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Optimal Environmental Conditions for Production of Plant Growth Regulators by Rizobacteria

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THIS STUDY was carried out to isolate and identify some plant growth promoting rhizobacteria (PGPR). The optimum incubation condition, *i.e.* temperature and fermentation period, for plant growth regulators (PGRs) production were limited. Obtained data revealed that rhizosphere of cereal crops showed higher PGPR isolates compared to rhizosphere of other crops. Among the examined isolates, fifteen were highly efficient for auxins production. The most potent isolates for indoles production were chosen and these isolates were identified as *Azotobacter chroococcum* (R19) and *Bacillus megaterium* var. *phosphaticum* (R44). The optimum incubation temperature for highest production of auxins, gibberellic acid (GA_3) and cytokinins were 32 and 30°C for *A. chroococcum* and *B. megaterium* var. *phosphaticum* , respectively. In addition, the highest production of these phytohormones were obtained by the two strains after four and two days fermentation period, respectively.

Keywords: Plant growth promoting rhizobacteria, Auxins, Gibberellic acid, Cytokinins, *A. chroococcum*, *B. megaterium* var. *phosphaticum*, Incubation temperature, Fermentation period.

The microorganisms can provide benefits to plants via symbiotic relationship with plants and free living bacteria which live in the soil but are often near roots and are usually referred as plant growth promoting rhizobacteria (El-Khawas *et al.,* 2000). Phytohormones are mainly produced by plant pathogens, fungi, bacteria and actinomycetes. For example the ability to myccorrhizal fungi have synthesize cytokinin. Microorganisms that interact with plants can also synthesize phytohormones similar to those produced by the plant as growth regulators, such as auxins, gibberellins and cytokinins. Among these microorganisms bacteria belonging to genera *Azotobacter*, *Azospirillum and Bacillus* are most producers of the previous phytohormones (Teixeira *et al*., 2007). Several soil bacteria, particularly those belonging to phosphate solubilizing bacteria, are also known to produce growth-promoting substances like indole acetic acid and gibberellic acid (Ponmurgan & Gopi , 2006).

With regard to the effect of incubation temperature and period, Arshad & Frankenberger (1991) stated that maximum IAA and zeatin production was obtained after 7 days of incubation by *A. beijerinckii*, *A. chroococcum* and *A. vinelandii* . However, 60 hr was the optimum incubation period for maximum production by *Bacillus megaterium* var. *phosphaticum* (Srinivasan *et al.*, 1996). El-Khawas & Adachi (1999) found that the optimal incubation period for auxins production was 72 and 48 hr for *A. brasilense* and *K. pneumoniae*, respectively.

Rodelas *et al*. (1999) found that the best temperature for PGRs production by *Azotobacter* spp. was 30°C ± 2. Ahmad *et al*. (2005) reported that IAA production by three strains of *Azotobacter* spp. were given as high amounts at 30˚C for one week. Teixeira *et al*. (2007) found the highest amount of IAA production by *Azotobacter* spp. was obtained at 28˚C after 48 hr of incubation.

The aim of this research is to isolate and identify some microorganisms which produce plant growth regulators and study the effect of different incubation temperatures and fermentation periods to elucidate the optimum conditions for abundant growth regulators.

Materials and Methods

Isolation of plant growth promoting rhizobacteria (PGPR)

Isolation of different bacterial isolates was carried out on different specific bacteriological media named Ashby's modified medium (Abdel-Malek & Ishac, 1986), Yeast extract mannitol agar medium (Vincent, 1970), King's medium (King *et al*., 1954), Modified nutrient agar medium (Jacobs & Gerstein, 1960), Semi – solid malate medium (Dobereiner, 1978) and Modified Bunt and Rovira agar medium modified by Abdel-Hafez (1966). One hundred bacterial isolates were obtained from rhizosphere of different crops such as wheat, rose, clover, rice, water grass, bean, maize and banana.

Purification of isolates

The isolates were subcultured on their specific media for purification then maintained as stock cultures at 4-5°C for subsequent studies.

Detection of plant growth regulators (PGRs)

Production of auxins was detected by using Salkowski's reagent method according to Gilickmann & Dessaux (1995).

Screening of potent bacterial isolates

One-hundred isolates were tested for indole production as an indicator of overall PGRs production, according to the method of Salkowski for identification of indoles was applied. Fifteen isolates showed high readings by the Salkowski test, suggesting these isolates may produce PGRs compounds. These isolates were screened again by culturing on their specific media for different intervals (4, 7, 10 and 14 days), and two isolates wich gave the highest reading by the indoles test, were chosen for further study.

