soils and the intestinal tract of animals, and capable of producing swollen polar endospores**.**

Cyanobacteria were identified which was carried out using the following characteristics (thallus color, thallus morphology and dimension, heterocyst, vegetative and reproductive cells).

The first isolate **(Fig. 9)** belong to *Nostoc* sp. trichome straight, cylindrical cell, as length as width or slightly larger than width, 3.2-3.8 µ width and 4.1-4.5 µ length, heterocysts both terminal and intercalary, larger than the vegetative cells, a kinetes oblong or oval, 4.1-6.3 µ. These are in accordance with those obtained by **Afify *et al.* (2018)**. The second isolates **(Fig. 10)** belong to *Anabaena* sp. thallus green, cells barrel-shaped, as long as width 4-5 µ. Heterocysts terminal and intercalary, akinetes 3-6 µ. These results are in agreement with **Shariatmadari and Riahi, (2010).**

The third isolates **(Fig. 11)** belong to *Synechococcus* sp. range from 2 to 10 µm in diameter, lack flagella, the cells were solitary, non-motile, don't have a Heterocysts **(Jo *et al*., 2020).**

4.5. Nitrogenase activity

The acetylene reduction assay (nitrogenase activity) was used as an index of the rate for nitrogen fixation. The examined isolates were screened *in vitro* for their N_2-fixing ability.

Table 4. *Azotobacter* sp. count measured by MPN technique using ashby's medium.

composte soil samples	Count of *Azotobacter* sp. (cfu x 10^5)	Count of *Azospirillum* sp. (cfu x 10^5)	Count of *Clostridium* sp. (cfu) x 10^4	Count of cyanobacteria (cfu x 102)
S1(Damietta)	46	15	19.9	7.4
S2(Kafr Saad)	110	29	13.8	15
S3(Faraskour)	46	21	10.9	20
S4(El-Zarka)	110	46	10.3	20

