Egypt. J. Microbiol. Special Issue "13th Conf. of Microbiol.", pp.45-61 (2010)

Effect of Carbon Source and Precursors on the Production of Plant Growth Regulators by *Azotobacter chroococcum* (R19) and *Bacillus megaterium* var. *phosphaticum* (R44)

A. GH. Rahal, R. A. Zaghloul*, N. A. Neweigy*, Ehsan A. Hanafy* and Rasha M. El-Meihy*
Soils; Water and Environment Research Institute, Agricultural Research Centre, Giza and *Faculty of Agriculture, Moshtohor, Benha University, Qalubia, Egypt.

AN EXPERIMENT was carried out to examine the effect of carbon source, tryptophan and adenine concentrations on the production of growth regulators by *A. chroococcum* (R19) and *B.megaterium* var. *phosphaticum* (R44). Mannitol and glucose were the best carbon sources for the production of plant growth regulators (PGRs) by *A. chroococcum* and *B. megaterium* var. *phosphaticum*, respectively. *A. chroococcum* produced higher amounts of zeatin and kinetin compared to those produced by *B. megaterium* var. *phosphaticum* while *B. megaterium* var. *phosphaticum* produced higher amounts of (9R) benzyl adenine and (9G) benzyl adenine compared with those produced by *A. chroococcum*. Production of auxins, gibberellic acid (GA_3) and cytokinins was increased with increasing tryptophan concentration. The highest amounts of PGRs produced by the two strains were obtained with tryptophan at 1000µM.

Highest amounts of PGRs produced by *A. chroococcum* and *B. megaterium* var. *phosphaticum* at 10 and 100 µM of adenine, respectively. Also, the produced amounts of gibberellic acid and cytokinin than that produced by *B. megaterium* var. *phosphaticum*. Generally, obtained data showed that the application of the optimal conditions together gave highest amounts of PGRs as compared with the other individual factors. This result is logic and was anticipated.

Keywords: PGPR, PGRs, Carbon sources, Tryptophan, Adenine, TLC, GLC, *Azotobacter, Bacillus.*

L-tryptophan is considered as the precursor for indole-3- acetic acid and other auxins production by microorganisms. Furthermore, L-tryptophan is considered as a substrate for GA_3 production. Adenine is considered the most suitable one among some precursors for cytokinins production by microorganisms.

Vessey (2003) and Morsy (2005) stated that glucose at 10 g L^{-1} was the best carbon source for PGRs production by plant growth promoting rhizobacteria (PGPR). L-tryptophan serves as a substrate for auxins and GA_3 production (Khalid *et al.* , 2004 and Zahir *et al.*, 2005). Adenine is considered the most suitable one among some precursors for cytokinins production by microorganisms (Arshad & Frankenberger, 1991). PGPR isolated from rhizosphere of various crops have the ability to produce auxins as secondary metabolities, various metabolic pathways such as :(1) Indole -3- acetamide pathway (2) Indole-3-pyrovic acid pathway (3) Tryptamide pathway (4) Tryptophan side chain pathway and (5) Indole -3-acetonitrile pathway are involved in the production of IAA (Pallai , 2005).

One of the common biosynthetic pathway utilize adenine as the precursor of free cytokinins production in PGPR (*Azotobacter* spp.). Also addition of precursors such as adenine and isopentyl alcohol to the culture medium of *A. chroococcum* resulted in enhanced growth of *Raphanus sativus* under genotobiotic and greenhouse conditions (Pallai , 2005).

Therefore, this research aims to determine the best carbon source and precursors for maximum growth regulators production by *A. chroococcum* and *B. megaterium* var. *phosphaticum.*

Material and Methods

Effect of different carbon sources on PGRs production

Specific media were prepared for either *A. chroococcum* and *B. megaterium* var. *phosphaticum* and supplemented with different carbon sources namely fructose, sucrose, mannitol, glucose and a mixture of them .Concentration of each applied sugar was 2 and 0.5 % for *A. chroococcum* and *B. megaterium* var. *phosphaticum*, respectively.

Ashbey's medium (Abdel-Malek & Ishac, 1986) was inoculated with 24-48hr old culture of *A. chroococcum* and incubated at 32°C for 4 days whereas, modified Bunt and Rovira medium (Abdel-Hafez , 1966) was inoculated with *B. megaterium* var. *phosphaticum* and incubated at 30°C for 2 days. The produced PGRs were detected by thin layer chromatography (TLC) and determined by gas chromatography (GC) to find out the most suitable carbon source for maximum PGRs production .

Effect of different concentrations of DL - tryptophan on PGRs production

Specific media for the investigated bacterial strains were provided with different concentrations of tryptophan (1000–0.01 µM) then, sterilized, inoculated with strains and incubated at 32°C for 4 days and at 30°C for 2 days for *A. chroococcum* and *B. megaterium* var. *phosphaticum*, respectively. The produced PGRs were detected by TLC and determined by GLC

according to Mazur & Homme (1993) to determine the most suitable concentration of tryptophan that can give high PGRs production .

Effect of different concentrations of adenine on PGRs production

Flasks containing the specific media were supplemented with different concentrations of adenine (1000 µM to 0.0001 µM*)*. Then, sterilized and inoculated with the tested strains of (24-48 hr old culture) and incubated at 32°C for 4 days and at 30°C for 2 days for *A. chroococcum* and *B. megaterium* var. *phosphaticum*, respectively. The produced PGRs was detected by TLC and determined by GLC as mentioned before to determine the most suitable concentration of adenine that can give high production of PGRs.

Optimal conditions for PGRs production

The optimal conditions for PGRs production by *A. chroococcum* are: Incubation temperature (32°C); incubation period (4 days); tryptophan concentration (1000 µM) ; adenine concentration (10 µM) and mannitol as a suitable carbon source. Whereas, the optimal conditions for PGRs production by *B. megaterium* var. *phosphaticum* are: Incubation temperature (30°C); incubation period (2 days); tryptophan concentration (1000 µM) ; adenine concentration (100 µM) and glucose as a suitable carbon source.

