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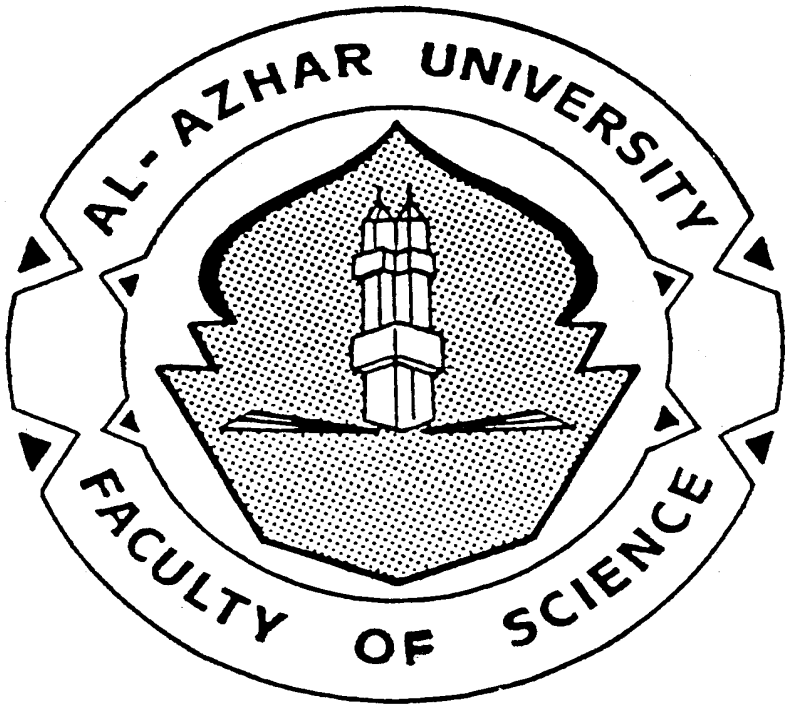

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PRODUCTION OF PLANT GROWTH PROMOTING SUBSTANCES (PGPS) BY SOME *AZOTOBACTER CHROOCOCCUM* ISOLATES

*ARAFA, RAWHIA A.M.; *SIDKEY, NAGWA M.;
**ABD EL-GHANY, BOUTHAINA F. AND **EL-SHAZLY, MONA M.

**Fac. of Science(Girls) Al-Azhar Univ., Cairo, Egypt*
*** Desert Research Center, Cairo, Egypt*

Abstract

A number of 34 N_2 fixing *Azotobacter* isolates have been isolated from Egyptian soils cultivated with different plants from different localities.The ability of the isolates for nitrogen fixation was tested. Only six isolates were selected ,choosen as the most promising isolates in nitrogen fixation and were characterized as *Azotobacter chroococcum*. Maximizing the growth of the *Azotobacter chroococcum* isolates was done by modifications of Ashby's medium as follows : starch was the most suitable carbon source, yeast extract and potassium nitrate were the most favorable organic and inorganic nitrogen sources, respectively; the best pH was 7 and shaking conditions increased the growth. The optimum temperature for growth was found to be 30°C; it exhibited cell proliferation and increased N_2 fixation of the tested *Azotobacter chroococcum* isolates. The plant growth promoting substances "PGPS" were determined in the six *A. chroococcum* isolates under study by using high performance liquid chromatography "HPLC". All the isolates were found to produce zeatin, kinetin, adenine, auxins and gibberellins. Isolate M_{GO2} (obtained from olive plant grown in El-Grawla, Matrouh) gave the highest production of gibberllins, moderate for cytokinins and low for auxins. Also, it gave moderate production of total PGPS, but it was less active in N_2-fixation. On the other hand, isolate M_{OM2} (obtained from mint plant grown in El-Obiyed, Matrouh)gave high total PGPS production, it exhibited high production of auxin and cytokinin and moderate production of gibberllins and N_2-fixation . Isolate M_{KZ1}, (obtained from Zea plant grown in El-Kaser, Matrouh) had the highest activity in N_2-fixation and lowest total hormonal production . Inoculation with a mixture of the three isolates M_{GO2}, M_{OM2} and M_{KZ1} gave the highest total "PGPS" production; this effect could be due to the complementation between *Azotobacter* isolates as regards both the hormonal production and nitrogen fixation. So, the observed plant improvement could be attributed not only to the increased N_2 fixation but also to the stimulated production of growth promoting substances.

Refreed by : Abdel-Moneim E. Ouda & Fawzia A.S. Ebad

Introduction

Investigations carried out during the last few decades proved that biofertilization is considered to be one of the most important field practices due to its effect in reducing environmental pollution caused by accumulation of harmful chemicals, decreasing agriculture costs and maximizing crop yield. Nitrogen is the most important nutrient input required for crop production. Biological nitrogen fixation (BNF), the microbial conversion of atmospheric N_2 to the plant in a usable form, is a recent trend. BNF is not the only main beneficial process of *Azotobacter* to the plants as the organism may also supply the plants with plant growth promoting substances. Some investigators mentioned that bacterial phytohormones production has more beneficial effect than bacterial N_2-fixation. (Rai, 1991; Graham, 1992; Delgado *et al*, 1994; Faid, 1994; Jimenez- Zurdo *et al*, 1995; Abdel Azeem, 1998; Serraj and Sinclair, 1998; Carranca *et al,* 1999; Cordovila *et al,* 1999; El-Haddad *et al,* 1999; Desouky, 2000; El-Sheshtawy, 2000; Hassan, 2000 and Faid, 2001).

