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Genetic Improvement of *Azotobacter chroococcum* Strain and Their Efficiency on Growth and Quality of Sweet Melon (cv. Kahera 6) Seedling

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Abstract: Attempts to induce genetic improvement in *Azotobacter chroococcum* wild type strain, identify their new mutants were conducted in this present work. The study was extended to evaluate their efficiency to promote some growth and quality traits of sweet melon (*Cucumis melo* L.) cv. Kahera 6 seedlings. After treatment of *Azotobacter chroococcum* wild type with different concentrations (50, 100 and 200µg/ml**)** of the mutagen N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG) for three times (30, 60 and 120 min), it was observed that the numbers and percentages of treated colonies were decreased by increasing the concentration and time of exposing to mutagen. The obtained number of *Azotobacter chroococcum* mutants was 15 auxotrophs which symbolized from FM1 to FM15. Among them, ten mutants were identified according to their specific auxotrophic requirements. It was found that 6 mutant cells had a single mutation: Isoleucineless (Isoleu$^-$), Aspartic acid less (Asp$^-$), Histidineless (His$^-$), Prolineless (Pro$^-$), Glutamic acid less (Glu$^-$) while the other mutant cells were carried a double mutation: Vit.B1$^-$/Thr$^-$, Thr$^-$/Thi$^-$, Orn$^-$/ Asp$^-$ and Vit.B9$^-$/Vit.B6$^-$. All strains were tested for their antibiotic resistance, pH and salinity tolerance properties. The obtained results revealed that there was possible genetic variation among them. Concerning the effect of wild type and their mutant strains in growth and quality of sweet melon seedling, it was found that almost of mutants were superior to wild type and untreated control treatments in germination percentages and growth traits particularly the four mutant strains FM12, FM13, FM8 and FM9. Inoculation of sweet melon plants with almost of tested *Azotobacter chroococcum* strains particularly the three mutants (FM12, FM13 and FM8) increased the uptake of leaves N, P and K and photosynthetic pigments contents as compared to wild type and untreated control treatments. Finally, it could be concluded that treatment of *Azotobacter chroococcum* wild type strain with different MNNG concentrations may produce a new genetic parameter (Genotype) which could be used as a desirable bio-inoculates to improve growth and quality of sweet melon seedlings.

Key words: Genetic enhancement, *Azotobacter sp*., auxotrophs, chemical mutagens, vegetative growth.

INTRODUCTION

Under sustainable agricultural systems, plant growth promoting microorganisms are considered as one of the important and alternative tools to replace the application of chemical fertilizer and pesticides (**Alengebawy *et al.*, 2021**). Plant growth promoting rhizobacteria (PGPR) are free-living aerobic soil-borne bacteria that include various bacterial genera such as, *Azotobacter, Pseudomonas, Bacillus, Agrobacterium, Rhizobium, Enterobacter, Azospirillum* and *Klebsiella* (**Lucy *et al.*, 2004** and **Đurić *et al.*, 2011)**. These bacteria positively effect on plant growth and health through increasing the availability of different nutrients in the soil, secreting various types of plant growth hormones, protecting plants against phytopathogens, improving physico-chemical properties of the soil, overcoming the abiotic stress e.g. drought, humidity, salinity, and acidity, bioremediation of numerous toxic heavy metals and degrading the composites of synthetic chemical (**Yousef, 2018** and **Warrad *et al.*, 2020**).

Among of all PGPR genera**,** those belonging to genus *Azotobacter* play a great role, due to their widely distributed in different environments, such as water, soil and sediments, so it is much used in bio-fertilizer formulations (**Mirjana *et al,* 2006** and **Dhaker *et al.*, 2017)**. *Azotobacter chroococcum* species is an aerobic free-living nitrogen fixer that able to enhances the plant growth and development by producing phytohormones and increasing solubility of mineral phosphates by producing hydrogen cyanide, siderophore and its anti-microbial activity against different phytopathogens by producing antimicrobial substances **(Behl *et al.*, 2003).**

Azotobacter chroococcum is also able to promote crops growth under different soil types and climatic conditions **(Gurikar *et al.*, 2016).**There were many attempts to genetically improve their benefit characters to increase their productivity in agricultural process (**Benemann *et al.*, 1971; Ramos and Robson, 1989**; **Lakshminarayana, 1993; PalSaha and Paul, 2003** and **Abd Al-Hadi *et al.*, 2013**).