Identification of potent selected isolates

The two bacterial isolates which showed highly phytohormones production were grown on Ashby's modified medium (Abdel-Malek & Ishac, 1986) and modified Bunt and Rovira medium (Abdel-Hafez, 1966). Identification of the isolates was achieved according to Bergy's Manual of Determinative Bacteriology (1994).

Effect of incubation temperature on PGRs production

An experiment was carried out to limit the optimum incubation temperature for PGRs production. Conical flasks (500 ml) containing 200 ml of specific media for *A. chroococcum* or *B. megaterium* var. *phosphaticum* were used. The pH of the media was adjusted to 6.8 and 7.2 for the two strains, respectively.

The flasks were inoculated with standard inoculum of the tested bacterial strains. Inoculated flasks were incubated at different incubation temperatures (30, 32, 34, 36 and 38°C) for 4 days. At the end of the incubation period, the produced PGRs were extracted, identified and determined by GLC.

Effect of incubation periods on PGRs production

Another experiment was carried out to know the optimum incubation period for PGRs production. Both strains were grown in their selective media after adjusting the pH to 6.8 and 7.2 for *A. chroococcum* and *B. megaterium* var. *phosphaticum*, respectively. Inoculated flasks were incubated at 32 and 30°C for the two strains, respectively. Flasks were incubated for different intervals (2, 4, 6, 8 and 10 days). At the end of each incubation period, PGRs produced by every strain were extracted, identified and determined by GLC.

Analysis procedures

Analysis of plant growth regulators was achieved by gas liquid chromatography in Soil Microbiology Department, Soil, Water and Environment Research Institute, Agricultural Research Centre, Giza, Egypt.

Gas-liquid chromatography (GLC) assessment

Extracted samples were silylated before GLC 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 100 µL of N,O-bis-(trimethylsilyl) acetamide (BSA) 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 (TMSi), then the residue was dissolved in 0.2 ml absolute methyl alcohol (Mazur & Homme, 1993 and Rahal *et al.*, 2006). One µL of each TMS derivative sample was injected into a Gas chromatograph. Retention time (R_t) for each peak of the authentic standard materials was recorded and the amounts of separated compounds were calculated automatically by a computer attached to the gas chromatograph.

Standard compounds used in GLC analysis

Indole-3-acetic acid, Indole-3-butyric acid and *t*-zeatin were obtained from Sigma; Adenine from Merck, Germany; tryptophan was obtained from Lopa,

India and GA_3, (9R) Benzyl adenine, (9G) Benzyl adenine and isopentyl alcohol were obtained from Genetic Engineering Research Ins. El-Sadat City, Egypt.

Results and Discussion

Isolation of plant growth promoting rhizobacteria (PGPR)

Data in Table 1 showed that auxin production was evident in many of the one-hundred isolates which obtained from the rhizosphere of different crops namely wheat, rice, maize, banana, clover, water grass, rose and bean. The wheat rhizosphere obtained the highest number of auxin producing bacteria, while banana rhizosphere had the lowest amounts.

TABLE 1. Indole acetic acid (IAA) production by different bacteria isolated from the plant root rhizosphere of different crops.

Isolates number	Sample source	Auxins production	Isolates number	Sample source	Auxins production	Isolates number	Sample source	Auxins production
1	Wheat	+	35	Maize	+	69	Water grass	-
2		++	36		++	70		-
3		+++	37		+++	71		-
4		+	38		-	72		+
5		++	39		++	73		++
6		+	40		+	74		++
7		+	41		+++	75		-
8		-	42		-	76		+
9		++	43		++	77		-
10		+	44		+++	78		+
11		+++	45		-	79		-
12		+++	46		+++	80		++
13		++	47		++	81		+
14		+	48		++	82	Rose	+
15		+	49	Banana	-	83		+
16		+++	50		-	84		+
17		++	51		-	85		-
18		+	52		-	86		-
19		+++	53		-	87		-
20	Rice	+	54		-	88		+
21		-	55	Clover	+	99		-
22		+++	56		+	90		+
23		+	57		+	91		-
24		++	58		+++	92		+
25		++	59		+	93	Bean	++
26		-	60		+	94		+
27		+	61		+++	95		-
28		+++	62		-	96		-
29		+	63		-	97		+
30		+	64		-	98		-
31		+	65		+	99		-
32		++	66		+++	100		+
33		+	67		-	-----		-----
34		+++	68		++	-----		-----

O.D. reading : (-)< 100 (+) =100-300 (++) = 300-400 (+++) > 400.