Growth regulators are organic compounds other than nutrients secreted by all plants and several kinds of soil microorganisms in small amounts during their life cycle. Auxins, gibberellins, cytokinins etc.. which are produced in minute quantities from bacterial cells, diffuse from their site of production, and exert highly specific effects on the inoculated plant (Abdel-Gawad, 2003).

Azotobacter spp. are among the most important extensively studied N_2-fixing bacteria .They are important due to their symbiotic N_2-fixation for cereal crops including wheat . Several investigators proved that azotobacters are the most predominating diazotrophs in Egyptian soils, and represent the highest densities in comparison to *Azospirilla and Klebsillae* (Ragab and Leith, 1993; Hassouna *et al,* 1995; Shenouda and Abdel Ghany, 1996; Abdel-Gawad, 1999). Many factors that affect the growth and N_2-fixing ability of *Azotobacter* were studied by several authors. The nitrogen fixing ability of *Azotobacter* strains may vary considerably depending on a variety of cultivation conditions viz. the composition of the nutrient medium, pH, temperature, aeration, carbon source, trace elements and moisture (Iswaran and Sen, 1960; Becking, 1962; Krylova, 1963; Dalton and Postagate, 1969; Torosvik, 1973; Bahadur and Lripathi, 1976 and Alexander, 1977).

Gonzalez *et al,* (1986) and El-Shanshoury (1994) reported that bacterial phyto-hormones production has been repeatedly postulated to be responsible for the observed plant growth stimulation upon *Azotobacter* inoculation. They also added that the production of phytohormones seems to play an important role in the *Azotobacter* -plant interactions, disease control and plant growth.

Lugtenberg *et al.* (1991) reported that the mechanisms used by microbes to stimulate plant growth include biofertilization (increasing the supply of mineral nutrients to the plant), biological control (elimination of the plant enemies including microbial pathogens, insects and weeds) and direct plant growth promotion (e.g. by delivering plant growth hormones to the plants). Mrkovacki and Millic (2001) stated that *Azotobacter* synthesizes auxins, cytokinins, and gibberellic acid-like substances, and that these growth materials are the primary substances controlling the enhanced plant growth. They also mentioned that the hormonal substances which originate from the rhizosphere or root surface affect the growth of the closely associated higher plants, thus guarantee the high effectiveness of inoculants as microbiological fertilizers.

Materials and Methods

Survey of soil microorganisms in the Egyptian soils :

Sixty five samples (soil and rhizosphere samples) were collected from different localities (in seven Egyptian Governorates) and different crops including olive, mint, clover, *Zea mays* (corn), wheat, fig, onion, bean, tomato and rice. This was done during the period from year 2000 to 2001. These samples were used for obtaining of the *Azotobacter isolates*

Media used for isolation of Azotobacter :

In this respect , Ashby's medium (Abdel-Malek and Ishac, 1968) was used . The composition of such medium (g/l) was as follows : mannitol, 10; sucrose, 10; K_2HPO_4, 0.5; $MgSO_4$ $7H_2O$, 0.2; NaCl,0.2; $CaSO_4$,0.1; $CaCO_3$, 5; $MnSO_4.7H_2O$, traces; $FeCl_3$, traces; $Na_2MoO_4.2H_2O$, traces; agar-agar 15. All the ingredients were dissolved in 1L distilled water then the medium was adjusted to pH 7.0.

The medium was used for preliminary isolation, purification and propagation of *Azotobacter* isolates in addition to determinations of the most probable number (MPN) of azotobacters. The inoculated tubes were incubated at 28±2°Cfor 10 days.

Isolation and Purification of Azotobacter isolates:

Thirty four isolates were obtained from soil samples. All the isolates were subjected to purification tests by successive streaking on nitrogen free modified Ashby's medium (Abdel-Malek and Ishac, 1968).

Selection of Azotobacter isolates :

The *Azotobacter* isolates were tested for nitrogen fixation ability according to micro-Kjeldahl method described by Jackson (1958). Six *Azotobacter* isolates were selected and grouped into three groups according to their ability for N_2 fixation. Low, moderate and highly active N_2- fixing isolates of *Azotobacter* (2 isolates for each level) were subjected to further studies.

Maximizing Azotobacter growth and nitrogen fixation.

1-The most suitable carbon source:

In this regard, three different carbon sources (starch, glycerol and sucrose) were individually added to the modified Ashby's basal medium (Abdel-Malek and Ishac, 1968) after being depleted for both carbon sources (mannitol and sucrose). The bacterial growth on such three carbon sources was compared with the growth obtained on the modified Ashby's basal medium that contained both mannitol and sucrose. Growth was measured by three parameters viz. the optical density (O.D.), the most probable number (MPN) and the total nitrogen (T.N.) .

2-The most suitable nitrogen source:

In this respect, four different nitrogen sources (peptone, yeast extract, ammonium sulphate and potassium nitrate) were added to the modified Ashby's basal medium (nitrogen-free) containing the most suitable carbon source. Growth of *Azotobacter* isolates on these nitrogen sources was compared with the growth observed on Ashby's nitrogen-free medium .

