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Full Length Research Paper

Isolation of Rhizobacteria and Micro Algae from Saline Soil and Production of Plant Growth Promoters

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Twenty-six bacterial and four algal isolates were isolated from rhizosphere of rice and sugar beet plants, grown in saline soil in Sahl El-Hussinian Station, El-Sharkia Governorate, Egypt and *in vitro* screening was done for different plant growth promotion activities i. e. indole acetic acid (IAA), gibberellic acid (GA) and carbohydrate production. Bio-control ability of bacterial isolates was screened by antagonistic activity against certain fungal pathogens. Results revealed that bacterial isolates No. 7 and 26 produced high amount of IAA ($78.63 \mu\text{g ml}^{-1}$ and $78.73 \mu\text{g ml}^{-1}$, respectively), while isolate No. 5 was most potent isolate for GA ($173.91 \mu\text{g ml}^{-1}$) and carbohydrate ($187.18 \mu\text{g ml}^{-1}$) production. The active bacterial isolates (No. 5, 7, 26) in plant growth promoting substances production showed high antagonistic activity against certain pathogenic fungi (*Fusarium* sp. and *Rhizoctonia* sp.). Additionally, algal isolate No. 3 was active in production IAA ($29.26 \mu\text{g ml}^{-1}$), while isolates No. 2 and 1 were efficient in GA ($83.91 \mu\text{g ml}^{-1}$) and carbohydrate ($178.81 \mu\text{g ml}^{-1}$) production, respectively. The most efficient rhizobacterial isolates (No. 5, 7 and 26) were selected and identified using Bio-log technique and 16S rRNA as *Bacillus subtilis* and *Pseudomonas aeruginosa*, respectively. Based on 16S rRNA sequence analysis both bacterial isolates (No. 7 and 26) identified as *Pseudomonas aeruginosa*. The most potent algal isolates (No. 1, 2 and 3) for plant growth promoting substances production were chosen and identified according to classical morphological characters as *Anabaena* sp., *Nostoc muscorum* and *Anabaena variabilis*, respectively. The optimum incubation period for highest production of IAA and GA by efficient bacterial and algal isolates was three and twenty days, respectively, while highest carbohydrate production was obtained after four and twenty five days, respectively.

Keywords: Soil salinity, Isolation, Purification, Identification, Phytohormones, Carbohydrate, Biocontrol, incubation periods.

INTRODUCTION

Salinity is one of the major abiotic factors that limit plant

growth and productivity in many regions of the world due to increasing use of poor quality of water for irrigation and soil salinization (Zhang and Shi, 2013), where it also a major problem that restricts yield of almost 40 million hectares of

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irrigated land, which is approximately one-third of the irrigated land on earth. It was estimated that about 50% of the arable land will be affected by salt stress by the year 2050 (Munns and Tester, 2008). About 20% of all irrigated land is affected by soil salinity, decreasing crop yields (Kader, 2010). In Egypt, Elbordiny and El-Dewiny (2008) reported that fifty five percent of the cultivated lands of northern Delta region are salt-affected, twenty percent of the southern Delta and middle Egypt region and twenty five percent of the Upper Egypt region are salt-affected soils. The southern part of El-Hussinia plain, Sharkia Governorate, Egypt covering an area of about 141.6 Km². Abdel-Hamid (2014) mentioned that saline soils possess high levels of sodium and chloride contents and thus exert adverse abiotic stress on plants. Salinity induces osmotic and ionic imbalance inside plant's cell, thus affecting plant growth and metabolism.

The application of plant growth promoting rhizobacteria PGPR bio-inoculants, such as *Azospirillum*, *Agrobacterium*, *Pseudomonas* and several Gram-positive *Bacillus* species, is an environmentally friendly, energy efficient and economically viable approach to reclaiming salinity-affected land and increasing biomass production (Berg, 2009). In recent years considerable attention has been paid to PGPR to replace agrochemicals (fertilizers and pesticides) for the plant growth promotion by a variety of mechanisms that involve soil structure formation, decomposition of organic matter, recycling of essential elements, solubilization of mineral nutrients, producing numerous plant growth regulators, degrading organic pollutants, stimulation of root growth, crucial for soil fertility, biocontrol of soil and seed borne plant pathogens and in promoting changes in vegetation Sivasakhti *et al.* (2014).