Data clearly showed that the isolates obtained from cereal crops as wheat and rice produced higher amount of auxins than that obtained from the rhizosphere of the other crops. Among the obtained isolates, fifteen were more efficient for auxins production therefore, these isolates were screened in subsequent experiment to select the most potent ones.

These results are in agreement with those obtained by EL-Khawas *et al*. (2000) who reported that the rhizospheres of maize, wheat, barely, sorghum and sugar cane are rich in auxin producing bacteria. In addition, microorganisms isolated from the rhizosphere of cereals have greater potential for auxins production than those from other crops (Khalid *et al.*, 2004).

Screening of the most potent bacterial isolates

The fifteen isolates which showed high amounts of auxins were screened by culturing on specific media. Inoculated media with the potent isolates were incubated at 30˚C for different incubation periods (4,7, 10 and 14 days) . At the end of various incubation periods, the indole amounts produced by isolates were determined spectrophotometerically. All tested isolates produced considerable amounts of indoles (Table 2). The bacterial isolates No. 19 and 44 produced the most indole at the different incubation periods. Therefore, these two isolates were identified and chosen for further studies.

TABLE 2. Optical density values of fifteen isolates tested for indole production at different incubation intervals.

Isolates Number	Intervals			
	4 days	7 days	10 days	14 days
3	0.486	0.467	0.431	0.402
11	0.446	0.446	0.548	0.566
12	0.427	0.511	0.254	0.323
16	0.513	0.557	0.616	0.438
19	1.127	0.864	0.672	0.783
22	0.533	0.787	0.419	0.400
28	0.484	0.602	0.620	-
34	0.426	0.867	0.417	0.474
37	0.577	0.575	0.422	-
41	0.463	0.435	0.706	0.467
44	1.583	0.999	0.980	0.800
46	0.405	0.433	0.446	0.400
58	0.428	0.502	0.451	0.122
61	0.668	0.464	0.464	0.429
66	0.511	0.481	0.451	0.439

Identification of the two more potent isolates

The superior two bacterial isolates No. 19 and 44, were purified and subjected to morphological and physiological studies according to Bergey's Manual of Determinataive Bacteriology (1994).

From the morphological characters, staining properties, spore formation and physiological properties presented in Table 3, the two bacterial isolates were be identified as *Azotobacter chroococcum* (R19) and *Bacillus megaterium* var. *phosphaticum* (R44) according to Bergey`s Manual of Determinative Bacteriology (1994).

TABLE 3. Morphological and physiological characteristics of the two isolates (No. 19 and 44).

Characters	**Isolate number (19)**	**Characters**	**Isolate number (44)**
Form (shape)	Ovoid shaped in pairs	Form (shape)	Rod shape
Gram stain	-	Gram stain	+
Carbon sources		Spore formation	+
Sucrose	+	Acid formation from	
Mannitol	+	Glucose	+
Benzoate	+	Mannitol	-
Motility	+	Arabinose	+
Nitrate reduction	+	Xylose	+
Catalase production	+	V.P. test	+
Hydrolysis of starch	+	Indole production	+
Production of non diffusible pigment	Brown pigment	Hydrolysis of	
		Starch	+
		Gelatin	+
		Casein	+
		Growth at pH	
		6.8	+
		5.7	+
		Catalase production	+
		Citrate utilization	+
		Growth at	
		10°C	+
		40°C	-
		50°C	-
		Phos. solubilization test	+
Identification	*Azotobacter chroococcum*	Identification	*Bacillus megaterium* var. *phosphaticum*

Retention time of standard compounds used in GLC analysis

Technical growth regulator agents were analyzed by gas-liquid chromatog raphy to limit their retention times (R_t) to be as reference to those obtained from sample extracts. The obtained values are presented in Table 4.

TABLE 4. Retention time of standard phytohormones compounds.

Compounds	Retention time (R_t) (min)
Indole acetic acid (IAA)	2.25
Indole butyric acid (IBA)	1.197
Gibberellic acid (GA_3)	3.52
Kinetin (KIN)	5.56
Zeatin (Z)	6.44
(9R)Benzyl adenine (BAP)	5.63
(9G)Benzyl adenine (BAP)	6.21
Isopentyl alcohol (IP)	4.42

Effect of different incubation temperatures on PGRs production

The amounts of auxins, gibberellin (GA_3) and cytokinins produced by *A. chroococcum* and *B. megaterium* var. *phosphaticum* at different incubation temperatures were determined by GLC. The results clearly indicated that both *A. chroococcum* and *B. megaterium* var. *phosphaticum* produced considerable amounts of PGRs under different tested incubation temperatures (Table 5). Data also emphasized that the highest amounts of auxins, gibberellin (GA_3) and cytokinins by *A. chroococcum* and by *B. megaterium* var. *phosphaticum* were observed at 32°C and 30°C incubation temperature, respectively.