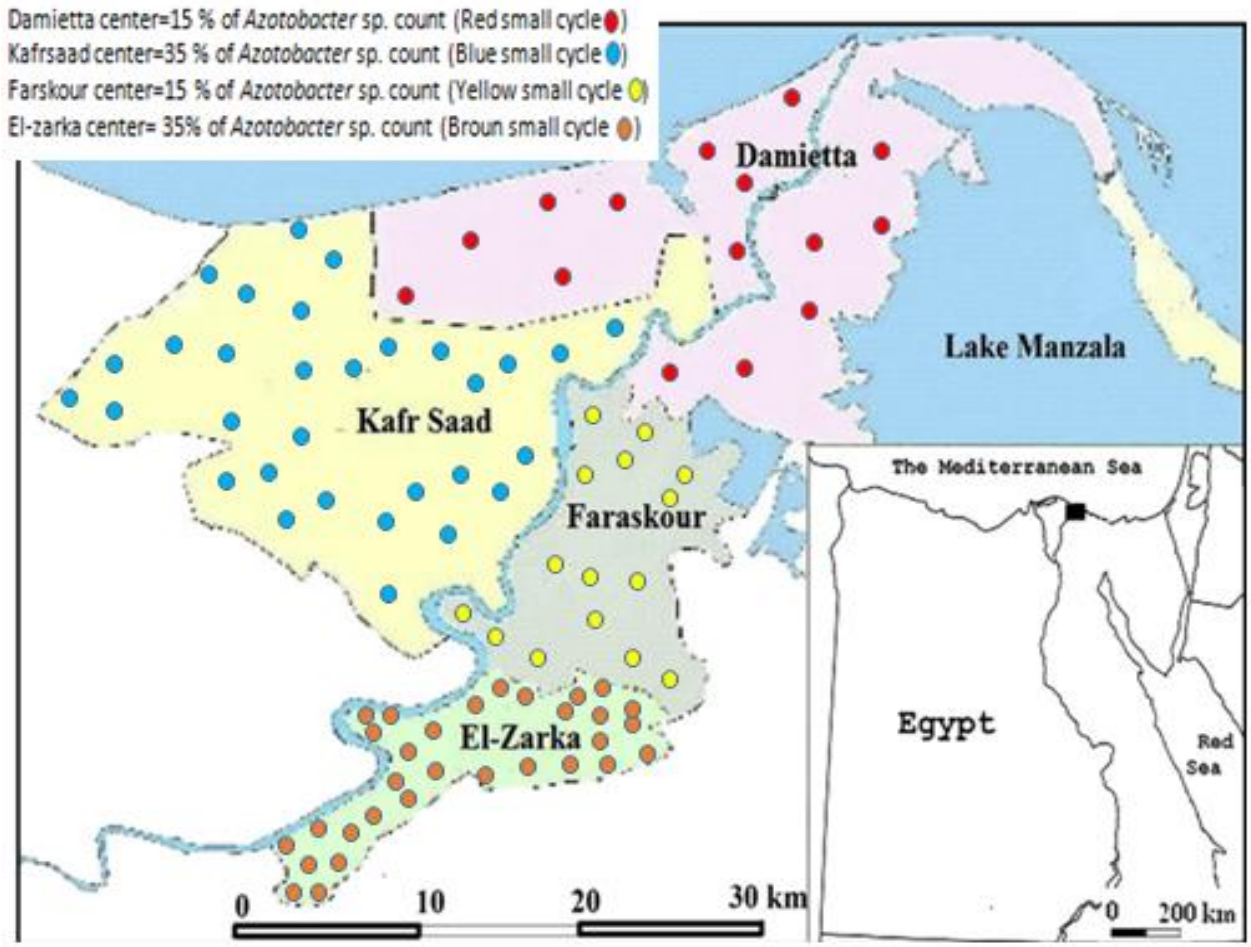


Fig. 2. Spatial distribution of *Azotobacter* spp. in Damietta Governorate centers