Algae play an economic role in soil reclamation increases soil fertility and improve the plant conditions under certain environmental factors. According to Mazur *et al.* (2001) algae have beneficial effect on plant growth. Recently, Priyanka *et al.* (2016) reported that cyanobacteria, the cosmopolitan, photosynthetic prokaryotes have been reported to grow extensively on both saline and alkaline soils that commonly called as "Usar". These are capable of thriving in condition which is considered to inhabitable tolerating desiccation, high temperature, extreme pH and high salinity illustrating their capacity to acclimatize to extreme environments. (Alam *et al.*, 2014) stated that the oxygen produced by algal photosynthesis prevents anaerobiosis in the root system of the crop. Furthermore, algae recently have been reported to release plant-growth promoters in plant cultivation systems. Cyanobacteria are known by their ability to excrete growth-promoting substances such as hormones (Auxin and Gibberellins-like), vitamins and amino acids. They also increase the water- holding capacity through their jelly structure, increase in soil biomass after their death and decomposition, preventing weeds growth.

The aim of this research is to isolate and identify some rhizospheric bacteria and algae from saline soil which produce plant growth regulators and study the effect of different incubation periods to elucidate the optimum time for abundant growth regulators.

MATERIALS AND METHODS

Isolation of rhizospheric bacteria and algae from soil

For Plant Growth Promoting Rhizobacteria (PGPR) isolation from rhizospheric of rice and sugar beet plants, grown in saline soil (the soil adhered to root surface and in between root) that obtained from Sahl El-Hussinian Station, El-Sharkia Governorate, Egypt, serial dilution techniques were used. The isolation carried out on King's medium (King *et al.* , 1954) at 30°C for 24-48 hours. After successful growth of microorganisms the individual colonies were picked up and purified.

For Plant Growth Promoting Rhizospheric Algae (PGPRA) isolation nitrogen free BG11 nitrogen-free medium (Bischoff and Bold, 1963) was used for isolation of blue green algae (cyanobacteria) and Bold's basal medium (Rippka *et al.*, 1979) used for isolation of green algae at 28 ± 2°C under light intensity 2500-3500 LUX for 3 weeks. The algal colonies were picked out using a sterilized inoculating needle for propagation, purification and identification.

Purification of bacterial and algal isolates

The pure cultures of bacteria were subcultured several times in a new plates by using the streak plate method (trypticase King's agar medium) for purification then maintained as stock cultures at 4°C for subsequent studies. On the other hand, the uni algal cultures were purified as described by Pringsheim (1949).

Bacteria free algal cultures:

To obtain bacteria free cultured each algal culture was serially purified by washing (Hoshaw and Rosowski, 1973) and antibiotic treatment, this method suggested by (Felfoldy and Zsuzsa, 1959).

Detection of indole acetic acid production for bacterial isolates

The modified method described by (Bric *et al.*, 1991) was used to detect indole acetic acid (IAA) and /or IAA analog qualitatively. Organism producing IAA and /or IAA analogs was distinguished from organisms producing other indoles (producing yellow to yellow – brown pigment) by their characteristic pink to red color.

Quantitative determination of plant growth promoting substances in culture media of bacteria and algae