Specific media were inoculated with standard inoculum of 24 – 48 hr old culture of *A. chroococcum* or *B. megaterium* var. *phosphaticum*, respectively. Then incubated at 32°C for 4 days and at 30°C for 2 days for both cultures, respectively. Then PGRs were extracted and determined by GLC as mentioned before .

Analysis methods

The analysis of plant growth regulators was identified by thin layer chromatography and determined by gas liquid chromatography in Soil Microbiology Department , Soils, Water and Environment Res. Inst., Agric. Res. Centre, Giza, Egypt .

Thin layer chromatography (TLC) assessment

Detection of IAA using TLC was accomplished by spotting 10 µL of the methanol extracts on fluorescent silica gel plates 0.25 mm thickness (Merch, Germany). The spots of the respective culture extracts and phytohormone standard solutions were developed in the following solvent systems:

A) Freshly prepared solvent mixture of chloroform : ethyl acetate: acetic acid (60: 40: 5) for auxins and gibberellins (Salamone *et al.*, 2001 and Ahmad *et al.*, 2005).

B) Freshly prepared solvent mixture of chloroform: methanol (9: 1) for cytokinins (Holl *et al.*, 1988).

After the development the TLC plates were dried at 50 °C for 5 min, then they were exposed to UV radiation at 350 nm wave length to detect the compounds and measure their R_f values (Torres-Rubio *et al.*, 2000).

Gas-chromatography (GC) assessment

Extracted samples were silylated before GC analysis as follow:
Aliquots of 0.5 to 1 ml of each fraction sample and standard solutions were placed individually in 5 ml test tubes and evaporated to dryness, then 100µL of N,O-bis-(trimethylsilyl) acetamide (BSA) [Sigma] was added to prepare trimethylsilyl derivatives (TMSi). The test tubes were immediately capped and heated to (50 – 60°C) for 30 min and evaporated to dryness to remove the access of (BSA), then the residue was dissolved in 0.2 ml absolute methanol alcohol (Mazur & Homme, 1993 and Rahal *et al.*, 2006).

One µL of each TMS derivative sample was injected into a split-splitless HP 5890 series II Gas chromatography equipped with a flame ionization detector, and capillary column HP_5 (0.32 mm internal diameter, 25 m long and 0.1 µm film thikness of methyl silica gum).

The GC temperature program was (185°C - 250°C) at rate of 10°C min^{-1}, then 2 min at 250°C, injector and detector temperatures were 250°C and 270°C, respectively . The nitrogen carrier gas flow rate of at 5 ml. min^{-1} and the flow rate of hydrogen and air for the flame ionization detector was 30 and 300 ml. min^{-1}, respectively .

Retention time (R_t) for each peak of the authentic standard materials was recorded and the amounts of separated compounds were calculated automatically by computer unit and printed .

Standard compounds used in TLC and GC analyses

Indole -3- acetic acid (IAA) , Indole-3- butyric acid (IBA) and *t*-Zeatin were obtained from Sigma; Adenine (Merch , Germany); Tryptophan was obtained from Lopa, (India) and GA_3, (9R) Benzyl adenine {(9R)BAP}, (9G) Benzyl adenine {(9G)BAP} and isopentyl alcohol (IP) were obtained from Genetic Engineering Research Inc. (El-Sadat City).

Results and Discussion

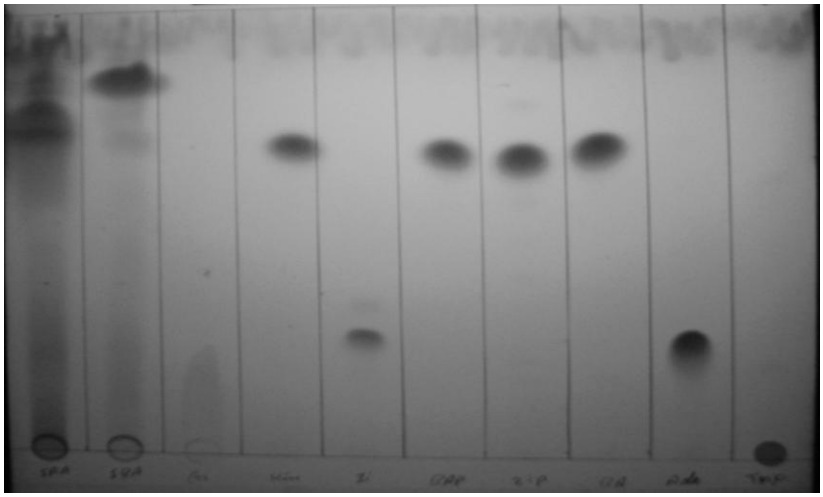
Retention flow of pure growth regulators, adenine and tryptophan

Technical growth regulator agents, adenine and tryptophan were developed by TLC to determine their retention flow (R_f) values to be as reference to those obtained from sample extracts (Martinez-Toledo *et al.*,

1988 ; Srinivasan *et al.*, 1996; Salamone *et al.*, 2001 and Torres-Rubio *et al.*, 2000). The obtained values are presented in Table 1 and shown in Fig. 1 a & 1 b.

TABLE 1. Retention flow (R_f) values of the pure phytohormones – like substances developed by thin layer chromatography (TLC) with two different development systems.