To generate new genetic characters (genotypes) of any microorganism, it can be done by mutations induction or genetic recombination. Although, almost of mutants are usually eliminated by selection, but some mutations which are useful, can be detected by screening and reserved indefinitely (**Adrio and Demain, 2006**). Genetic improvement of bacterial strains could be achieved by mutagenesis (**Riyanti *et al.*, 2012**). Numerous mutagenic agents like, N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG), nitrous acid (HNO 2), mitomycin C (MC), ultraviolet radiation (UV), and X-rays have been used to induce genetic variations in various microorganisms (**Drake and Baltz, 1976; Del Galloa *et al*., 1987; Tripura *et al*., 2007** and **Mutwakil, 2011**).

So the main objects of the present investigation was inducing genetic improvement to *Azotobacter chroococcum* wild type strain, identifying their new mutants and testing their efficiency to improve some growth and quality traits of sweet melon seedling.

MATERIALS AND METHODS

1- Bacterial strain

Wild type strain of *Azotobacter chroococcum* was kindly obtained from microbial genetics Lab., faculty of agriculture, Minia University (**Dakhly and Abdel-Mageed, 1997**).

2- Mutagenic treatment

Overnight liquid cultures (1.4×10^9 cells/mL) of *Azotobacter chroococcum* (Wild type) strain were centrifuged at 5,000 rpm for 10 min at 4°C. Cells were washed twice with double distilled water (ddH_2O) then the cells were re-suspended in 10 mL (ddH2O), placed on ice for 10 min. Treatment with MNNG were done according to (**Miller, 1992**) with some modifications. The stock solution of MNNG was prepared in acetone then diluted in (ddH_2O). Different concentrations (50, 100 and 200 mg/ml) of MNNG were prepared from stock solution. The mutagenic treatment were done by add 0.1 mL from different concentrations of MNNG solution to 1.9 mL resuspended cells, the control treatment was 0.1 mL (ddH_2O). The cell suspension was incubated for tree different time (30, 60 and 120min) at 28°C in shaking water bath to determine the best mutagenic conditions. After incubation, the cells were harvested and washed twice before re-suspending in 2 mL (ddH_2O). Serial dilutions (10^{-4}, 10^{-5} and 10^{-6}) of treated and untreated cells were prepared in (ddH_2O) and 0.1mL aliquots of diluted samples were

speared on Ashby-Mannitol agar CM, (Mannitol 1.5%, $CaCO_3$ 0.5%, $MgSO_4$ 0.02%, NaCl 0.02%, KH_2PO_4 0.02%, $FeSO_4$ 0.0005% and Agar 1.5%) plates (**Gomare *et al.*, 2013**) and viable cells were counted. After four days of incubation at 28°C, the grown colonies were counted and the percentage of survivals for each treatments were determined by dividing the number of viable cells after MNNG treatment to the number of viable cells of control treatment and the survival curve for each treatments was made by plotting MNNG concentration and time of exposure versus percent survival. After three days of incubation at 28^0C, colonies were tested for possible mutation on minimal medium (MM) and Ashby- Mannitol agar medium (CM) plates during five days **(Gomare *et al.*, 2013**). The colonies which showed growth on complete media but not on minimal media were considered as auxotrophic (mutants).

3- Characterization of Auxotrophic Mutants

According to growth of the treated bacteria in complete medium (CM) and minimal medium (MM) plates, it was classified as prototrophs and auxotrophs. Colonies of *Azotobacter chroococcum* that failed to grow on MM plates were screened using the scheme of modified cross-pool auxanography **(Holliday, 1956)** to identify their specific auxotrophic requirements. The MM was supplemented with different nutrients (amino acids, vitamins, purine, and pyrimidine) with different combinations. The amino acids and vitamins were used with (1 mg/L) singly and in combination: DL-Alanine, DL-2-amino-*n*-butyric acid, L-arginine monohydrochloride, DL-Aspartic acid, Biotin, Choline, L-Cysteine hydrochloride, L-Cysteine, Folic acid, L-glutamic acid, Glycine, L-Histidine monohydrochloride, DL-*iso*-leucine, L-leucine, L-Lysine monohydrochloride, DL-Methionine, Nicotinic acid, Ornithine, DL-3,4-Dihydroxyphenylalanine, L-Hydroxy-proline, Pyridoxine, Riboflavin, Dl-Serine, Thiamine, DL-Tryptophan, L-Tyrosine, Dl-Ornithine monohydrochloride, DL-Threonine, DL-Serine and DL- Valine (**Amino acid reference collection, BDH chemicals Ltd, Poole, England**). The bacterial isolates that grown clearly on the supplemented MM and form colonies similar to that on CM were confirmed as auxotrophic for the relevant component.