Rhizobacterial and algal isolates were tested for their quantitative capabilities to produce carbohydrates and plant growth promoting substances i.e., indole acetic acid and gibberellins in culture media. All bacterial isolates were cultivated in King's broth medium supplemented with 0.1 mg ml⁻¹ tryptophan. After three days, bacterial cultures were centrifuged at 4000 rpm for 15 min to separate supernatant. The extraction was carried out according to the method of Shindy and Smith (1975). The supernatant (30 ml) were adjusted to PH 2.8 with 1 % HCl. The aqueous residue was partially purified by partition with ethyl acetate three times with equal volumes of it (acid fractions) and the remaining aqueous phase was discarded. This fraction contained acid hormones such as IAA and GA, (Shindy and Smith, 1975). Algal isolates were grown in BG-11 nitrogen - free for blue-green algae and Bold's basal medium for green algae supplemented with 0.5 mg ml⁻¹ tryptophan, after 3 weeks the algal cultures were centrifuged at 1000 rpm at 4°C for 10 min to separate supernatants. For estimation of extracellular IAA and GA the pH of the supernatant was adjusted to 2.8 (using 1.0 M HCl) and extracted three times with three volumes (1:3 v/v) of ethyl acetate Ahmed *et al.* (2010).

Determination of indole acetic acid (IAA) and total gibberellins (GA)

Indole acetic acid produced by bacterial and algal isolates was determined by using the method of Glickmann and Dessaux (1995), while production of GA by bacterial and algal isolates was detected according to (Udagwa and Kinoshita, 1961).

Determination of total carbohydrate

The bacterial isolates and algae were culture as mentioned before, and then the bacteria and algal cultures were centrifuged at 4000 rpm and 10000 rpm, respectively for 15 min to separate supernatant. The total carbohydrate content of either insoluble or soluble sugars was determined as glucose by the phenol- sulphuric acid method (Dubois *et al.*, 1956).

Antagonistic activity of PGPR

The possible interaction between the PGPR agents and pathogenic fungi (*Fusarium* sp. and *Rhizoctonia* sp.), responsible for root rot in a variety of legume and non-legume crops was monitored on potato dextrose agar (PDA) medium using a modified agar – plate inhibition zone technique (Silosuch *et al.*, 1994).

Identification of selected bacterial and algal isolates

The efficient bacterial isolates (No.5, 7 and 26) that produce high amount of IAA, GA, total carbohydrates and were have high antagonistic activity were selected and identified. One of this bacterial isolates (isolate No.5) was identified by Bio-log Technique at Plant Pathology Research Institute, Agriculture Research Center, Ministry of Agriculture, Giza, Egypt. The other two bacterial isolates (No. 7 and 26) were identified by 16S rRNA sequence analysis at Sigma Scientific Services Company, Lebanon Square, El Giza, Egypt, and also the three selected bacterial isolates undergo to Gram staining according to (Vincent, 1970). Additionally the purified three algal isolates were identified according to Desikachary (1959).

RESULTS AND DISCUSSION

Twenty-six bacterial isolates and four algal isolates were obtained from rhizosphere of rice and sugar beet plants, grown in saline soil in Sahl El-Hussinian Station, El-Sharkia Governorate, Egypt. The isolated bacteria were preliminary qualitative analysis of the bacterial isolates for indole acetic acid (IAA) production and the results revealed that the isolates Nos. 7 and 26 appeared the most active IAA producers which indicated by the highest reaction color intensities displayed by these strains when grown on its proper media. The quantitative analysis data on the amounts of IAA produced *in vitro* by the examined bacterial isolates showing that the most active IAA producers were isolates Nos. 7 and 26 producing 78.63 and 78.73 µg ml⁻¹ IAA respectively followed by isolate No. 5 producing 48 µg ml⁻¹ Table (1). On the other hand, among all bacterial isolates, isolate No. 5 was found to be good producer of gibberellic acid GA, which producing 173.91 µg ml⁻¹ Table (1). However, the present result also showed that the range of carbohydrate production was 9.04 to 187.18 µg ml⁻¹ and isolate No. 5 was superior in the production of carbohydrate that producing 187.18 µg ml⁻¹ followed by isolates Nos. 7 and 26 releasing 171.13 µg ml⁻¹ Table (1). Additionally, all bacterial isolates were tested for antagonistic activity against pathogenic fungi (*Fusarium* sp. and *Rhizoctonia* sp.) and the results showed that 18 bacterial isolates have antagonistic activity against *Fusarium* sp. and 23 bacterial isolates active against *Rhizoctonia* sp. The isolates Nos. 7, 26 and 5 were the most active isolates against *Fusarium* sp. and *Rhizoctonia* sp. as well as isolate No. 13 was active against the latter (Table 2). This result was in similarity with Sandip *et al.* (2012) who reported that the antagonistic activity of *P. fluorescense*, *P. aeruginosa* and *B. subtilis* was confirmed against three plant pathogen fungi *Fusarium oxysporum*, *Aspergillus niger* and *Alternaria alternata* through *in vitro* assay.