3-Effect of aeration:

The modified liquid medium developed from the previous series of experiments was inoculated either static or under submerged culture conditions using a reciprocal shaker with an agitation rate of 160 stroke per minute. After incubation at 30°C for 14 days growth densities of *Azotobacter* isolates were determined . .

4- The most suitable pH :

The modified medium containing the most suitable carbon and nitrogen sources was adjusted to different pH's (6.0, 6.5, 7.0, 7.5 and 8.0), incubated and incubated 30°C. The microbial growth was then estimated .

5- The most suitable incubation temperature :

The modified liquid medium developed from the previous series of experiments was inoculated and incubated at 25,30, 35 and 40°C. Growth densities of the *Azotobacter* isolates were determined 14 days from the beginning of incubation.

Characterization of the selected Azotobacter isolates :

The study of the growth characteristics was carried out according to Cowan (1977). Morphological, physiological and taxonomical characters were studied according to Bergey's manual of systematic bacteriology (1984) and Bergey's manual of determinative bacteriology (1994).

Determination of Nitrogenase (N_2-ase) activity of the selected Azotobacter isolates :

The selected isolates were tested(in the Agriculture Research Center) for nitrogenase activity (as a confirmatory test) by acetylene reduction technique (Scholhorn and Burris, 1967) .

Determination of growth promoting activity :

In this respect the method described by *Zimmer et al.* (1988) was used. In such method, wheat *(Triticum aestivum)* grains were soaked, allowed to germinate for 24 hours till just the protrusion of the radicle. Four germinated grains were then transferred to a test tube containing a strip of filter paper soaked with 3.0 mls of the metabolite of each of the *Azotobacter* isolates . Then seven days later the growth of the seedlings, represented by radicale and plumule lengths, was measured.

Determination of radicle colonization ability by the selected Azotobacter isolates:

Radicle colonizing ability of *Azotobacter* isolates were determined by the plate test method according to Kortemma *et al* (1994).The radicle colonization (i.e the adherence of the organism to the radicle) was calculated as percentage of the radicle length colonized by the organism to the total radicle length.

Determination of auxins, gibberellins and cytokinins production by the selected Azotobacter chroococcum isolates and by wheat seedlings :

This was determined by HPLC according to the method described by Tien *et al* (1979) and the modified method of Rizzollo *et al.* (1991). The HPLC technique was carried out in Fac.of Agriculture, Ain Shams University. The chromatograms of HPLC were produced by injection of 5-10 µl of the filtered extract into a 10µl Schimatzu reverse phase column (C_{18} 4mm by 30 cm) . The solvent system was 50% methanol, flow rate was 1.5 ml /min. and the operating pressure was 1.600 lb/ inch^2 (108 atm.); detection was at 280 nm. Retention time for peaks were compared to those of authentics of IAA, GA_3 and cytokinins (zeatin, kinetin and adenine) standards . Quantification was made by area integration through the Schimatzu Data Module microprocessor.

Extraction, separation and bioassay of growth regulating substances :

Extraction: Extraction of plant growth substances from the bacterial isolates, was carried out as described by Abdel- Gawad (1999). In this regard, the bacterial medium of 7 days old culture (100 ml) was centrifuged at 2,000 xg for 30 minutes. The clear supernatant was mixed with an equal volume of methanol and stirred for 3 hours at 4°C in brown jars. The mixture was evaporated under vacuum (40°C) till dryness and the residue was then dissolved in 5 ml of 70% methanol and kept in glass stoppered brown bottles.

Fractionation :The extracts of the bacterial isolates (obtained from the previous step) were separated into their major components of growth promoting substances by paper partition chromatography. Strip-loading method was carried out by a graduated micropipette. The chromatograms were then examined for fluorescence by ultraviolet chromatolite lamp. Some of the chromatograms were sprayed with chemical reagents specific for indole compounds.These reagents were Ehrlich's reagent (Leopold,1955; Block *et al.*,1958 and Powell,1959) and nitrous/nitric reagent (Powell,1959). Still some other chromatograms were used for auxin bioassay using Hordeum coleoptile straight growth test .

Results and Discussion

In the present investigation, isolation of thirty four Azotobacter isolates was carried out from different locations (rhizosphere and soil samples), in six Egyptian Governorates cultivated with different crops. The bacterial isolates were purified and their ability for N_2 fixation and plant growth regulators production was screened. Six *Azotobacter* isolates with different abilities for nitrogen fixation (low, moderate and highly active; 2 isolates of each level) were selected. Table (1) illustrates the symbols of these isolates, their localities, the associated cultivated crops, and the isolate ability to fix atmospheric nitrogen as being estimated by the content of total nitrogen .

The six isolates were subjected to characterization as illustrated in Table (2) according to their morphological, physiological and biochemical characteristics using the methods described by Bergy's manual of systematic-, and determinative bacteriology (1984) and (1994). It has been found that the six isolates had many of the characteristics of *A.chroococcum.* On the other hand , the six isolates showed some differences with respect to their abilities for nitrogen fixation, their potentialities for production of plant growth promoting substances, and also for some other physiological characteristics. Accordingly, the isolates could be considered as non- nodule nitrogen fixing *Azotobacter chroococcum* different strains.