4- Antibiotic resistance tests

To test the intrinsic antibiotic resistances of the wild type and new induced strains the complete medium (CM) supplemented with one of the eight following antibiotics (µg/ml): Colistin(CT)10, Enrofloxacin (ENR) 5, Oxacillin (OX)5, Ceftriaxone (CRT)30, Azitromycin (AZM) 15; Ampicillin (AMP)10, Oxytetracycline (O) 30 and Amikcin (AK)30 were used. All bacterial strains were inoculated overnight at 28°C on liquid culture medium tubes on rotary shaker. Then, by micropipette 0.1ml drops from culture were spotted on each plate and distributed with spreader. By using a sterile scalpel, two antibiotic discs were placed on each plate and incubated at 28°C for 3 days. Effects of antibiotic resistances were determined by measuring the zones around antibiotic discs to determine the resistances and sensitive of each antibiotic (**Clower and Hay, 1968**).

5- Morphological Characterizations

5-1. pH tolerant test

The ability of different *Azotobacter chroococcum* strains to grow under different pH degrees start from pH 5.0 to pH 12.0 was tested by adjusting the PH of Ashby-Sucrose agar plates using NaOH and HCL and the growth of the PH tolerant bacteria were checked after 24 to 72 hours of incubation at 28°C as compared to wild type bacteria (**Akhter *et al.*, 2012**).

5-2. Salt tolerant test

Salinity test was done by growing different *Azotobacter chroococcum* strains at different salt concentration (5 to 10%) of NaCl add to Ashby-Sucrose agar medium and observing the bacterial growth and tolerance to exceeding salt concentration comparing to the control, the colonies were observed after 5 days of incubation at 28°C (**Akhter *et al.*, 2012**).

6. Evaluation of plant growth promoting activity on sweet melon plants

Pot experiment was carried out at the greenhouse of field crop department, faculty of agriculture, Minia University, with clear assist of Prof. Dr. Abobaker Tantawy.

6.1. Surface sterilization of sweet melon seeds

Seeds of sweet melon (cv. Kahera 6) were obtained kindly from the Horticulture department, Faculty of agriculture, Mina University, Egypt. Surface sterilization was done to kill all the coated microorganisms surround the seeds by washing it with 70 % ethanol for 1 min then 3 % Sodium hypochlorite for 3 min followed by 70 % ethanol wash for 1 min. Finally, the seeds were rinsed three times in sterile distilled water and blot dried.

6.2. Preparation of bacterial inoculum

All the tested sixteen bacterial strains were grown for 48 h at 28°C on Ashby-Sucrose broth with constant shaking at 150 rpm to approximately 10^7 cfu/ml the harvesting of bacteria was done by centrifugation at 13,000 rpm for 10 min, then the bacterial cells were resuspended in sterile distilled water (**Thompson, 1996**).

6.3. Seed bacterisation

Appropriate quantity of sweet melon seeds were soaked in ten milliliter of the prepared bacterial suspension containing 10^7cfu/ml for 1h and dried under shade. The control seeds were soaked in sterile distilled water.

6-4. Pot experiments

To determine the efficiency of all tested bacterial strains to promote growth parameter of sweet melon seedlings under greenhouse conditions, plastic pots (12 cm width) were prepared and filled with mixture of (sterilized soil/sand in 1:1 ratio + 100 g of sterilized vermiculite + 4g of rock phosphate) the final weight was 3kg. Five sweet melon seeds were sown in each pot; in completely randomized experimental design, five replicate pots were specified for each treatment. Coated seeds planted in pots under soil surface and irrigated weakly. After 30 days of planting, sweet melon seedling were collected and the percentage of seed germination was estimated. Different growth parameters were measured such as: shoot and root length, leaves number and fresh and dry weight for shoot and root. The vigor index was estimated according to the equation of **Abdul Baki and Anderson (1973)**.