Table 1: Production of Plant Growth Promoters (PGPs) by bacterial and algal isolates

No. of isolates	IAA ($\mu\text{g ml}^{-1}$)	GA($\mu\text{g ml}^{-1}$)	Carbohydrate ($\mu\text{g ml}^{-1}$)	No. of isolates	IAA ($\mu\text{g ml}^{-1}$)	GA ($\mu\text{g ml}^{-1}$)	Carbohydrate ($\mu\text{g ml}^{-1}$)
1	0.44	1.27	79.35	14	3.1	18.45	94
2	3.31	6.64	58.26	15	21.71	0.91	78.9
3	40.44	14.82	87.3	16	6.4	0.2	44.54
4	36.29	0.73	109.04	17	4.15	6.64	98.9
5	48	173.91	187.18	18	1.61	2.73	80.51
6	0.44	22.1	80.67	19	0.16	33	90.59
7	78.63	81.2	171.13	20	4.26	40.27	81.05
8	1.81	51.2	71.6	21	17.67	26.64	122.45
9	4.26	2.64	105.1	22	14.90	0.45	68.34
10	0.86	3.91	44	23	27.9	12.1	83.38
11	25.75	32.1	9.04	24	10.33	28.45	64.31
12	21.18	53.91	112.6	25	22.35	33	103.07
13	0.51	9.4	71.52	26	78.73	80.27	171
Algal isolates							
1	21.4	61.18	178.81	3	29.26	31	160.124
2	14.90	83.91	162.14	4	1.5	3.23	77.72

Table 2: Antagonistic activity of plant growth promoting rhizobacteria (PGPR)

No. of isolates	Inhibitory effect		No. of isolates	Inhibitory effect	
	<i>Fusarium</i> sp.	<i>Rhizoctonia</i> sp.		<i>Fusarium</i> sp.	<i>Rhizoctonia</i> sp.
1	-	+	14	++	++
2	+	+	15	+	++
3	-	++	16	++	++
4	+	++	17	+	+
5	+++	+++	18	-	-
6	+	++	19	+	+
7	+++	+++	20	-	-
8	++	++	21	-	+
9	+	-	22	+	++
10	-	++	23	+	+
11	+	+	24	-	+
12	+	++	25	-	++
13	+	+++	26	+++	+++

+++ More inhibition; ++ moderate inhibition; +Less inhibition; - Negative effect

The most efficient bacterial isolates Nos. 5, 7 and 26 were subjected to identification. The primary identification by Gram staining showed that isolate No. 5 appeared as a rod-shaped Gram-positive bacterium Figure. (1, a) while isolates Nos. 7 and 26 appeared as Gram-negative and rod-shaped bacteria produced yellowish green pigment on King's medium Figure. (1, b and c). According to 16S rRNA isolates Nos. 7 and 26 were identified as *Pseudomonas aeruginosa* Figure. (2). The sequence of

16S rDNA was obtained after DNA extraction and PCR amplification result in 1500bp Figure. (3). This result was similar to the result recorded by Sasirekha *et al.* (2012) who recorded that *P. aeruginosa* produced $80 \mu\text{g mL}^{-1}$ (5 fold) increase in IAA production on tryptophan supplementation (0.1 g L^{-1}) when compared to control (without tryptophan). On the other hand, Apastambh *et al.* (2016) found that eight isolates of fluorescent *pseudomonas* were positive for IAA production, which