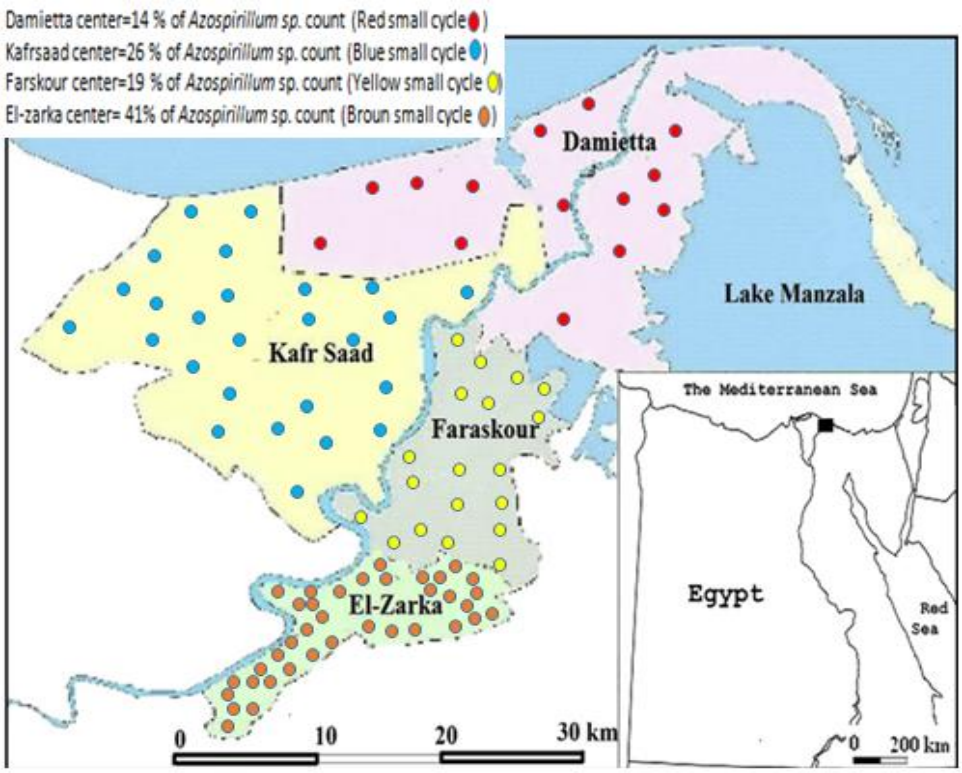


Fig.3. Spatial distribution of *Azospirillum* spp. in Damietta Governorate centers