Mean root length (cm) + Mean shoot length (cm) × % Seeds germination

The photosynthetic pigments (Chlorophyll a, chlorophyll b and carotenoid) were determined from the second, third and fourth fresh leaves of seedlings from the top. 0.5g from leaves was soaked into 5 ml 100% acetone in a test tube with stopper. After 5 days of incubation in refrigerator at 4°C, the supernatant colored solution was decanted carefully in 25 ml volumetric flask, crushed with a blunt glass rod and 5 ml fresh acetone was added to the test tube and left for 15 min. Then the supernatant solution was gained by centrifuging at 13,000 g for 5 min. Finally, the volume was made up to 10 ml with fresh acetone. Chlorophyll a, b and carotenoids were measured at 663, 645 and 440.5 nm wavelength, respectively, with spectrophotometer (**Lichtenthaler and Wellburn 1983**). Nitrogen, Phosphorus and Potassium (N, P and K) contents were measured due to the methodology of (**Dawwam *et al.*, 2013**).

RESULTS AND DISCUSSION

The possibility to induce new desirable mutants in *Azotobacter chroococcum* wild type strain and their role as a promising bio-inoculate for improving growth and quality of sweet melon seedling will be shown in the following results.

1- The effect of different MNNG mutagen concentration on number and percentages of *Azotobacter chroococcum* survivals

The presented data in Table 1 and Figure 1 showed that treatment of *Azotobacter chroococcum* wild type with different concentration (50, 100 and 200µg/ml) of MNNG mutagen for three times (30, 60 and 120min) was accompanied with reducing the numbers and percentages of *Azotobacter chroococcum* survivals as compared to the untreated control. It was observed that the numbers and percentages of treated colonies were decreased with increasing the concentration and time exposing of mutagen (Figure

1). The highest number of survivals was obtained by untreated control (13150 cells with 100%). While, the lowest value (590 cells) were found after 120min in plates treated with 100mg/ml MNNG mutagen with 4.5% survivals. There was no survivals colony after treatment with 200mg/ml of MNNG for 120min.

Table (1). Number and percentages of *Azotobacter chroococcum* survivals after treatment with different concentrations of MNNG (0, 50, 100 and 200 µg/ml) for 30, 60 and 120 min

Strain	MNNG conc. (µg/ml)	Time (min)	No. and percentage of survivals	
			No	%
Azotobacter chroococcum	Control	30	13150	100.0
	50		11089	84.3
	100		9124	69.4
	200		6570	50.0
	Control	60	13150	100.0
	50		8478	64.5
	100		5150	39.2
	200		3420	26.0
	Control	120	13150	100.0
	50		2110	16.0
	100		590	4.5
	200		0	0.0

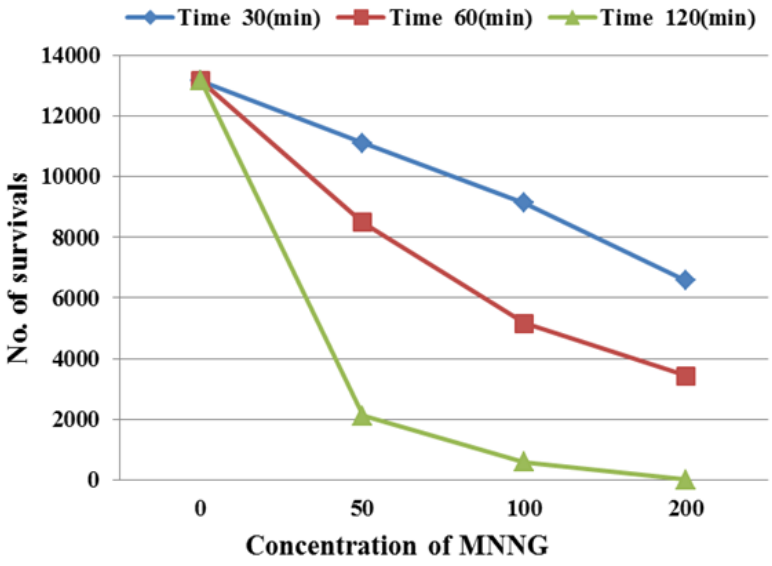

Figure (1). Number of survived *Azotobacter chroococcum* colons treated with different MNNG concentrations (0, 50, 100 and 200µg/ml) at three incubation times (30, 60 and 120 min).