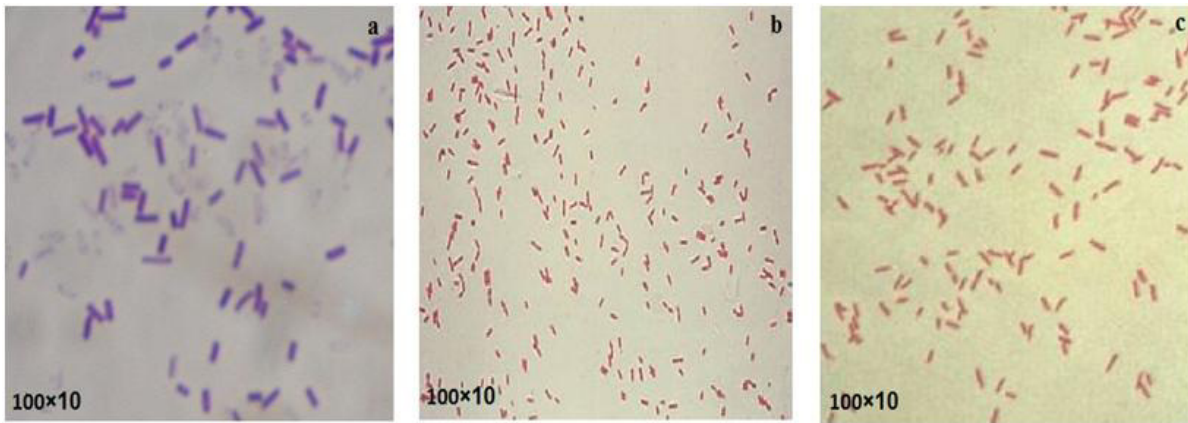


Figure 1: Gram staining of bacterial isolates, a) *Bacillus subtilis* b and c) *Pseudomonas aeruginosa*.

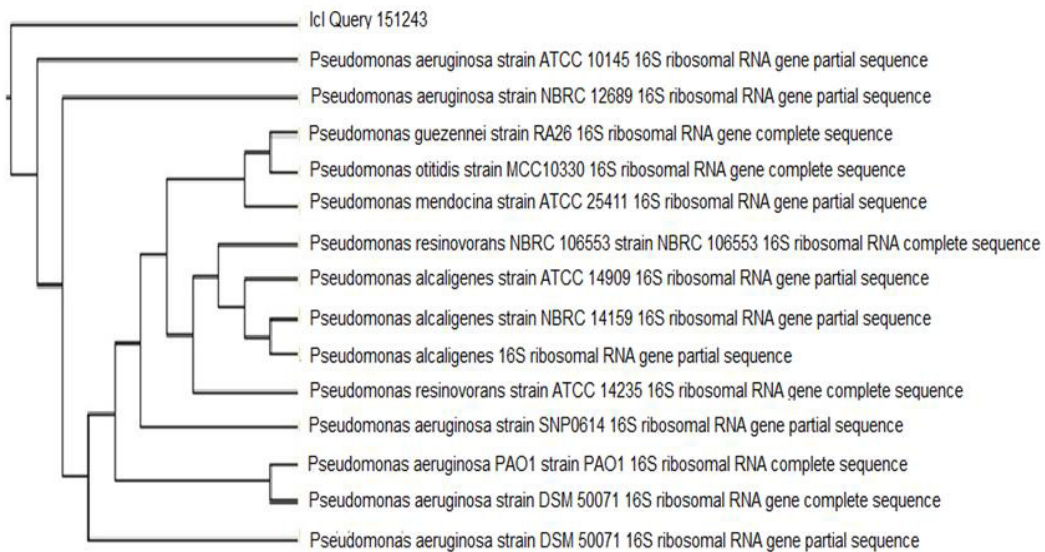


Figure 2: Phylogenetic tree illustrating the genetic relationship of *Pseudomonas aeruginosa* strain.