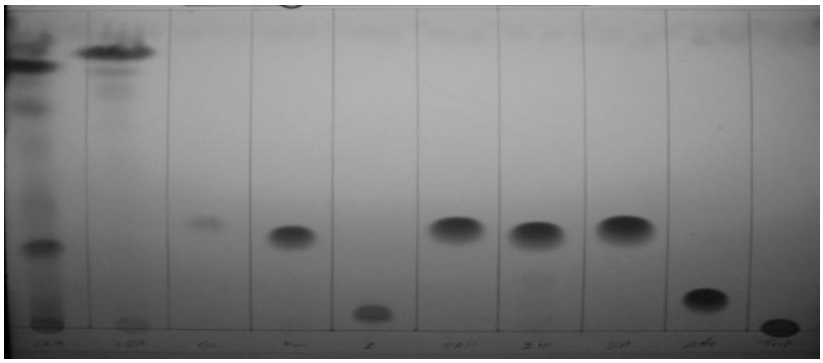
Compounds	Retention flow (R_f) (cm)	
	In auxins development system (1)	In cytokinins development system (2)
Indole acetic acid (IAA)	0.25 , 0.38 , 0.55 , 0.69	0.71 , 0.75
Indole butyric acid (IBA)	0.86	0.81
Gibberellic acid (GA_3)	0.33	0.16
Zeatin (Z)	0.05	0.3
Kinetin (KIN)	0.28	0.66
(9R) Benzyl adenine (BAP)	0.31	0.65
(9G) Benzyl adenine (BAP)	0.29	0.64
Isopentyl alcohol (IP)	0.28	0.63
Adenine (ADE)	0.12	0.24
Tryptophan (TRY)	zero	zero



1- IAA : Indole acetic acid 6- (9G) BAP : Benzyl adenine .

2- IBA : Indole butyric acid . 7- IP :Isopentyl alcohol
3- GA_3 : Gibberellic acid . 8- (9R) BAP : Benzyl adenine
4- KIN : Kinetin . 9- ADE : Adenine
5- Z : Zeatin 10- TRY : Tryptophan

Fig. 1a. TLC separation for authentic phytohormones- like substances using auxins separation development system.



1- IAA : Indole acetic acid 6- (9G) BAP : Benzyl adenine .
2- IBA : Indole butyric acid . 7- IP :Isopentyl alcohol
3- GA_3 : Gibberellic acid . 8- (9R) BAP : Benzyl adenine
4- KIN : Kinetin . 9- ADE : Adenine
5- Z : Zeatin 10- TRY : Tryptophan

Fig. 1b. TLC separation for authentic phytohormones – like substances using cytokinins separation development system .

Effect of carbon sources on PGRs production

Qualitative analysis of PGRs produced in presence of different carbon sources

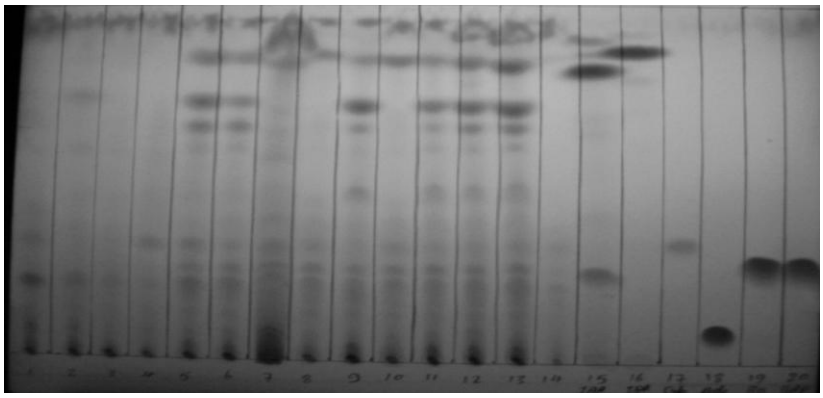
Figure 2a shows the TLC separation of growth regulators extracted from cultures grown with different sugars as a carbon source. The results indicated that *A. chroococcum* produced IAA, IBA and GA_3 when mannitol or sucrose was used individually as a carbon source. Whereas, IAA and GA_3 were the only product when glucose or fructose was used.

Concerning the PGRs produced by *B. megaterium* var. *phosphaticum* , data in Fig. 2a revealed that three compounds having R_f 0.24, 0.33 and 0.86 were identified as IAA , IBA and GA_3 as the main product when glucose was used as a carbon source. While, IAA and GA_3 were the main products when mannitol, fructose and sucrose were used as a carbon source.

Data in Fig. 2b showed the TLC separation of PGRs produced by *A. chroococcum* and *B. megaterium* var. *phosphaticum* in presence of different carbon sources using cytokinins development system. TLC chromatogram show that *A. chroococcum* produced kinetin compound when glucose or fructose was added as a carbon source, while, zeatin was produced when mannitol or sucrose was used. IBA compound was produced only by *A. chroococcum* when the

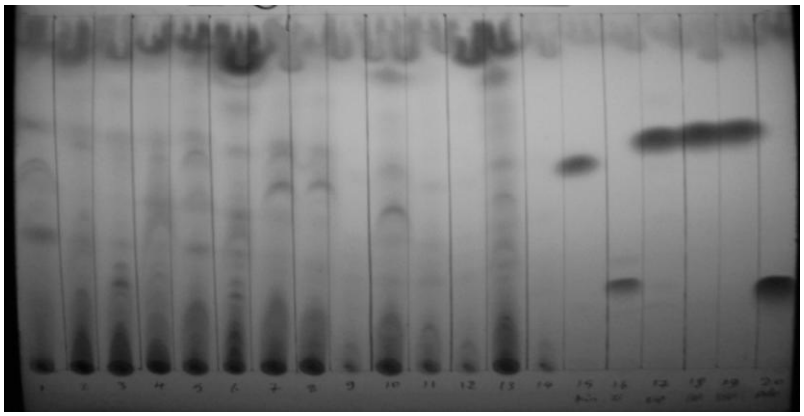
mixture of either (mannitol + glucose) or (sucrose + fructose) were used as carbon sources.

Concerning the PGRs produced by *B. megaterium* var. *phosphaticum*, data in Fig. 2 b indicated that three compounds having R_f 0.33, 0.65 and 0.66 were produced when glucose was used as a carbon source. These compounds were identified as Z, (9R)BAP and KIN, respectively. Zeatin was the only detected compound when mannitol was used as a carbon source.