Growth and N_2-fixation by the Azotobacter chroococcum isolates:

In a trial to maximize the growth and N2- fixation ability of the *Azotobacter chroococcum* isolates under study, different conditions were used. In this respect, Ashby's medium (Abd el-Malek and Ishac, 1968) was subjected to several modifications .

The obtained results clearly indicated that starch was the most suitable carbon source for the growth and N_2 fixation of the six A. *chroococcum* isolates (Table 3). This result is in agreement with Jenson and Peterson (1954), Abdel-Azeem (1998), Faid (2001)and Abdel-Gawad (2003).

Concerning the relation between the growth of *Azotobacter* and different N-sources (organic and inorganic) yeast extract and potassium nitrate were found to be the most favorable sources for the growth and proliferation of the *A. chroococcum* isolates (Table 4). This result is compatible with the finding of Faid (2001). Also, in this regard, it has been documented that ammonium sulphate, ammonium chloride and nitrate salt can be used as N-sources for the growth of *Azotobacter* (El-Safty, 1979; Thompson and Skerman, 1979).

On the other hand, the obtained results clearly indicate that submerged culture technique considerably stimulated the growth and recorded maximal N_2 fixation for the tested *A. chroococcum* isolates (Table 5).This result could be due to the stimulatory effect of shaking on microbial growth evolved from one or more of the following : increasing the contact between the growing cells and the substrates of the growth medium, increasing O_2 penetration into the growth medium and getting rid of gases (formed as a result of metabolic activities) which may be poisonous for microbial growth (Peppler, 1967; Mostafa, 1983 and Faid, 2001).

It is also obvious (Table 6) that the best pH was 7 for the different Azotobacter *chroococcum* isolates since it recorded higher viability and stimulated N_2 fixation. Similar results were obtained by Mostafa (1983) and Tippannavar *et al.* (1989). The obtained results also, clearly indicated that, the optimum incubation temperature for the growth of the *Azotobacter chroococcum* isolates was 30°C since the growth rate decreased above and below this temperature (Table 7). Our results are in accordance with those of Nerraj *et al.* (1996) who observed that 30°C was the best temperature for the growth of *A. chroococcum.*

Based on the results of the present work the modified Ashby's medium (Abdel-Malek and Ishac, 1968) was remodified to contain the following (g/l): starch, 20; K_2HPO_4,0.5; $Mn.SO_4$, 0.2; $CaSO_4$, 0.1;$CaCO_3$, 5; $MgSO_4.7H_2O$, traces; $FeCl_3$, traces; $Na_2MoO_4.2H_2O$, traces; yeast extract, 3. The medium was then adjusted to pH 7,inoculated , and incubated at 30°C under shaking condition (160 stroke /min.) for 10 days.The previous conditions resulted in improvement of both the growth and nitrogen fixation of the selected *A. chroococcum* isolates

The selected isolates were evaluated according to their nitrogenase activity, the degree of their colonization to the radicle of wheat seedlings, and also due to the magnitude of their improvement to the growth of wheat seedlings. The obtained data (Table 8) clearly revealed a parallelism between the abilities of the *A.chroococcum* isolates for N-fixation and their improving effects of the growth of wheat seedlings represented by the observed increases in the lengths of the radicles and plumules of the seedlings .

The growth regulators production by the selected *Azotobacter chroococcum* isolates were determined. Table (9) showed that the selected six isolates contained zeatin, kinetin and adenine in their alkaline portion of the extract whereas the acidic portion contained gibberellic acid and indole acetic acid. Quantities of such growth substances varied among the different isolates . The data reveal that the highest production of zeatin, kinetin and adenine was for the isolate M_{OM2} (which is moderate in N_2-fixation). Accordingly this isolate showed the highest total alkaline plant growth promoting substances (44.121 mg/100ml). The same isolate also showed the highest IAA content (0.969 mg/100ml). On the other hand, the highest GA_3 content (4.739 mg/100ml) was recorded in the isolate M_{GO2} (which is the lowest in N_2-fixation) . Isolate M_{KZ1} (the highest in N_2-fixation) proved to be the lowest with respect to plant growth promoting substances production . Based on the values of the total plant growth promoting substances (calculated by summation of both alkaline and acid portions) the three isolates (M_{OM2} , M_{GO2} and M_{KZ1}) proved to be high ,moderate and the low with respect to their potentiality for the production of the plant growth promoting substances .These isolates exhibited values of total plant growth promoting substances of 48.490, 20.814 and 8.065 mg/100ml in the given order. It is worth to mention that these three isolates were moderate, low and high with respect to their abilities for N_2-fixation, respectively. And so, these three isolates were choosen for further work with the aim of chromatographic separation of the different indole compounds (Table 10). The work was extended to the treatment of wheat seedlings with the metabolites of these isolates and estimating the contents of IAA,GA_3 and cytokinins in the treated as well as in the control seedlings (Table 11).