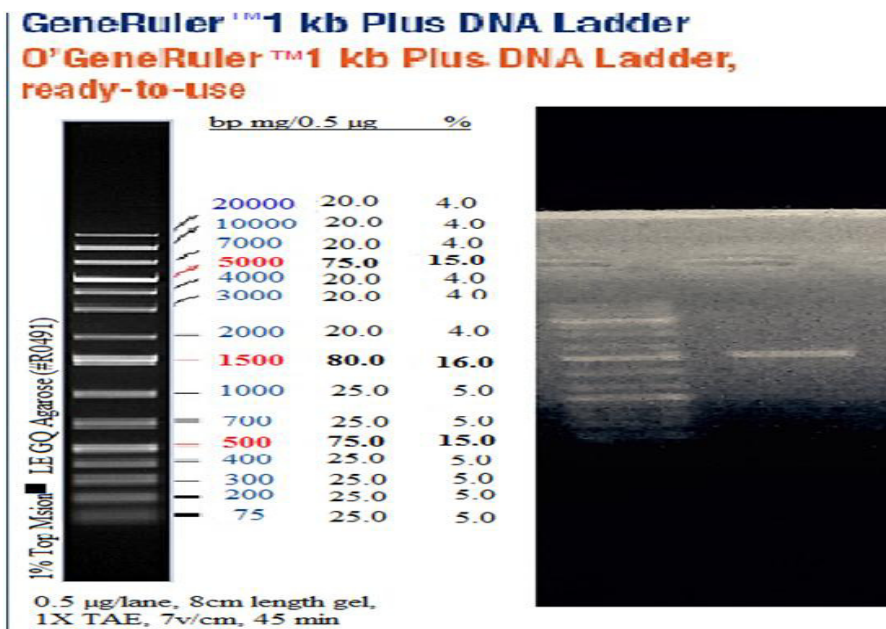


Figure 3: Right: Electrophoresis marker; Left: Separation of PCR product of 16S rDNA on 1% agarose gel and the sharp band produced by the strain 16S rDNA on the right. 1kb marker used is shown on the left.

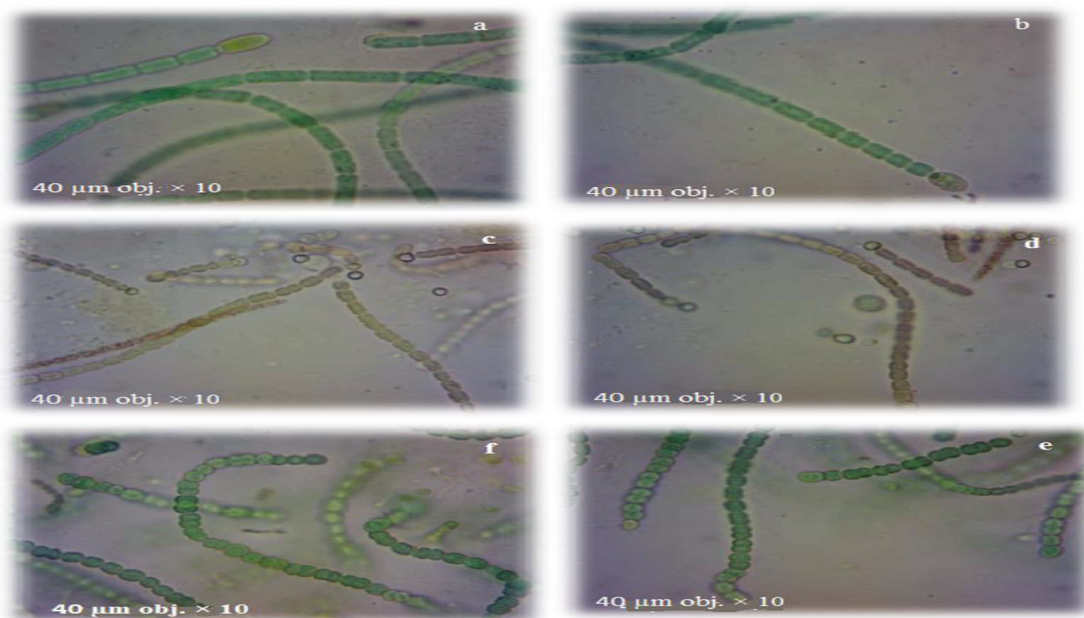


Figure 4: Selected algal isolates, a and b) Trichomes of *Anabaena* sp. showing twisted and coiled, heterocyst appears "empty," was ellipsoidal and terminal. c and d) Trichome of *Nostoc muscorum* showing the thallus including vegetative cells, heterocyst spherical and terminal. e and f) Trichome of *Anabaena variabilis* showing the thallus including vegetative cells.