1-*Bacillus* (glucose) 6-*Azotobacter* (mannitol) 11-*Azotobacter* (sucrose + glucose)
2-*Bacillus* (sucrose) 7-*Azotobacter* (fructose) 12-*Azotobacter* (mannitol + fructose)
3-*Bacillus* (mannitol) 8-*Azotobacter* (glucose) 13-*Azotobacter* (mannitol + glucose)
4-*Bacillus* (fructose) 9-*Azotobacter* (sucrose + mannitol) 14-*Azotobacter* (glucose + fructose)
5-*Azotobacter* (sucrose) 10-*Azotobacter* (sucrose + fructose)

Fig. 2a. TLC separation of auxins and gibberellins produced by *A. chroococcum* and *B. megaterium* var. *phosphaticum* grown in presence of different carbon sources using auxins development separation system.



1-*Bacillus* (glucose)
2-*Bacillus* (sucrose)
3-*Bacillus* (mannitol)
4-*Bacillus* (fructose)
5-*Azotobacter* (sucrose)
6-*Azotobacter* (mannitol)
7-*Azotobacter* (fructose)
8-*Azotobacter* (glucose)
9-*Azotobacter* (sucrose + mannitol)
10-*Azotobacter* (sucrose + fructose)
11-*Azotobacter* (sucrose + glucose)
12-*Azotobacter* (mannitol + fructose)
13-*Azotobacter* (mannitol + glucose)
14-*Azotobacter* (glucose + fructose)

Fig. 2b. TLC separation of cytokinins produced by *A. chroococcum* and *B. megaterium* var. *phosphaticum* grown in presence of different carbon sources using cytokinins development separation system.

Quntitative analysis of the produced PGRs

Table 2 shows the PGRs amounts produced as a result of different sugar application. Results indicated that both investigated strains produced PGRs under all applied carbon sources. Data also showed that mannitol was the most convenient carbon source for PGRs production by *A. chroococcum*, whereas, glucose was the best carbon source for PGRs production by *B. megaterium var. phosphaticum*.

TABLE 2. Quntitative analysis of the PGRs produced under application of different carbon sources (mg . L^{-1}).

Carbon sources	Auxins		Gibberellin	Cytokinins				
	IAA	IBA	GA_3	Z	KIN	(9R) BAP	(9G) BAP	IP
Azotobacter chroococcum								
Glucose	14.0	37.7	38.3	53.9	82.9	30.7	ND	ND
Fructose	8.40	26.7	44.8	ND	82.6	52.2	ND	ND
Sucrose	15.0	34.3	25.7	54.7	81.5	39.5	ND	5.90
Mannitol	18.6	39.3	66.2	64.7	97.4	79.2	0.72	7.30
Bacillus megaterium var. *phosphaticum*								
Glucose	18.6	46.2	94.6	55.9	64.6	66.4	ND	4.40
Fructose	3.40	38.3	6.13	37.9	45.6	55.2	1.97	3.90

Sucrose	**5.30**	**45.4**	**21.1**	**35.9**	**56.7**	**60.0**	**3.90**	**ND**
Mannitol	**ND**	**ND**	**0.00**	**41.2**	**64.6**	**66.4**	**ND**	**4.40**

Abbreviations : as those stated for Table 1 .

Generally, the data show that *A. chroococcum* produced higher amounts of IAA than *B. megaterium* var. *phosphaticum* along with application of any carbon sources, whereas, the contrary occurred for IBA production by both tested strains. The effect of carbon sources on cytokinins production, data revealed that *A. chroococcum* produced higher amounts of zeatin and kinetin than that produced by *B. megaterium* var. *phosphaticum*. Also, the produced amounts of cytokinins by both strains under application of different carbon sources were higher than those produced from auxins and gibberellins (GA_3) .

Similar results were observed by Martinez-Toledo *et al.* (1988) who found that *A. chroococcum* grown on both of the two medium states (supplemented with 0.5% glucose or amended with maize root exudates) stimulated the production of all phytohormones.

Morsy (2005) studied the effect of different carbon sources (glycerol, glucose, fructose, mannose, xylose, arabinose and sucrose) for maximum IAA and GA_3 production by three strains of *B. subtilis* 47, 82 and 104. The author found that glucose, fructose and glycerol were the best carbon sources , respectively.

Effect of tryptophan on PGRs production

Qualitative analysis of PGRs produced in presence of different tryptophan concentrations

Figure 3a shows the separation of PGRs compounds produced by *A. chroococcum* and *B. megaterium* var. *phosphaticum* under application of different tryptophan concentrations using auxins development separation system. Obtained data showed that the application of tryptophan with concentrations (1000 – 100 µM) activated the production of IAA and IBA by *A. chroococcum*. The application of tryptophan with 0.01 M increased the production of IAA and GA_3. On the other hand, *B. megaterium* var. *phosphaticum* produced Z , IAA , GA_3 and IBA when tryptophan was applied with concentration 10 µM. Also, the same compounds exept IBA were detected with the application of other tryptophan concentrations.

Figure 3b emphasize the PGRs produced by *A. chroococcum* and *B. megaterium* var. *phosphaticum* under different tryptophan concentration detected by using cytokinin development separation system . Obtained results showed that IP , (9R)BAP , KIN and IAA compounds were produced by *A. chroococcum* when tryptophan was applied with 1000 µM. The same

compoundes exept KIN were detected when tryptophan was applied with 0.01 µM .

Figure 3 (a & b) showed that growth regulators GA_3, Z and KIN produced by *B. megaterium* var. *phosphaticum*, when tryptophan was applied with 1000 µM, whereas its application with 100 µM tryptophan, GA_3, Z, (9G) BAP and IBA were produced with the other applied concentrations of tryptophan. *B. megaterium* var. *phosphaticum* produced Z, (9R)BAP, IP, IAA and KIN. Identification of these PGRs were achieved by TLC according to their retention flow rates.