These findings were in accordance with that of **Arshad *et al.*, 2010** who indicate that decreasing the number and percentages of survivals colonies are depending on dose and exposure time of mutagens. Also **PalSaha and Paul (2003)** reported that *Azotobacter chroococcum* MAL-201 (MTCC 3853) exhibited high degree of sensitivity after treatment with MNNG mutagen due to the sharp decrease in number and percentage of survival cells.

2- The mutagenic effect of different MNNG mutagen concentrations on *Azotobacter chroococcum* wild type strain

The numbers, frequencies and types of mutants that found after exposing the wild type of *Azotobacter chroococcum* to different concentrations of MNNG for different times were shown in Table 2. The number of tested cells which appeared in complete medium (CM) and in the same time did not grown in minimal medium (MM) were ranged from (250 to 320cell) with a total number 2320 cells during all tested treatments. The total number of *Azotobacter chroococcum* mutants which obtained after treatments with MNNG mutagen was 15 auxotrophs with frequency 0.65%. These mutants were symbolized from FM1 to FM15 (Table 3). The highest number of mutant cells (5cell) with frequency 1.92% was found in cells treated with 200mg/ml for 30min while the lowest value was one cell that found after treated with 200mg/ml for 60min with frequency 0.35%. Cells exposing to the two doses 50 and 100mg/ml for 30min and100mg/ml for 120min of MNNG mutagen didn't gave mutants. All the 15 mutants were identifying according to their specific auxotrophic requirements **(Holliday, 1956)**. Among of them, ten mutants were identified while the other five gave unclear results so it was excluded from the identification but it used in the other experiments. It was found that 6 mutants had a single mutation: Isoleucineless (Isoleu$^-$), Aspartic acid less (Asp$^-$), Histidineless (His$^-$), Prolineless (Pro$^-$), Glutamic acid less (Glu$^-$) while the other mutants were carried a double mutation: Vit.B1$^-$/Thr$^-$, Thr$^-$/Thi$^-$, Orn$^-$/ Asp$^-$ and Vit.B9$^-$/Vit.B6$^-$ (Table 3).

Table (2). Numbers, percentages and types of *Azotobacter chroococcum* mutants obtained after treatment with different concentrations of MNNG (50, 100 and 200 µg/ml) for 30, 60 and 120 min

Time (min)	MNNG conc. (µg/ml)	No. tested colonies	No. of mutants	Mutants freq. %	Type of auxotrophic mutation								
					Single mutation					Double mutation			
					Isoleu$^-$	Asp$^-$	His$^-$	Pro$^-$	Glu$^-$	Vit.B1$^-$ /Thr$^-$	Thr - /Thi-	Orn$^-$/ Asp$^-$	Vit. B9$^-$/ Vit.B6$^-$
	50	275	0	0	-	-	-	-	-	-	-	-	-
30	**100**	320	0	0	-	-	-	-	-	-	-	-	-
	200	260	5	1.92	-	1	1	-	-	1	-	-	-
	50	250	3	1.20	-	-	-	1		-	1	-	-
60	**100**	350	3	0.86	-	-	-	-	1	-	-	1	-
	200	280	1	0.35	-	-	-	-	-	-	-	-	1
	50	275	3	1.09	1	1	-	-	-	-	-	-	-
120	**100**	310	0	0	-	-	-	-	-	-	-	-	-
	200	0	0	0	-	-	-	-	-	-	-	-	-
Total		2320	15	0.65	-	-	-	-	-	-	-	-	-

Abbreviations: Isoleucineless (Isoleu$^-$), Aspartic acid less (Asp$^-$), Histidineless (His$^-$), Prolineless (Pro$^-$), Glutamic acid less (Glu$^-$), Vitamin B1 less (Vit.B1$^-$), Threonineless (Thr$^-$), Thiamineless (Thi$^-$), Ornithineless (Orn$^-$), Vitamin B9 less (Vit.B9$^-$), and Vitamin B6 less (Vit.B6$^-$).

It is well known that MNNG is a common mutagenic agent and it widely used to induce desirable mutations in bacteria **(PalSaha and Paul, 2003; Thiab and Jasim, 2009; Mutwakil, 2011** and **Abd Al-Hadi *et al.*, 2013)**. The induction of mutations by MNNG could be resulted from its effect by adding alkyl (methyl group) to numerous sites on nucleic acids and generate mispairing lesion which effect badly on DNA replication, transcription and DNA repair mechanisms (**Stonesifer** and **Baltz, 1985** and **Abbas *et al.*, 2004)**.