ranged from 89 to 108 µg ml⁻¹. The isolate No. 5 was further identified by bio-log Technique as *Bacillus subtilis*. This result was in agreement with the previous study of Fekry (2014) who reported that *Bacillus subtilis* produce 125µg ml⁻¹ of GA. The result was harmony with Patil and

Shirsath (2015) who found that the concentration of carbohydrate in the exopolysaccharide in malt extract-glucose-yeast extract-peptone (MGYP) broth medium (used for the enrichment of the microorganisms) produced by *Bacillus subtilis* and it was found to be 495µg ml⁻¹.

Table 3: production of plant growth promoters PGPs by selected bacterial and algal isolates at different incubation periods

PGPs	IAA($\mu\text{g ml}^{-1}$)		GA($\mu\text{g ml}^{-1}$)		Carbohydrate($\mu\text{g ml}^{-1}$)				
Isolates Days	<i>Bacillus subtilis</i>	<i>Pseudomonas aeruginosa</i>	<i>Bacillus subtilis</i>	<i>Pseudomonas aeruginosa</i>	<i>Bacillus subtilis</i>	<i>Pseudomonas aeruginosa</i>			
1	8.15	13.09	32	22.09	159.43	155.7			
2	20.06	25.54	65.73	41.18	167.18	164.5			
3	53.202	78.78	173	83	187	171.52			
4	46.5	70.86	118.45	64.36	247.8	222.8			
5	32.14	63.414	80.73	54.82	232.95	203			
Cyanobacterial isolates									
	<i>Anabaena</i> sp.	<i>Nostoc muscorum</i>	<i>Anabaena variabilis</i>	<i>Anabaena</i> sp.	<i>Nostoc muscorum</i>	<i>Anabaena variabilis</i>	<i>Anabaena</i> sp.	<i>Nostoc muscorum</i>	<i>Anabaena variabilis</i>
5	1.82	2.78	3.15	3.41	6.00	2.18	146.87	99.50	81.80
10	5.22	4.45	8.95	21.10	19.36	8.45	156.29	126.60	122.30
15	14.58	8.31	15.91	34.82	41.20	18.45	167.60	151.83	148.85
20	23.84	15.91	29.61	61.20	83.91	34.82	179.58	162.91	159.74
25	14.16	12.88	18.31	57.64	80.27	30.27	231.33	193.73	195.20
30	9.4	7.13	14.22	49.56	73.04	25.11	226.11	188.1	189.95

Furthermore algal isolates were screened for their ability to produce IAA. Among the four isolates, isolate No.3 showed maximum IAA ($29.26\mu\text{g ml}^{-1}$) and isolate No.4 showed minimum IAA ($1.5\mu\text{g ml}^{-1}$) production extracellularly (Table1). The results also showed that all isolates have ability to produce GA in varying extent. GA production ranges from $3.23\mu\text{g ml}^{-1}$ to $83.91\mu\text{g ml}^{-1}$. Isolate No. 2 was the most active in GA production, that producing $83.91\mu\text{g ml}^{-1}$ Table (1). On the other hand algal isolates showed that the isolate No. 1 was the most active in carbohydrate production that producing $178.81\mu\text{gml}^{-1}$ Table (1).

Based on the description of Desikachary (1959) the isolate No.1 was *Anabaena variabilis* Fig. (4, a and b) the same trend of results were found by Anuj and Rajinder (2014) which reported that *Anabaena variabilis* produced highest amount of auxin (IAA). While isolate No.