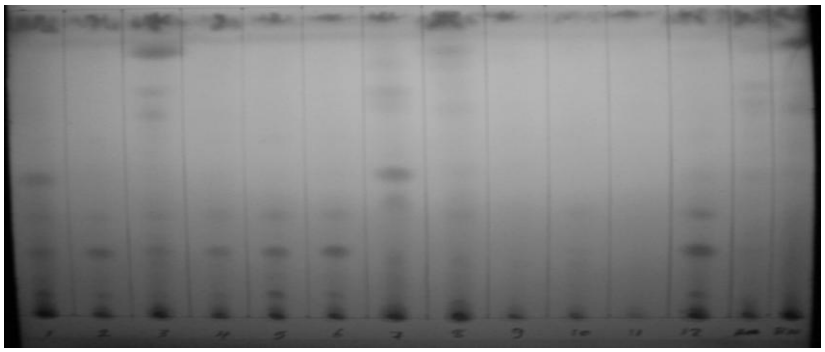
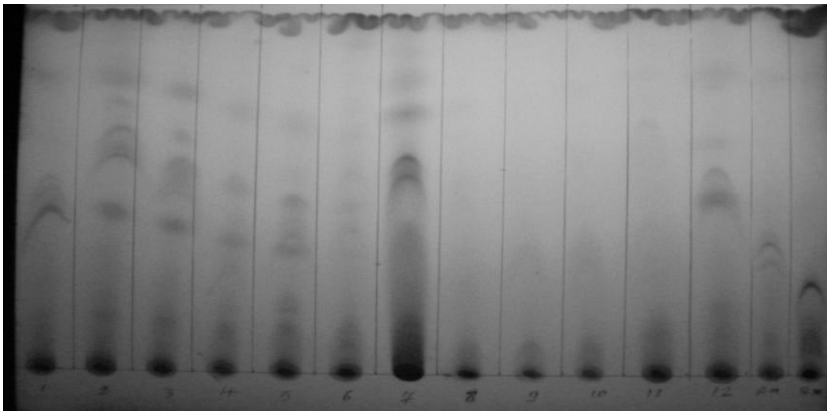


Fig. 3a. TLC separation of auxins and gibberellins produced by *Azotobacter chroococcum* and *Bacillus megaterium* var. *phosphaticum* grown in presence of different tryptophan concentrations using auxins development separation system.

1-	*Bacillus* (10^{-3})	5-	*Bacillus* (10^{-7})	9-	*Azotobacter* (10^{-5})
2-	*Bacillus* (10^{-4})	6-	*Bacillus* (10^{-8})	10-	*Azotobacter* (1)
3-	*Bacillus* (10^{-5})	7-	*Azotobacter* (1000)	11-	*Azotobacter* (0.1)
4-	*Bacillus* (10^{-6})	8-	*Azotobacter* (100)	12-	*Azotobacter* (0.01)



1-*Bacillus* (10^{-3})	5-*Bacillus* (10^{-7})	9-*Azotobacter* (10^{-5})
2-*Bacillus* (10^{-4})	6-*Bacillus* (10^{-8})	10-*Azotobacter* (1)

3-*Bacillus* (10^{-5}) 7-*Azotobacter* (1000) 11-*Azotobacter* (0.1)
4-*Bacillus* (10^{-6}) 8-*Azotobacter* (100) 12-*Azotobacter* (0.01)

Fig. 3b. TLC separation of cytokinins produced by *A. chroococcum* and *Bacillus megaterium* var. *phosphaticum* grown in presence of different tryptophan concentrations using cytokinins development separation system.

Quantitative analysis of the PGRs produced under different tryptophan concentrations

Data in Table 3 clearly showed that the production of auxins, gibberellic acid and cytokinins increased with increasing tryptophan concentration. These results were observed with *A. chroococcum and B. megaterium* var. *phosphaticum* strains.

TABLE 3. Quantitative analysis of the PGRs produced in presence of different tryptophan concentrations. (mg . L^{-1}).

Tryptophan concentrations *(molar)*	Auxins		Gibber-ellin	Cytokinins				
	IAA	IBA	GA_3	Z	KIN	(9R) BAP	(9G) BAP	IP
	Azotobacter chroococcum							
0.01	2.80	ND	12.4	23.0	13.2	ND	ND	ND
0.1	3.34	12.6	12.7	24.4	18.5	ND	ND	ND
1	5.52	10.4	17.8	30.0	22.3	ND	ND	ND
10	5.71	18.0	23.3	31.9	33.8	ND	ND	0.91
100	6.64	25.3	45.6	33.7	48.1	ND	0.30	1.92
1000	10.3	37.1	85.5	42.5	49.4	17.6	1.44	4.75
	Bacillus megaterium var. phosphaticum							
0.01	ND	ND	ND	14.8	14.0	12.0	ND	ND
0.1	8.11	10.7	16.3	18.5	18.2	24.0	ND	ND
1	8.53	18.0	21.4	19.2	26.6	29.3	0.46	0.87
10	9.30	18.8	44.0	23.1	33.7	41.9	0.94	1.24
100	11.2	18.9	62.2	36.5	54.2	49.6	2.32	2.22
1000	13.6	24.9	76.4	41.3	93.5	68.3	2.81	6.32

Abbreviations : as those stated for Table 1 .

These results are in harmony with those obtained by Khalid *et al.* (2004) and Zahir *et al.* (2005) who found that L-tryptophan serves as a physiological precursor for auxins in plant and microbes of the rhizobacterial isolates (*Azospirillum* sp., *Bacillus* sp. and *Pseudomonas* sp.), 83% produced auxins in absence of L- tryptophan, whereas 100% produced auxins in presence of L-tryptophan. Most isolates of rhizobacteria were likely to be due to L- tryptophan serving as an auxins precursor rather than any precursor.

The maximum amounts of auxins, gibberellic acid and cytokinins produced by the two strains were recorded when 1000 µM of tryptophan was applied, while, the minimum amounts of these compounds were produced when 0.01 molar of tryptophan was used.

Concerning the effect of different tryptophan concentrations on cytokinins production, data in Table 3 showed that *A. chroococcum* produced higher amounts of zeatin compared to those produced by *B. megaterium* var. *phosphaticum*. On the contrary, *B. megaterium* var. *phosphaticum* strain produced higher amounts of kinetin, (9R) benzyl adenine, (9G) benzyl adenine and isopentyl alcohol compared to those produced by *A. chroococcum* under all applied different concentration of tryptophan.