Table (3). Symbols and auxotrophic requirements of *Azotobacter chroococcum* mutants used in the present work

No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Mutant symbol	FM1	FM2	FM3	FM4	FM5	FM6	FM7	FM8	FM9	FM10	FM11	FM12	FM13	FM14	FM15
Auxotrophs	Thr^- /Thi -	Isoleu -	-	-	Asp -	Orn^-/ Asp^-	His -	-	Pro -	-	-	$Vit.B1^-$ /Thr^-	$Vit.B9^-$ / $Vit.B6^-$	Asp -	Glu^-

3. Antibiotic resistance test of *Azotobacter chroococcum* wild type (W.T.) and their mutants on the presence of different antibiotics

The antibiotic resistance properties of wild type and 15 auxotrophs of *Azotobacter chroococcum* were done on complete medium CM supplemented with one of eight antibiotics Figure (2) and Table (4). The presented results showed that all of tested *Azotobacter chroococcum* strains had different levels of sensitivity to Amikacin (30) and Enrofloxacin ENR5 except the mutant strain FM13which was resistant to Enrofloxacin ENR5. The wild type (W.T.) strain exhibited different level of sensitivity to all tested antibiotics except Colistin (10). All the tested strains were sensitive to Ceftriaxone CRT30 except the three mutant strains (FM1, FM3 and FM9) which were able to grow well in the presents of this antibiotic.

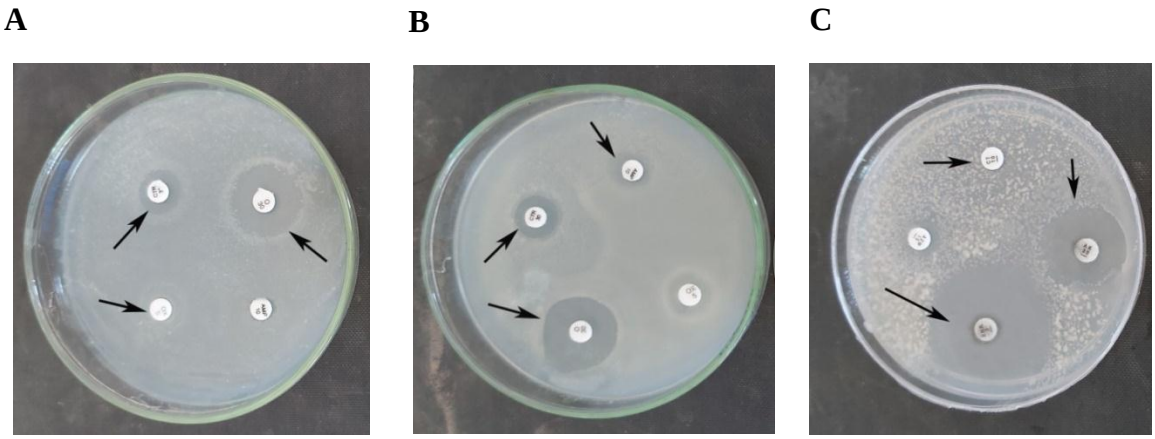

Figure (2). Effect of different antibiotics on growth of some *Azotobacter chroococcum* mutants used in this study. (A) Colistin CT (10), Oxacillin OX5, Ampicillin AMP10 and Oxytetracycline O 30 on FM10, (B) Ceftriaxone CRT30, Oxacillin OX5, Ampicillin AMP10 and Oxytetracycline O 30 on FM2, (C) Colistin (10), Amikacin AK (30), Azithromycin AZM (15) and Enrofloxacin ENR 5on FM8 strain. Arrows referred to inhibition zone.