2 was *Nostoc muscorum* Fig. (4, c and d), this result was in consonance with the work of Mostafa *et al.* (2010) who reported that *N. muscorum* produced GA in amount $0.286\text{ g }100\text{ ml}^{-1}$. However, isolate No. 3 was *Anabaena* sp Fig. (4, e and f), this result was in agreement with Romi *et al.* (2015) who revealed that *Anabaena* sp. BTA35 produced ($1.12\pm 0.06\text{ mg ml}^{-1}$) of exopolysaccharide and the total amount of exopolysaccharide (EPS) includes the released polysaccharides in the medium or slime polysaccharides.

The highest amounts of *phytohormones* (IAA and GA) and carbohydrate produced by *P. aeruginosa* and *B. subtilis* were detected at three days and four days incubation periods, respectively (Table 3). *B. subtilis* produced higher amounts of GA and carbohydrate compared to *P. aeruginosa*. The present result was conformity with the previous study of Morsy

(2005) who reported that the maximum production of IAA and GA by three strains of *Bacillus subtilis* was at 30°C after three days. This result also agree with that of Sirajunnisa *et al.* (2013) who concluded that maximum productivity of EPS by *Bacillus subtilis* was achieved at central point conditions of 35°C and pH 7 at 72h with 2 % inoculums (5.56 g L^{-1}). On the other hand, *P. aeruginosa* produced high amount of IAA in King's supplemented with tryptophan compared to *B. subtilis* Table (3). This result was similar to the results recorded by Bedhya *et al.* (2015) who revealed that IAA production by fluorescent *Pseudomonas* spp. increased gradually till three days of incubation, followed by decline after four days, this might be due to the release IAA degrading enzymes such as IAA oxidase and peroxidase as

was reported earlier in *Rhizobium* sp. from *Cajanus cajan* (Datta and Basu, 2000).

The amount of IAA, GA and carbohydrate produced by selected cyanobacterial strains increased gradually with increase the time of incubation till reach to specific time at which high amount of plant growth promoting substances were produced by all cyanobacteria strains Table (3). The results clearly indicated that *Anabaena variabilis* produced considerable amount of IAA (29.61 $\mu\text{g ml}^{-1}$) after 20 days, after this time of incubation the IAA productivity decreased. Our result was similar to that reported by Anuj and Rajinder (2014) who recorded *Anabaena variabilis* produced highest amount of auxin in the media supplemented with 100 $\mu\text{g ml}^{-1}$ tryptophan after 20 days of inoculation. Furthermore the highest amount of GA produced by *Nostoc muscorum* was detected at 20 days incubation periods (83.91 $\mu\text{g ml}^{-1}$). This was in harmony with Abd El-All and Abou El Kheir (2014) found that *N. muscorum* and *A. oryzae* produced high amount of GA 2859 mg l^{-1} and 8839 mg l^{-1} respectively after 30 days when grown on BG11 medium in a growth chamber under continuous illumination (3000 lux) at $25^{\circ}\text{C} \pm 2$. Result in Table (3) revealed that the highest total carbohydrate content of 231.33 $\mu\text{g ml}^{-1}$ was due to *Anabaena* sp. followed by 195.20 $\mu\text{g ml}^{-1}$ and 193.73 $\mu\text{g ml}^{-1}$ for *Anabaena variabilis* and *Nostoc muscorum*, respectively after 25 days. The same trend of result were found by Romi *et al.* (2015) who revealed that the accumulation of exopolysaccharide (EPS) by *Anabaena* sp. BTA35 increased during growth over time and the maximum amount was obtained from the cultures on day 28; several days after the organisms had entered stationary phase growth. EPS production increased linearly for the first 20 days after inoculation, followed by a more rapid increase as the cultures approached stationary phase.

CONCLUSION

In recent years, more and more attention has been paid worldwide to use plant growth promoter substances produced by bacteria and algae to intensify organic plant production, particularly in the context of changing climate and increasing saline soil. Thus, it is concluded that bacteria and algae isolated from the rhizospheric saline soil sample were found to be useful in the production of plant growth promoting substances and these species of *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Anabaena* sp., *Nostoc muscorum* and *Anabaena variabilis* would be promising agents to help crop productivity.

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