Table (10) revealed that the three isolates (M_{GO2} , M_{OM2} and M_{KZ1}) contained indole acetic acid (IAA,R_f 0.3-0.4), indole acetamide (IAM,R_f 0.6-0.7) and indole acetonitrile (IAN,R_f 0.8-0.9). It was observed that isolate M_{OM2} also contained an additional indole compound isolated at R_f 0.5-0.6.This compound proved to be indole carboxylic acid (ICA). *Using the Hordeum* coleoptile straight growth test for the previously mentioned compounds revealed promoting effects represented by the increases in the length of the coleoptile sections that were observed in most of the cases .

The obtained results (Table 11) revealed that wheat seedlings treated with the three *Azotobater* isolates individually or in a mixture contained three growth promoting substances (IAA ,GA_3 and cytokinins). The quantities of such substances varied with respect to the type of treatment. Table (11) showed also that isolate M_{OM2} was the most active concerning IAA and cytokinin as represented by the

higher contents of these substances in the analyzed wheat seedlings. Isolate M_{GO2} proved to be the most active with respect to GA_3 content in the seedlings. Considering the contents of total plant growth promoting substances revealed that the three isolates can be descendingly arranged in the following order, M_{OM2} (9.89 mg/10 g plant tissue), M_{GO2} (7.4) and M_{KZ1} (4.95). It is worth to mention that a good confirmation was observed between the results of tables (9) and (11), meaning that the arrangement of the three isolates, based on the total of plant growth promoting substances, was the same in the two tables. Treating the seedlings with the mixture of the three isolates showed the highest pormotive effect as being represented by the value of 20.79 mg/10 g plant tissue . Results of the present work are in agreement with those of Brown and Susan (1968); Brown (1972) and Pharis and King (1985) who reported that gibberellins secreted by *Azotobacter* caused better growth and development of the inoculated seedlings when being compared with those of the aseptically non *Azotobacter* inoculated seedlings. They added that the promoting effect could be due to the synthesis of further gibberellins in the root zone thus the treated seedlings differed in their morphology from the untreated controls .

Table (11) revealed that inoculation with the mixture of the three isolates showed the highest ability for hormonal production by the seedlings. This effect could be explained as *Azotobacter* isolates may complement each other with respect to supplying the plant with its needs of nitrogen and growth promoting substances. This explanation is based on the obtained results that isolate M_{GO2} was the highest in GA_3 production whereas M_{OM2} was the most active in IAA and cytokinins. Isolate M_{KZ1} was the highest in N_2 - fixation .

In conclusion, the combination of the microbial isolates together in a mixture provides better results which may be attributed not only to the N_2-fixation process, but also to the production of growth promoting substances especially gibberllins. Several authors observed that *Azotobacter spp.* were able to improve plant nutrition via nitrogen fixation and also by inducing the synthesis of biologically active compounds such as vitamins, auxins, cytokinins, gibberellins, nicotinic acid, pantothenic acid, pyridoxine, biotin, IAA and other compounds . Such compounds are so essential for the enhancement of growth and yield of the plants. Also, *Azotobacter spp.* are able to produce antibacterial and antifungal compounds (Gordon and Robert, 1950; Krupine and Byul, 1960; Vancura, 1961, Brown and Susan, 1968; Cleland, 1969; Mishustin, 1970; Brown, 1972; Scott, 1972; Pandey and Kumar, 1989; Arshade and Franken Berger, 1991; Lugtenberg *et al.*, 1991; Rabie *et al.*, 1995; Mrkovacki and Millic 2001and Abdel-Gawad, 1999, 2003).

Table (1): Relation of the *Azotobacter* isolates (associated with different cultivated crops) obtained from various localities to nitrogen fixation.

Isolates symbols	Locality	Cultivated crop	T.N (ppm)	Selected isolates according to their abilities to fix nitrogen (T.N.)
M_{GO1}	**El-Grawla-Matrouh**	**Olive**	**13.3**	**Low active isolates**
M_{GO2}	**El-Grawla-Matrouh**	**Olive**	**15.5**	
M_{Gm}	El-Grawla-Matrouh	Mint	41.66	
M_{KC1}	El-Kaser-Matrouh	Clover	20.38	
M_{KC2}	El-Kaser-Matrouh	Clover	21.2	
M_{KC3}	El-Kaser-Matrouh	Clover	24.75	
M_{KZ1}	**El-Kaser-Matrouh**	**Zea mays (corn)**	**95.25**	**Most active isolate**
M_{OO}	El-Obiyed-Matrouh	Olive	32.01	
M_{OF}	El-Obiyed-Matrouh	Fig	39.17	
M_{OM1}	El-Obiyed-Matrouh	Mint	43.7	
M_{OM2}	**El-Obiyed-Matrouh**	**Mint**	**46.46**	**Moderate active isolate**
M_{OM3}	El-Obiyed-Matrouh	Mint	46.01	
M_{ON}	El-Obiyed-Matrouh	Onion	48.71	
M_{OM}	El-Obiyed-Matrouh	Mint	53.21	
M_{SF1}	Samla-Matrouh	Fig	32.86	
M_{SF2}	Samla-Matrouh	Fig	34.2	
M_{RM}	**Om-El-Rakhem- Matrouh**	**Mint**	**42.92**	**Moderate active isolate**
M_{Rb}	Om-El-Rakhem Matrouh	Bean	43.7	
Q_{Z1}	Moshtohor -Qalyubia	Zea mays(corn)	20.28	
Q_{Z2}	Moshtohor -Qalyubia	Zea mays(corn)	20.32	
Q_{b1}	Moshtohor -Qalyubia	Bean	43.9	
Q_{b2}	Moshtohor -Qalyubia	Bean	43.78	
Q_{b3}	Moshtohor -Qalyubia	Bean	46.18	
Q_Z	Moshtohor -Qalyubia	Zea mays(corn)	65.8	
T_Z	Tanta –Garbyia	Zea mays(corn)	33.31	
B_{Z1}	Banha- Qalyubia	Zea mays(corn)	40.7	
B_{Z2}	Banha- Qalyubia	Zea mays(corn)	41.5	
KH_W	El-Khatatba –Monofyia	Wheat	56.4	
BE_T	Behira	Tomato	59.4	
N_T	Noubaria Alexanderia	Tomato	62.65	
MA_W	Mariout- Alexanderia	Wheat	71.68	
F_W	**Fayoum- Fayoum**	**Wheat**	**80.63**	**Most active isolate**
KA_{R1}	Kafr El-Zayat – Garbyia	Rice	34.86	
KA_{R2}	Kafr El-Zayat - Garbyia	Rice	36.21	