TABLE 5. Effect of incubation temperatures on PGRs production (mg.L^{-1}).

Incubation temperatures	Auxins		Gibbe-rellin	Cytokinins				
	IAA	**IBA**	**GA_3**	**ZE**	**KIN**	**(9R) BAP**	**(9G) BAP**	**IP**
	Azotobacter chroococcum							
30°C	2.61	22.6	18.7	30.7	60.0	21.6	ND	2.00
32°C	2.91	22.6	24.5	38.0	65.3	19.4	0.54	2.34
34°C	2.24	21.0	18.9	17.5	48.3	18.8	0.32	1.91
36°C	2.00	ND	15.0	15.5	33.1	10.7	0.21	1.90
38°C	1.84	ND	13.6	13.7	26.8	9.90	ND	1.14
	Bacillus megaterium var. *phosphaticum*							
30 °C	3.60	38.4	25.0	40.2	73.9	16.5	0.32	2.04
32 °C	1.93	36.3	16.3	31.6	40.5	9.40	ND	1.35
34 °C	1.24	35.1	14.1	23.5	39.2	8.01	ND	1.00
36°C	1.20	32.0	15.2	23.0	37.3	6.32	ND	0.97
38°C	0.84	30.4	16.9	23.0	36.9	7.54	ND	1.46

Abbreviations: as those stated for Table 4.

In contrast, the lowest amounts of PGRs were produced at 38°C incubation temperature by both *A. chroococcum* and *B. megaterium* var. *phosphaticum* strains. Concerning the effect of incubation temperatures on production of auxins, in Table 5 it is clearly that the amount of indole butyric acid (IBA) produced was higher than indole acetic acid (IAA). Except at 36°C and 38°C incubation temperatures for *A. chroococcum*, the amounts of IBA produced were

higher than of IAA. Also, at all tested incubation temperatures *B. megaterium* var. *phosphaticum* produced more IBA than IAA. In addition, *A. chroococcum* produced higher amounts of IAA at all applied incubation temperatures compared to that produced by *B. megaterium* var. *phosphaticum* except at 30°C., while, *B. megaterium* var. *phosphaticum* produced higher amounts of IBA at all tested incubation temperatures compared to *A. chroococcum*.

Phytohormones production by *A. chroococcum* was increased with the increasing incubation temperature to reach a maximum values at 32°C. While, above 32°C decreasing phytohormones production view observed on the lowest amounts of phytohormones were detected at 38°C. *B. megaterium* var. *phosphaticum* also showed the highest phytohormones production at 30°C and then decreased thereafter.

Both bacterial strains produced higher amounts of cytokinins than either auxins or gibberellin (GA_3). This result was in agreement with those obtained by Rahal *et al.* (2006). Kinetin-among the produced cytokinins compounds was produced in highest amounts. *B. megaterium* var. *phosphaticum* produced high amounts of zeatin at all incubation temperatures as compared to those produced by *A. chroococcum*, while *A. chroococcum* produced higher amounts of cytokinins {(9R) BAP, (9G) BAP and IP} at all incubation temperatures compared to by *B. megaterium* var. *phosphaticum* . Rodelas *et al*. (1999) found that the best temperature for PGRs production by *Azotobacter* sp. was 30°C ± 2. Ahmad *et al.* (2005) reported that IAA production by three strains of *Azotobacter* spp. gave high amounts at 30˚C ± 2 for one week using shaking. Monteiro *et al*. (2005) found that 30°C was the best incubation temperature for phytohormones producing by *Bacillus* spp. Morsy (2005) reported that the maximum production of IAA and GA_3 by three strains of *Bacillus subtilis* was at 30˚C for three days. Teixeira *et al*. (2007) found the highest amount of IAA produced by *Azotobacter* spp. was obtained at 28˚C after 48 hr of incubation.

Effect of different incubation periods on PGRs production

Data in Table 6 showed that the highest amounts of phytohormones (PGRs) produced by *A. chroococcum* and *B. megaterium* var. *phosphaticum* were detected at four and two days incubation periods, respectively. *B. megaterium* var. *phosphaticum* also, produced higher amounts of IAA and IBA compared to *A. chroococcum*.