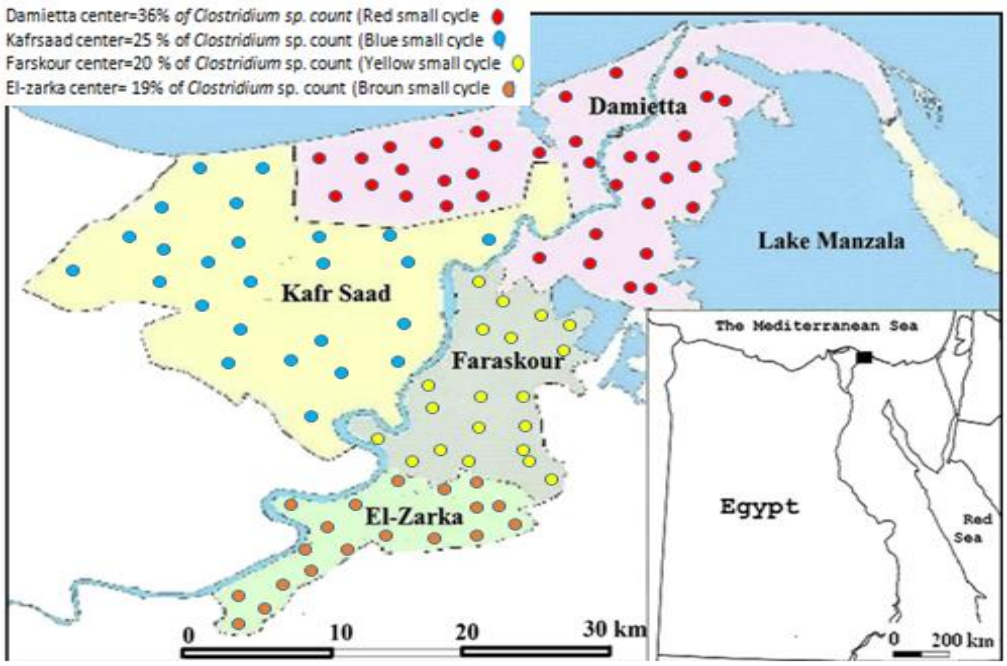


Fig.4. Spatial distribution of *Clostridium* sp. in Damietta Governorate centers

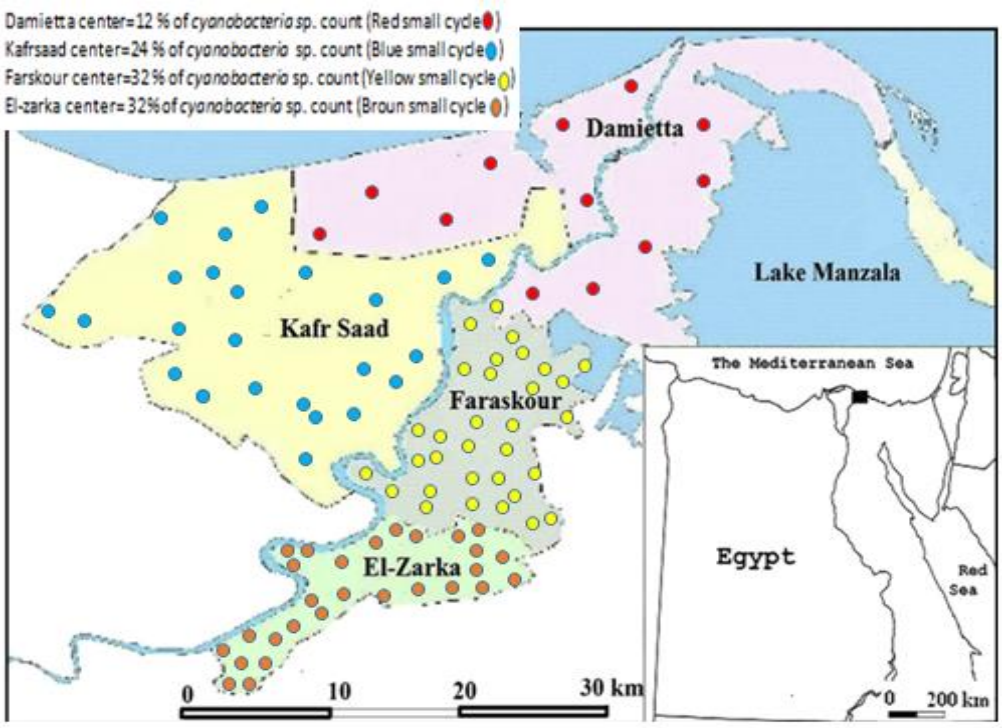
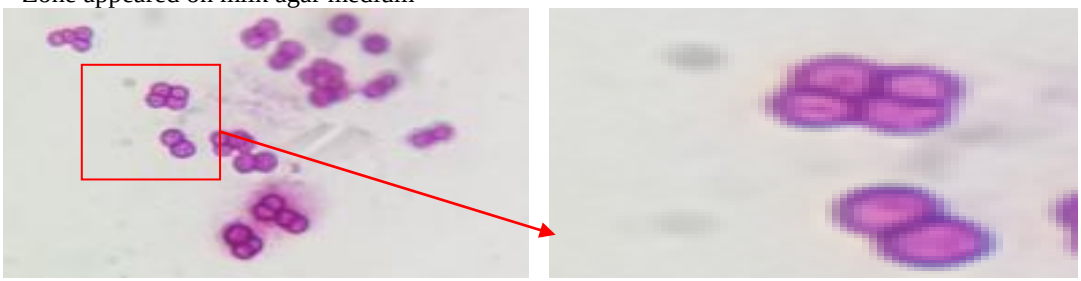


Fig.5. Spatial distribution of cyanobacterial count in Damietta Governorate centers

Table 5. Morphological and physiological characteristics of N-fixing bacterial isolates obtained on modified Ashby's medium

Isolates code	Cell Shape	Arrangement	Gram staining	Spore staining	Capsule staining	Motility	Catalase test	Voges-Proskauer Test (V.P)	*Starch hydrolysis (mm)	**Protein hydrolysis (mm)
MAM-1	Oval	Diploid	-	-	+	-	+	-	13	16
MAM-2	Oval	Diploid	-	-	+	-	+	-	16	8
MAM-3	Oval	Diploid	-	-	+	-	+	-	22	17
MAM-4	Oval	Diploid	-	-	+	-	+	-	17	15