T.N. = Total Nitrogen

Table (2): Characterizatin of the *Azotobacter* isolates

Isolates characters	*Azotobacter* isolates						*Azotobacter chroococcum*
	M_{GO1}	M_{GO2}	M_{OM2}	M_{RM}	F_W	M_{KZ1}	
Pigment	Brown	Brown	Brown	Brown	Brown	Brown	Pigmented
Cell morphology	Ovoid	Ovoid	Ovoid	Ovoid	Ovoid	Ovoid	Ovoid
Gram reaction	-ve	-ve	-ve	-ve	-ve	-ve	-ve
KOH test (3%)	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Motility	+	+	+	+	+	+	+
O_2 requirement	+	+	+	+	+	+	+
Pellicle formation	+	+	+	+	+	+	+
Nitrogen fixation (Aerobically)	+	+	+	+	+	+	+
Nitrogen fixation at pH 6	+w	+w	+	+	++	++	++
pH 6.5	+	+	++	++	+++	+++	+++
pH 7	++	++	+++	+++	+++	+++	+++
pH 7.5	+	+	++	++	+++	+++	+++
pH 8	+w	+w	+	+	++	++	+
N- fixation with molybdenum	+	+	+	+	+	+	+
Growth at 20°C	+w	+w	+	+	+	+	+
25°C	+	+	++	++	++	++	++
30°C	++	++	+++	+++	+++	+++	+++
35°C	+	+	++	++	+++	+++	+++
40°C	+w	+w	+	+	++	++	+
C. sources uilitization							
Glucose	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+
Starch	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+
Raffinose	+	+	+	+	+	+	+
Cellulose	-	-	-	-	-	-	-
Cellobiose	-	-	-	-	-	-	-
Glycerol	+	+	+	+	+	+	+
Benzoate	+	+	+	+	+	+	+
Sorbose	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-
Galactose	+	+	+	+	+	+	+
Xylose	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-
N. sources utilization							
Pot. Nitrate	+	+	+	+	+	+	+
Amm.sulphate	+	+	+	+	+	+	+
Amm. nitrate	+	+	+	+	+	+	+
Sod.nitrate	-	-	-	-	-	-	-
Amm.chloride	+	+	+	+	+	+	+
Amm.molybdate	-	-	-	-	-	-	-
Amm.hypo.sulphate	+	+	+	+	+	+	+
Peptone	+	+	+	+	+	+	+
Yeast extract	+	+	+	+	+	+	+
Lysine	+	+	+	+	+	+	+
Tryptophan	+	+	+	+	+	+	+
Arginine	+	+	+	+	+	+	+

Table (2) Continued

Isolates characters	*Azotobacter* isolates						*Azotobacter chroococcum*
	M_{GO1}	M_{GO2}	M_{OM2}	M_{RM}	F_W	M_{KZ1}	
Asparagine	+	+	+	+	+	+	+
Phenyl alanine	+	+	+	+	+	+	+
Iso-leucine	+	+	+	+	+	+	+
2Keto glutarate	+	+	+	+	+	+	+
Methionine	+	+	+	+	+	+	+
Thereonine	+	+	+	+	+	+	+
Antibiotic sensitivity							
Pencillin	R	R	R	R	R	R	R
Ampicillin	R	R	R	R	R	R	R
Streptomycin	R	R	R	R	R	R	R
Vibramycin	S	S	S	S	S	S	S
Phenol	R	R	R	R	R	R	R
Amikine	S	S	S	S	S	S	S
Zinnat	S	S	S	S	S	S	S
Garamycin	S	S	S	S	S	S	S
Flumox	R	R	R	R	R	R	R
Augmantin	S	S	S	S	S	S	S
Velosef	S	S	S	S	S	S	S
Septrine	S	S	S	S	S	S	S
Taraviol	S	S	S	S	S	S	S
Ofloxacin	S	S	S	S	S	S	S
Claforan	S	S	S	S	S	S	S
Fucidin	R	R	R	R	R	R	R
Tetracyclin	R	R	R	R	R	R	R
Oxytetracyclin	R	R	R	R	R	R	R
Gentamycin	S	S	S	S	S	S	S
Bacteriocin	R	R	R	R	R	R	R
Rifamycin	S	S	S	S	S	S	S
Sulfamethazole	S	S	S	S	S	S	S
Biochemical tests							
Nitrate reduction	+	+	+	+	+	+	+
Catalase production	+	+	+	+	+	+	+
Gelatin liquefaction	-	-	-	-	-	-	
Starch hydrolysis	+	+	+	+	+	+	+
Hydrolysis of Tween 80	-	-	-	-	-	-	
Nitrogenase activity	+	+	+	+	+	+	+