Effect of adenine concentrations on PGRs production

Qualitative analysis of the PGRs produced in presence of different adenine concentrations

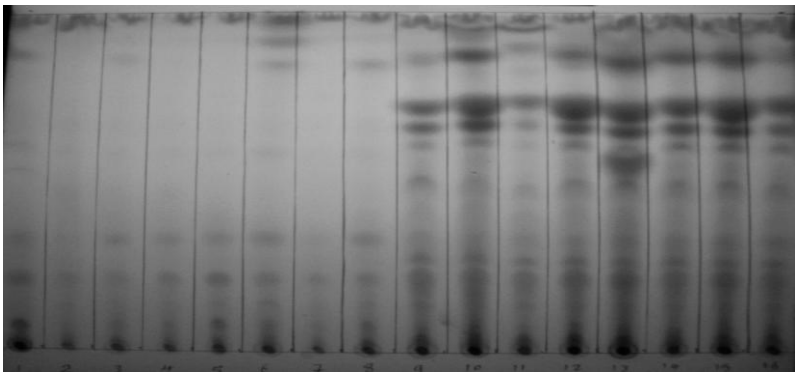
Figure 4 a shows different PGRs compounds produced by *A. chroococcum* and *B. megaterium* var. *phosphaticum* under application of different adenine concentrations, which were detected by thin layer chromatography using auxin development separation system.

***A. chroococcum* grown in presence of 1000 µM adenine, three compounds having R_f values (0.24, 0.32 and 0.86) were appeared and identified as IAA, GA_3 and IBA, respectively . Also, IAA and IBA were detected when applied the other concentrations of adenine.**

Moreover, data in Fig. 4 a showed that *B. megaterium* var. *phosphaticum* produced IAA , GA_3 and IBA when adenine was applied at concentrations of 1000 and 10 µM, whereas , Z , IAA , GA_3 and IBA were produced when it was added with 0.01 and 0.0001 µM. On the other hand, the separation of the extract components with of cytokinins development system indicated that the produced PGRs compounds by *A. chroococcum* and *B. megaterium* var. *phodsphsticum* had many different compounds shown in Fig. 4 b .

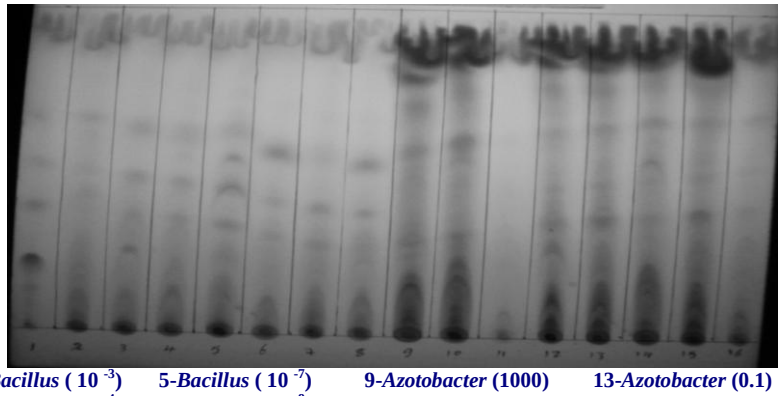
Obtained results indicated that the compounds (9G)BAP and IAA were detected in the extracted culture of *A. chroococcum* when 1000 µM of adenine was applied, while, Z and KIN detected when 100 µM of adenine was applied . Two another compounds were produced have R_f values (0.3 and 0.63) can be identified as Z and IP , respectively .

Concerning the produced compounds by *B. megaterium* var. *phosphaticum*, Fig. 4 b shows that, zeatin was produced under all investigated concentrations of adenine except the concentration 1 µM, since, KIN was the only produced compound. Also, Z , IP , ADE and (9G)BAP were detected when 1 µM adenine was applied.



1- *Bacillus* (10^{-3}) 5-*Bacillus* (10^{-7}) 9-*Azotobacter* (1000) 13-*Azotobacter* (0.1)
2-*Bacillus* (10^{-4}) 6-*Bacillus* (10^{-8}) 10-*Azotobacter* (100) 14-*Azotobacter* (0.01)
3-*Bacillus* (10^{-5}) 7-*Bacillus* (0.001) 11-*Azotobacter* (10^{-5}) 15-*Azotobacter* (0.001).
4-*Bacillus* (10^{-6}) 8-*Bacillus* (0.0001) 12-*Azotobacter* (1) 16-*Azotobacter* (0.0001).

Fig. 4a. TLC separation of auxins and gibberellins produced by *A .chroococcum* and *B. megaterium* var. *phosphaticum* grown in presence of different adenine concentrations using auxins development separation system.



1-*Bacillus* (10^{-3}) 5-*Bacillus* (10^{-7}) 9-*Azotobacter* (1000) 13-*Azotobacter* (0.1)
2-*Bacillus* (10^{-4}) 6-*Bacillus* (10^{-8}) 10-*Azotobacter* (100) 14-*Azotobacter* (0.01)
3-*Bacillus* (10^{-5}) 7-*Bacillus* (0.001) 11-*Azotobacter* (10^{-5}) 15-*Azotobacter* (0.001).
4-*Bacillus* (10^{-6}) 8-*Bacillus* (0.0001) 12-*Azotobacter* (1) 16-*Azotobacter* (0.0001).

Fig. 4b. TLC separation of cytokinins produced by *A. chroococcum* and *B. megaterium* var. *phosphaticum* grown in presence of different adenine concentrations using cytokinins development separation system.

Quantitative analysis of the PGRs produced in presence of different adenine concentrations

It is obvious from data in Table 4 that, the highest amounts of PGRs produced by *A. chroococcum* strain were detected when 0.01 µM of adenine

was used, whereas, the highest amounts produced by *B. megaterium* var. *phosphaticum* strain were detected when 100 µM of adenine was used .