***Zone appeared on starch agar medium
** Zone appeared on milk agar medium



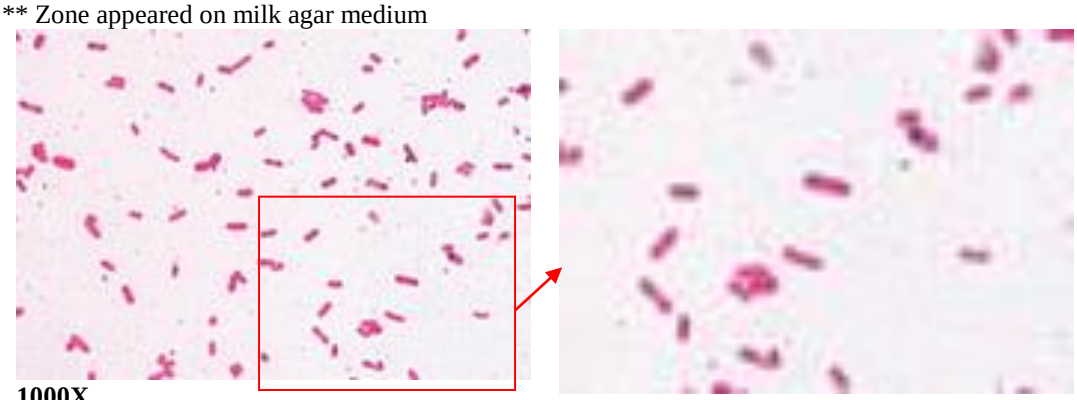
1000 X **3000 X**

Fig.6. Cell shape and arrangement of *Azotobacter* sp.

Table 6. Morphological and physiological characteristics of N-fixing bacterial isolates obtained on nitrogen deficient medium

Isolates code	Cell Shape	Arrange ment	Gram staining	spore staining	capsule staining	Motility	Catalase test	Voges-Proskauer Test (V.P)	Starch hydroly sis (mm)	Protein hydroly sis (mm)
NDM-1	Rod	Single	-	-	-	+	+	-	23	19
NDM-2	Rod	Single	-	-	-	+	+	-	9	12
NDM-3	Rod	Single	-	-	-	+	+	-	15	18
NDM-4	Rod	Single	-	-	-	+	+	-	15	22

*****Zone appeared on starch agar medium
** Zone appeared on milk agar medium



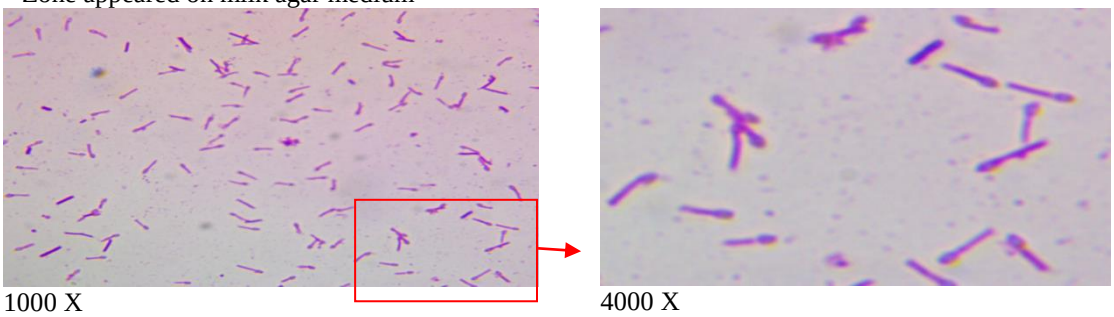
1000X **2000X**

Fig.7. Cell shape and arrangement of *Azospirillum* sp.

Table 7. Morphological and physiological characteristics of N-fixing bacterial isolates obtained on Winogradsky agar medium.

Isolates code	Cell Shape	Arrange ment	Gram staining	spore staining	capsule staining	Motility	Catalase test
WAM-1	Rod	Single	+	+	-	+	-
WAM-2	Rod	Single	+	+	-	+	-
WAM-3	Rod	Single	+	+	-	+	-
WAM-4	Rod	Single	+	+	-	+	-
WAM-5	Rod	Single	+	+	-	+	-

*****Zone appeared on starch agar medium
** Zone appeared on milk agar medium



1000 X 4000 X

Fig.8. Cell shape and arrangement of *Closridium* sp.