N.B. S = Sensitive R = Resistant + w = Weak growth
+ = Moderate + + = Good + + += Excellent

Table (3): Effect of different carbon sources on growth and nitrogen fixation of the *Azotobacter chroococcum* isolates

Azotobacter chroococcum isolates		Ashby's medium (basal medium			Starch			Sucrose			Glycerol		
		O.D.	MPN	T.N.	O.D.	MPN	T.N.	O.D.	MPN	T.N.	O.D.	MPN	T.N.
Less active	M_{GO1}	0.659	22.8	13.30	0.692	35.0	20.9	0.425	21.0.	12.6	0.160	5.0	7.51
	M_{GO2}	0.679	25.0	15.50	0.698	35.0	31.4	0.432	21.0	13.4	0.168	7.0	8.00
Moderate active	M_{RM}	0.763	38.0	42.92	0.867	47.0	43.6	0.563	23.0	31.0	0.180	7.0	10.53
	M_{OM2}	0.790	38.6	46.46	0.897	47.0	43.9	0.510	25.0	32.8	0.195	8.0	13.41
Most active	F_W	0.823	41.0	80.63	0.924	63.0	89.7	0.608	25.0	49.0	0.198	18.0	20.42
	M_{KZ1}	0.950	43.0	95.25	**0.995**	**68.0**	**99.1**	0.650	30.0	52.0	0.272	20.0	21.33

Table (4): Effect of different nitrogen sources on growth and nitrogen fixation of the *Azotobacter chroococcum* isolates .

Azotobacter chroococcum isolates		Ashby's modified medium			Nitrogen sources											
					Organic nitrogen sources						Inorganic nitrogen sources					
					Yeast extract			Peptone			Amm.sulphate			K.nitrate		
		O.D.	MPN	T.N.	O.D.	MPN	T.N.	O.D.	MPN	T.N.	O.D.	MPN	T.N.	O.D.	MPN	T..N
Less active	M_{GO1}	0.69	35.0	20.9	1.87	45.0	39.7	0.11	15.0	22.0	0.40	30.0	29.0	1.51	43.0	39.5
	M_{GO2}	0.70	35.0	31.4	1.91	45.6	41.0	0.21	19.0	23.2	0.50	32.0	30.0	1.60	44.0	40.9
Moderate active	M_{RM}	0.87	47.0	43.6	1.95	58.0	57.1	0.34	28.0	28.9	0.70	37.5	37.5	1.70	50.0	55.0
	M_{OM2}	0.90	47.0	43.9	1.95	58.5	58.6	0.44	35.0	29.8	0.80	38.0	39.0	1.79	50.0	56.2
Most active	F_W	0.92	63.0	89.7	1.98	76.0	92.0	0.45	35.0	38.0	0.90	50.0	50.0	1.87	73.0	90.5
	M_{KZ1}	0.99	68.0	99.1	**1.99**	**88.0**	**100.0**	0.46	37.0	38.0	0.95	52.0	53.0	**1.84**	**75.0**	**95.2**

Table(5):Effect of aeration on growth and nitrogen fixation of the *Azotobacter chroococcum* isolates .

Azotobacter chroococcum isolate		Static incubation			Shaking incubation		
		O.D (nm)	MPN ($\times 10^5$cell/ml)	T.N (ppm)	O.D (nm)	MPN ($\times 10^5$cell/ml)	T.N (ppm)
Less active	M_{GO1}	1.9	50	43	1.89	157	99.0
	M_{GO2}	1.95	52	47	1.99	159	107.0
Moderate active	M_{RM}	2.01	68	70	2.40	160	119.0
	M_{OM2}	2.10	70	75	2.70	175	199.7
Most active	F_W	2.40	85	110	2.77	177	229.0
	M_{KZ1}	2.60	99	130	**2.80**	**181**	**235.7**

Table (6): Effect of different pH values on growth and nitrogen fixation off the *Azotobacter chroococcum* isolates .

Azotobacer chroococcum isolates		pH 6			pH 6.5			pH 7			PH 7.5			pH8		
		O.D	MPN	T.N	O.D	MPN	T.N	O.D	MPN	T.N	O.D	MPN	T.N	O.D	MPN	T.N
Less active	M_{GO1}	0.14	20.0	11.5	0.22	22.0	21.0	1.9.0	50.0	43.0	1.70	42.0	41.0	0.11	18.0	10.6
	M_{GO2}	0.15	21.0	17.9	0.23	25.0	23.0	1.95	52.0	47.0	1.71	43.0	42.0	0.12	18.0	13.0
Modera te active	M_{RM}	0.18	26.1	19.0	0.27	29.0	33.0	2.01	68.0	70.0	1.95	57.0	63.0	0.14	20..3	16.1
	M_{OM2}	0.19	27.0	21.0	0.29	29.0	35.5	2.10	70.0	75.0	1.97	58.0	65.0	0.17	21.0	16.9
Most active	F_W	0.26	28.0	36.7	0.31	30.0	60.0	2.40	85.0	110	2.01	65.0	95.0	0.25	24.0	28.9
	M_{KZ1}	0.27	30.0	38.0	0.37	35.0	67.0	**2.60**	**99.0**	**130**	2.25	79.0	101	0.26	26.1	27.0

Table (7): Effect of different incubation temperatures on growth and nitrogen fixation of the *Azotobacter* isolates.