Similar trend of results was observed at all investigated incubation periods. In contrast, *A. chroococcum* produced higher amounts of cytokinins and gibberellic acid than those produced by *B. megaterium* var. *phosphaticum*. This result was also obtained at all determination periods.

TABLE 6. Effect of different incubation periods on PGRs production (mg. L^{-1}) .

Incubation periods (days)	Auxins		Gibberellin	Cytokinins				
	IAA	IBA	GA_3	ZE	KIN	(9R) BAP	(9G) BAP	IP
Azotobacter chroococcum								
2	6.6	ND	55.2	16.8	39.4	37.8	0.5	1.3
4	15.7	42.4	66.1	36.2	39.6	44.1	0.8	4.0
6	10.3	19.1	42.1	28.2	22.4	36.4	1.0	2.4
8	6.6	ND	40.5	ND	ND	38.4	0.4	ND
10	4.5	ND	16.1	ND	ND	ND	ND	ND
Bacillus megaterium var. *phosphaticum*								
2	28.4	59.6	49.8	16.8	30.3	16.8	1.3	ND
4	16.5	43.0	39.3	24.8	21.6	16.2	1.8	1.1
6	14.7	28.1	32.9	23.1	10.0	16.2	ND	0.8
8	14.6	11.7	22.4	21.4	ND	9.2	ND	0.1
10	12.0	ND	11.8	8.9	ND	5.4	ND	ND

Abbreviations : as those stated for Table 4.

Generally, the two investigated strains produced higher amounts of cytokinins compounds in comparison with the other produced phytohormones. Similar results were observed by Arshad & Frankenberger (1991) who stated that maximum phytohormones (IAA and Zeatin) production by *Azotobacter* strains namely, *A. beijerinckii*, *A. chroococcum* and *A. vinelandii* was obtained after 7 days of incubation. Srinivasan *et al.* (1996) found that the incubation for 60 hr was the optimum incubation period for maximum phytohormones production by *B. megaterium* var. *phosphaticum* and *B. polymyxa* L6. In contrast, Ahmad *et al*. (2005) reported that high amounts of IAA produced by three strains of *Azotobacter* spp. occured after incubation at 30˚C for one week with shaking. Similarly, Teixeira *et al*. (2007) found that the highest amount of IAA production by *Azotobacter* spp. was obtained at 28˚C after 48 hr of incubation.

Conclusion

It is clear that the soils of Egypt are rich in auxin-producing rhizobacteria. These bacteria produce amounts of auxins, gibberellins and cytokinins that one high enough to potentially impact plant growth. These microorganisms need to be tested as inocula to determine if they actually do to promote plant growth.

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أفضل الظروف البيئية لإنتاج منظمات النمو بواسطة البكتريا

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الهدف من هذا البحث هو عزل وتعريف بعض الميكروبات المنتجة لمنظمات النمو و دراسة تأثير بعض العوامل البيئية على معدل إنتاج هذه المواد مثل درجة الحرارة وفترة التحضين . و تم عزل مائة عزلة بكتيرية من ريزوسفير بعض النباتات المنزرعة مثل القمح ، الأرز ، الذرة ، البرسيم ، الجرجير ، الفاصوليا ، الموز ، الورد. أوضحت نتائج العزل أن ريزوسفير محاصيل الحبوب كان أكثرها إحتواءا لهذه الكائنات وبإختبار كفاءة هذه العزلات فى إنتاج الأوكسينات أوضحت النتائج أن عزلات ريزوسفير محاصيل الحبوب كانت الأعلى إنتاجا. أختير من هذه العزلات أكثرهم إنتاجا للإندول ووجدت أنها (عزلة) ثم إختير منهم عزلتين وهما الأعلى إنتاجا للإندولات هما رقم ١٩ و ٤٤ لإتمام الدراسة .

أوضحت نتائج التعريف أن هاتين العزلتين هما *A. chroococcum* (19) and *B. megaterium* var. *phosphaticum* (44) . كما أوضحت النتائج أن أنسب درجة حرارة تحضين لإنتاج أعلى كمية من الأوكسينات والجبريللينات واالسيتوكينينات هى ٣٢°م لميكروب *A. chroococcum* و ٣٠° م لميكروب *B. megaterium* var. *phosphaticum* . كذلك أظهرت نتائج هذا البحث أن أعلى إنتاج من منظمات النمو المختلفة بواسطة ميكروب *A. chroococcum* كانت عند فترة تحضين أربعة أيام بينما كانت يومين فقط لميكروب *B. megaterium* var. *phosphaticum* .