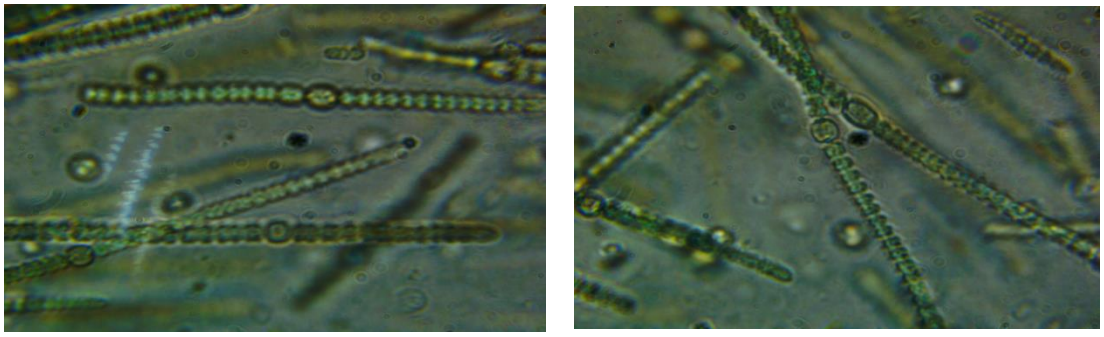


Fig.9. The first isolate of cyanobacteria

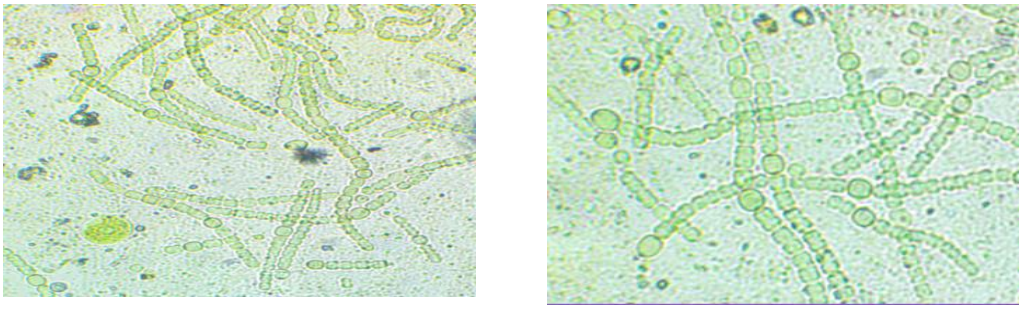


Fig.10. The second isolate of cyanobacteria

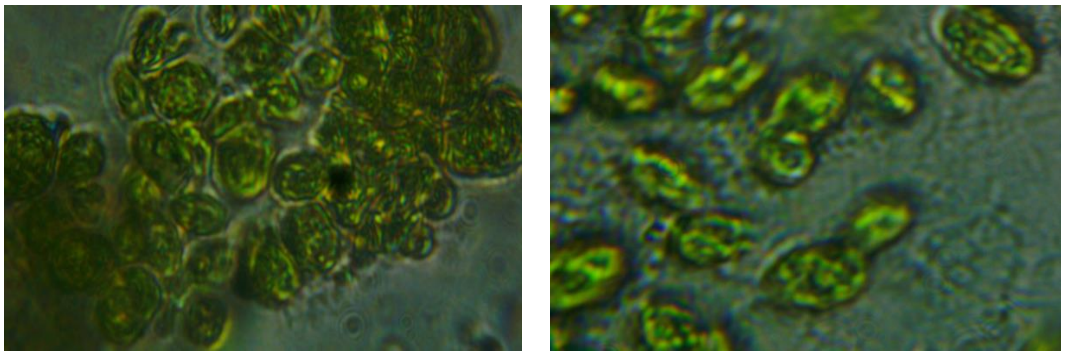


Fig.11. The third isolate of cyanobacteria

Table 8. Acetylene reduction values (nitrogenase activity) of the obtained bacterial isolates

Isolation Site (centers)	*Azotobacter* sp.	N_2-ase activity (nmole C_2H_2/h/l)	*Azospirillum* sp.	N_2-ase activity (nmole C_2H_2/h/l)	*Clostridium* sp.	N_2-ase activity (nmole C_2H_2/h/l)
Damietta	M.A.M-1	107	N.D.M-1	21	W.A.M-1	119
Kafr Saad	M.A.M-3	80	N.D.M-3	39	W.A.M-3	122
Faraskour	M.A.M-4	72	N.D.M-4	23	W.A.M-4	135
El-Zarka	M.A.M-5	130	N.D.M-5	51	W.A.M-5	125