In addition, the lowest production of PGRs by the two strains were detected when the application of adenine was decreased to 0.0001 µM. Moreover, at the optimum adenine concentration (0.1 and 0.01 µM) the production of IBA by *A. chroococcum* and *B. megaterium* var. *phosphaticum* was higher than IAA .

Also, the data indicated that *A. chroococcum* and *B. megaterium* var. *phosphaticum* strains produced higher amounts of kinetin and (9R) benzyl adenine than other cytokinins compounds. Similar trends of results was recorded with the different adenine concentrations. It is important to mention that *A. chroococcum* strain produce higher concentrations of gibberellic acid rather than those produced by *B. megaterium* var. *phosphaticum* strain. Also, *A. chroococcum* produced higher values of cytokinin compounds at maximum adenine concentration than that produced by *B. megaterium* var. *phosphaticum* strain.

TABLE 4. Quantitative analysis of the PGRs produced in presence of different adenine concentrations (mg . L^{-1}).

Adenine concentrations *(Molar)*	Auxins		Gibber-ellin	Cytokinins				
	IAA	IBA	GA_3	Z	KIN	(9R)BAP	(9G)BAP	IP
	Azotobacter chroococcum							
0.0001	4.70	ND	22.6	20.7	ND	36.9	0.50	ND
0.001	9.20	ND	35.7	57.3	45.6	70.4	1.40	ND
0.01	8.30	22.6	42.6	11.0	46.6	72.5	1.70	ND
0.1	10.6	23.7	45.3	71.7	52.8	71.9	2.80	ND
1	8.00	33.7	52.3	37.7	56.6	76.2	3.00	5.10
10	14.3	41.8	59.8	79.3	92.8	96.2	4.50	9.46
100	10.6	ND	48.0	51.7	86.6	82.3	2.00	9.20
1000	5.90	ND	42.7	76.8	69.9	70.3	1.00	12.0
	Bacillus megaterium var. phosphaticum							
0.0001	2.40	10.4	7.01	8.90	ND	13.7	ND	ND
0.001	0.40	11.3	9.43	16.4	12.3	21.3	ND	ND
0.01	1.40	22.3	13.0	17.3	61.5	30.1	ND	ND
0.1	1.00	22.4	13.3	39.7	63.9	35.2	ND	1.10
1	2.30	25.1	17.2	46.7	76.7	43.9	0.67	2.00
10	12.9	28.4	23.8	58.5	82.6	66.7	0.80	2.00
100	5.40	40.4	30.4	69.3	89.7	85.2	1.60	2.20
1000	4.50	ND	13.1	43.1	73.1	19.8	ND	ND

Abbreviations : as those stated for Table 1 .

Similar trend of results was observed by Nieto & Frankenberger (1989 a) who studied the effect of various concentrations (10, 1, 0.1 µM) of adenine on biosynthesis of cytokinins by *A. chroococcum*. He found that 10 µM was the best concentration for zeatin production.

Also , Arshad & Frankenberger (1991) found that the application of adenine at 10 M to *Azotobacter chroococcum* culture was the best one among several applied concentrations (1000 - 1 µM) for cytokinins production .

Optimal conditions and PGRs production by Azotobacter chroococcum and Bacillus megaterium var. phosphaticum

Obtained results recorded in Table 5 showed that the optimal conditions gave the highest production of PGRs. This result is logic and was anticipated. Data recorded in Table 5 clearly indicated that *B. megaterium* var. *phosphaticum* produced higher amounts of auxins rather than *A. chroococcum*, while, the reverse occurred with gibberellic acid production.

TABLE 5. Optimal conditions and PGRs production by *Azotobacter chroococcum* and *Bacillus megaterium* var. *phosphaticum* (mg. L^{-1}).

Compounds / Strains	Auxins		Gibber-ellin	Cytokinins				
	IAA	IBA	GA_3	Z	KIN	(9R) BAP	(9G) BAP	IP
Azotobacter chroococcum	71.8	56.7	145.6	107.9	93.9	100.4	9.3	17.0
Bacillus megaterium var. phosphaticum	79.6	102.0	102.3	98.1	97.4	69.3	10.0	11.3

Abbreviations : as those stated for Table 1.

Regarding the cytokinins production, obtained data revealed that zeatin and (9R) benzyl adenine were produced with higher amounts by *Azotobacter chroococcum* compared to those produced by *Bacillus megaterium* var. *phosphaticum* .

Conclusion and Recommendation

In view of the obtained results, it could be concluded that the nutritional compounds are affeced the PGRs production because the tryptophan is considered as a precursor for auxins and gibberellins biosynthesis. In addition, adenine is considered the most suitable precursor for cytokinins biosynthesis .

References

Abdel-Hafez, A.M. (1966) Some studies on acid producing micro-organisms in soil and rhizosphere with special reference to phosphate dissolvers. *Ph.D. Thesis,* Agric. Botany Dep., Fac. Agric., Ain Shams Univ., Cairo, Egypt, p. 31.

Abdel–Malek, Y. and Ishac, Y. Z. (1986) Evaluation of methods used in counting *Azotobacter* . *J. Appl. Bacteril.* 31, 267-269.

Ahmad, Farah, Ahmad, Iqbal and Khan, M.S. (2005). Indole acetic acid production by the indigenous isolates of *Azotobacter* and fluorescent *Pseudomonas* in the presence and absence of tryptophan. *Turk. J. Biol.* 29, 29-34.

Arshad, M. and Frankenberger, W.T. (1991) Microbial production of plant hormones. *Plant Soil,* 133(2), 1-8.

Holl, F. B. , Chanwany, C. P., Turkington, R. and Redley, R. A.(1988) Response of crested wheat grass (*Agropyron cristatum* L.), perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens* L.) to inoculation with *Bacillus polymyxa*. *Soil Biol. Biochem.* 20(1), 19-24.