Azotobacter chroococcum isolates		25°C			30°C			35°C			40°C		
		O.D	MPN	T.N	O.D	MPN	T.N	O.D	MPN	T.N	O.D	MPN	T.N
Less active	M_{GO1}	1.81	121	92	1.89	157	99.0	1.78	133	83	1.59	129	78
	M_{GO2}	1.86	137	101	1.99	159	107.0	1.82	148	104	1.68	136	86
Moder ate active	M_{RM}	2.05	144	114	2.40	160	119.0	2.13	153	108	1.92	141	96
	M_{OM2}	2.54	157	158	2.70	175	199.7	2.39	160	140	2.03	158	129
Most active	F_W	2.59	162	210	2.77	177	241.0	2.41	165	179	2.19	161	155
	M_{KZ1}	2.69	169	224	2.80	**181**	259.0	2.59	172	183	2.81	164	166

Table (8): Growth ,radicle colonization and nitrogen fixation of the the *Azotobacter chroococcum* isolates.

Azotobacter chroococcum isolates		N.fixation		Wheat seedling			Radicle colonization	
		T.N (ppm)	N.ase	Plumule length (cm)	Radicle length (cm)	Total seedling length (cm)	Radicle length cm	Radicle colonization %
Control		7.60	18.4	8.90	6.20	15.10	4.80	0.00
Less active	M_{GO1}	13.30	44.300	10.34	8.80	19.14	7.40	52.00
	M_{GO2}	15.50	46.984	12.90	10.32	23.22	7.65	52.40
Moderate active	M_{RM}	42.92	136.325	13.34	11.20	24.54	8.61	55.50
	M_{OM2}	46.46	156.945	14.33	11.83	26.16	8.92	56.56
Most active	F_W	80.63	348.627	14.70	14.26	28.96	9.75	63.589
	M_{KZ1}	**95.25**	**396.857**	**19.40**	**16.86**	**36.26**	**10.50**	**64.76**

Control = Media without inoculation **N.ase**.= nitrogenase ($\mu lC_2H_4h^{-1}l^{-1}$)

Table (9): Production of PGPS from the acidic and alkaline portions by the *Azotobacter chroococcum* isolates .

Azotobacter chroococcum isolates		Alkaline portion			Total alkaline PGPS (mg/100 ml)	Acidic Portion		Total acidic PGPS (mg/100 ml)	Total PGPS
		Zeatin	Kinetin	Adenine		GA_1	IAA		
Less active	M_{GO1}	2.068	0.193	0.095	2.356	3.690	0.109	3.799	6.155
	M_{GO2}	**1.328**	**6.355**	**8.195**	**15.878**	**4.739**	**0.197**	**4.936**	**20.814**
Moderate active	M_{RM}	1.811	14.615	0.619	17.045	1.217	0..296	1.513	18.558
	M_{OM2}	**5.066**	**24.774**	**14.281**	**44.121**	**3.400**	**0.969**	**4.369**	**48.490**
Most active	F_W	1.730	0.088	0.694	2.512	2.502	0.014	2.516	5.028
	M_{KZ1}	**2.990**	**2.375**	**0.089**	**5.454**	**2.575**	**0.036**	**2.611**	**8.065**

Table (10): Production of indole compounds by the selected *Azotobacter chroococcum* isolates and its effect on the length of the coleoptile sections

R_f range	Type of indole	Selected *Azotobacter chroococcum* isolates		
		M_{GO2}	M_{OM2}	M_{KZ1}
		Mean length of coleoptile sections (cm)		
0.3-0.4	IAA	1.32	1.48	1.20
0.5-0.6	ICA	-	1.25	-
0.6-0.7	IAM	1.21	1.35	1.17
0.8-0.9	IAN	1.18	1.29	1.10

Note= Initial length of the coleoptile sections was 1cm IAA= indole acetic acid
ICA= indole carboxylic acid IAM= indole acetamide IAN= indole acetonitrile

Table 11 .Determination of growth regulators content in wheat seedlings treated with either one of the metabolites of the three of the *Azotobacter* isolates or their mixture .

Treatments	IAA	GA_3	Total IAA+ GA_3	Cytokinin	Total PGPS
	mg/ 10 g plant tissue				
Control	0.22	0.03	0.25	2.30	2.55
M_{GO2}	0.35	2.38	2.73	4.67	7.40
M_{OM2}	1.04	0.90	1.94	7.95	**9.89**
M_{KZ1}	0.26	1.74	2.00	2.95	4.95
MIX	2.11	4.10	6.21	14.59	**20.80**

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