Table 8 show the amounts of acetylene reduced by *Azotobacter* sp., *Azospirillum* sp. and *Clostridium* sp. All isolates were able to reduce acetylene, where the high value of *Azotobacter* spp. (M.A.M-5) was 130 nmole C_2H_2/h/l N_2-ase activity while the high value of *Azospirillum* spp. (N.D.M-5) was 51 nmole C_2H_2/h/l N_2-ase activity. The high value of *Clostridium* spp. (W.A.M-4) was 135 nmole C_2H_2/h/l N_2-ase activity. Similar results were obtained by **Tejera *et al.*, (2005)** in the range from 76.79 to 189.6 n mole C_2H_2/ l/ h. and also as the range from 51.3 to 100 n mole C_2H_2/ l/ h. as found by **Abdel-Hamid *et al.*(2010)**.

CONCLUSION:

Based on this study the highest nitrogen fixing isolates of *Azotobacter* sp. , *Azospirillm* sp. and *Clostridium* sp. could be highly used in cultivation of soil in coastal environment such as Damietta Governorate soils as a bio fertilizer to reduce the chemical fertilizer and save the soil from chemical addition and keep the environmental clean

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS CONTRIBUTION

The authors developed the concept of the manuscript. All authors checked and confirmed the final revised manuscript.

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الملخص العربي

التوزيع المكاني لمحتوى النيتروجين والبكتريا المثبتة للنيتروجين في مراكز محافظة دمياط

حسين الفضالى, شريف القاضى ومحمد عبدالتواب

قسم البيوتكنولوجيا الزراعية –كلية الزراعة – جامعة دمياط

يتناول البحث التوزيع المكاني لمحتوى النيتروجين والبكتيريا المثبتة للنيتروجين، مثل .*Azotobacter* sp. , *Azospirillum* sp. .*Clostridium* sp. والسيانوبكتريا من منطقة الريزوسفير لمحصول البرسيم المصرى بمراكز محافظة دمياط، مصر. عشرين عينة تربة مختلف تم تجميعها ، خمس عينات مجمعة لكل مركز (دمياط، كفر سعد، فارسكور، الزرقا). تم تقدير محتوى النيتروجين في عينات التربة التى تم فحصها، وتم عد البكتيريا المثبتة للنيتروجين وعزلها في كل مركز. تم تعريف جميع العزلات اعتماداً على الصفات المظهرية والفسيولوجية وتقدير نشاط انزيم النتروجينيز بواسطة اختزال الأسيتيلين. أعلى قيمة لمحتوى النيتروجين كانت 448 ملجم/كجم-1 في مركز الزرقا (S4). ويعتبر مركزا الزرقا وكفر سعد الأعلى عددا بنسبة 35%، 32% في حالة بكتيريا *Azotobacter* sp. السيانوبكتريا على التوالي ويعتبر مركز الزرقا هو المركز السائد من حيث عدد .*Azospirillum* sp. بنسبة (41%). بينما يعتبر مركز دمياط هو الأعلى بنسبة 36% في حالة بكتيريا .*Clostridium* sp. قيمة نشاط انزيم النيتروجينيز اعلى عزلة M.A.M-5 (130) فى حالة .*Azotobacter* sp , بينما كانت اعلى عزلة N.D.M-5 (51) فى حالة .*Azospirillum* sp, , بينما كانت اعلى عزلة W.A.M-4 (135) فى حالة .*Clostridium* sp. بناء على هذه الدراسة فإن عزلات بكتريا sp *Azotobacter*, *Azospirillum* sp.و*Clostridium* sp الأعلى تثبيتا للنيتروجين يمكن استخدامها بشكل كبير في تسميد التربة في البيئة الساحلية مثل تربة محافظة دمياط كسماد حيوى وذلك لتقليل استخدام السماد الكيماوي وبذلك نحافظ على التربة الزراعية من زيادة الكيماويات ذات المردود السي على البيئة.