Khalid, A., Tahir, SH., Arshad, M. and Zahir, Z.A. (2004) Relative efficiency of rhizobacteria for auxin biosynthesis in rhizosphere and non-rhizosphere soils. *Aust. J. Soil Research,* 42, 921-926.

Martinez-Toledo, M.V., Rubia, T., Moreno, J. and Gonzalez-Lopez, J. (1988) Root exudates of *Zea mays* and production of auxins ,gibberellins and cytokinins by *Azotobacter chroococcum*. *Plant Soil,* 110, 149-152.

Mazur, H. and Homme, E. (1993) Presence of auxin indole-3-acetic acid in the Northen Adriatic Sea: Phytohormones and mucilage. *Marin Ecological Prog. Ser.* 99, 163-168.

Morsy, Ebtsam, M. (2005) Role of growth promoting substances producing microorganisms on tomato plant and control of some root- rot fungi. *Ph. D. Thesis,* Microbiology Dept., Fac. Agric., Ain Shams Univ., Cairo, Egypt, pp. 58-69.

Nieto, K. F. and Frankenberger, W.T. (1989 a) Biosynthesis of cytokinins by *Azotobacter chroococcum*. *Soil Biol. Biochem.* 21(7), 967-972.

Pallai, R. (2005) Effect of plant growth-promoting rhizobacteria on canola (*Brassica napus* L.) and lentil (*Lens culinaris* medik.) plants. *M.Sc. Thesis* , Sasskatoon, Canada.

Rahal, A.Gh., Mohamed, Faten M. and Abdel-Monium, M.M. (2006) Biological and chemical studies on some free nitrogen fixers isolation from different Egyptian soils. *Egypt. J. Appl. Sci.* 21(6),342-352.

Salamone, I.E.G., Hynes, R.K. and Nelson, L.M. (2001) Cytokinin production by plant growth promoting rhizobacteria and selected mutants. *Can. J. Microbiol.* 47, 404-411.

Srinivasan, M., Petersen, D.J. and Holl, F.B. (1996) Influnce of indole acetic- acid-producing *Bacillus* isolates on the nodulation of *Phaseolus vulgaris* by *Rhizobium etli* under gnotobiotic conditions. *Can. J. Micobiol.* 42, 1006-1014.

Torres-Rubio, Maria G., Valencia-Planta, Sandra A., Bernal-Castillo, J. and Martinez-Nieto, P. (2000) Isolation of enterobacteria, *Azotobacter* sp. and *Pseudomonas* sp. producers of indole-3-acetic acid and siderophores, from Colombia rice rhizosphere. *Revista Latino Ammericana de Microbiologia,* 42, 171-176.

Vessey, J. K. (2003) Plant growth promoting rhizobacteria as biofertilizers. *Plant Soil.* 255, 571-586.

Zahir, Z. A., Asghar, H.N., Akhtar, M.J. and Arshad, M. (2005) Precursor (L-tryptophan) inoculum (*Azotobacter*) interaction for improving yield and nitrogen uptake of maize. *J. plant Nutr.* 28, 805-817.

**(*Received* 13/7/2010;
accepted 1/11/2010)**

اختيار أفضل مصادر الكربون والمواد المحفزة لتحسين إنتاج منظمات النمو بواسطة *B.megaterium* var.*phosphaticum (R44)* و*A. chroococcum* (R19)

أحمد غنيم رحال ، راشد عبدالفتاح زغلول*، نسيم عبدالعزيز نويجى* ، إحسان أحمد حنفى* و رشا محمد الميهى*

***Egypt. J. Microbiol.* Special Issue "13th Conf. of Microbiol." (2010)**

معهد بحوث الأراضى والمياه والبيئة– مركز البحوث الزراعية – الجيزة و*كلية الزراعة– جامعة بنها – القليوبية– مصر.

فى هذا البحث تم دراسة تأثير مصادر الكربون والتربتوفان والآدينين على معدل إنتاج منظمات النمو بواسطة جنسى البكتريا *Azotobacter chroococcum* و*Bacillus megaterium* var. *phosphaticum* .

ولقد أوضحت النتائج أن أنسب مصدر كربون للحصول على أعلى إنتاج من منظمات النمو بواسطة *A. chroococcum* هو المانيتول ، بينما كان الجلوكوز هو أنسب مصدر كربونى لميكروب *B. megaterium* var. *phosphaticum* . أيضا أوضحت النتائج أن أعلى إنتاج من منظمات النمو وخصوصا الإندولات بواسطة كلا منهما لوحظ عند إضافة التربتوفان بتركيز ١٠٠٠ مولر بينما تناقص بإنخفاض تركيز التربتوفان فى بيئة النمو . كذلك أوضحت النتائج المتحصل عليها أن إضافة الآدينين بتركيز ١٠ مولر و ١٠٠ مولر إلى بيئة نمو كل من *A. chroococcum* و *B. megaterium* var. *phosphaticum* على التوالى أدى إلى الحصول على أعلى إنتاج من منظمات النمو.

عند إستخدام الظروف المثلى المتحصل عليها من التجارب السابقة فى إنتاج منظمات النمو بواسطة السلالتين تحت الدراسة تم الحصول على أعلى إنتاج من كل منظمات النمو التى درست وذلك بالمقارنة بإستخدام كل عامل بمفرده . أثبتت الدراسة أن ميكروب *B. megaterium* var. *phosphaticum* أنتج كمية أكبر من الأوكسينات مقارنة بميكروب *A. chroococcum* . بينما أنتج ميكروب *A. chroococcum* كمية أكبر من حامض الجبريلليك مقارنة بميكروب *B. megaterium* var. *phosphaticum* . كذلك وجد أن ميكروب *Azotobacter chroococcum* أنتج كمية كبيرة من الزياتين والبنزيل آدينين مقارنة بميكروب *Bacillus megaterium* var. *phosphaticum* , بينما كان إنتاج *Bacillus megaterium* var. *phosphaticum* من الكينيتين أعلى من*Azotobacter chroococcum* .