It was found that the three mutant strains (FM and FM5 and FM10) were resistant to Oxytetracycline O30, while the other strains showed sensitive properties against the same antibiotic. All strains were sensitive to Oxacillin OX5 except the five mutant strains (FM5, FM8, FM9, FM11 and FM15) which were resistant to it. More than half of all tested strains exhibited resistance against to the two antibiotics (Colistin CT10 and Ampicillin AMP10), while the rest were sensitive to them. Concerning the Azithromycin AZM15, five mutant strains (FM1, FM3, FM4, FM6 and FM10) were resistant, while the other strains were sensitive to the same antibiotic. Antibiotic resistance trait and replica plating could be used to confirm the nature of mutants **(PalSaha and Paul, 2003)**. Resistance to antibiotics is a mechanism which microorganisms protect itself from undesirable conditions. The

changes of antibiotic resistance traits could be related to presence of numerous genes which are responsible for generating certain type of enzymes that led to antibiotic detoxification and proteins that suppress antibiotic cellular transferring (**Hayes and Wolf, 1990**).

Table (4). Growth of the *Azotobacter chroococcum* wild type and their mutants on the presence of different antibiotics.

Strains	Colistin CT(10)		Enrofloxacin ENR 5		Oxacillin OX5		Ceftriaxone CRT30		Azithromycin AZM(15)		Ampicillin AMP10		Oxytetracycline O 30		Amikacin AK(30)	
	D/Z	R/S	D/Z	R/S	D/Z	R/S	D/Z	R/S	D/Z	R/S	D/Z	R/S	D/Z	R/S	D/Z	R/S
W.T.	0	R	28	S^{++}	22	S^{++}	3	S	15	S^{+}	18	S^{+}	28	S^{++}	14	S^{+}
FM1	0	R	30	S^{++}	20	S^{++}	0	R	0	R	0	R	0	R	13	S^{+}
FM2	0	R	28	S^{++}	11	S^{+}	12	S^{+}	12	S^{+}	8	S	21	S^{++}	12	S^{+}
FM3	11	S^{+}	35	S^{++}	8	S	0	R	0	R	0	R	18	S^{+}	14	S^{+}
FM4	18	S^{+}	25	S^{++}	20	S^{++}	34	S^{++}	0	R	15	S^{+}	32	S^{++}	18	S^{+}
FM5	0	R	22	S^{++}	0	R	12	S^{+}	15	S^{+}	0	R	0	R	13	S^{+}
FM6	0	R	28	S^{++}	13	S^{+}	12	S^{+}	0	R	0	R	18	S^{+}	12	S^{+}
FM7	8	S	2	S	12	S^{+}	12	S^{+}	12	S^{+}	0	R	21	S^{++}	15	S^{+}
FM8	0	R	35	S^{++}	0	R	18	S^{+}	9	S	15	S^{+}	12	S^{+}	21	S^{++}
FM9	0	R	45	S^{++}	0	R	0	R	12	S^{+}	0	R	22	S^{++}	15	S^{+}
FM10	8	S	18	S^{+}	5	S	13	S^{+}	0	R	0	R	28	S^{++}	16	S^{+}
FM11	0	R	18	S^{+}	0	R	10	S^{+}	13	S^{+}	0	R	0	R	15	S^{+}
FM12	9	S	28	S^{++}	16	S^{+}	21	S^{++}	2	S	22	S^{++}	3	S	18	S^{+}
FM13	8	S	0	R	22	S^{++}	21	S^{++}	18	S^{+}	20	S^{++}	28	S^{++}	18	S^{+}
FM14	10	S^{+}	25	S^{++}	13	S^{+}	18	S^{++}	11	S^{+}	11	S^{+}	21	S^{++}	18	S^{+}
FM15	0	R	3	S	0	R	9	S	13	S^{+}	0	R	17	S^{+}	13	S^{+}

Abbreviations: Resistant (R); Weakly Sensitive (S); Sensitive (S^{+}); Highly Sensitive(S^{++}); Diameter (mm) of Zone around Antibiotic (D/Z).

4- pH tolerance of *Azotobacter chroococcum* wild type and their mutants

The results of growth of all *Azotobacter chroococcum* under eight levels of pH (5, 6, 7, 8, 9, 10, 11 and 12) on Ashby-Mannitol agar medium are shown in **Table (5). Figure (3)** illustrate growth type of all tested strains under two pH levels (5 and 12) as an example of growth used in the present work. Data indicated that, all mutants were tolerant to pH levels less than 10 similar to the wild type, except the FM2 mutant which weakly grew at pH 5. All tested strains survived well at pH 11 whereas, FM2 mutant grew weakly at the same level. All the mutants grew well at pH12 similar to wild type strain, but the two mutants (FM2 and FM13) exhibited weakly growth at the same level and only the mutant FM6 couldn't survived at pH12 level.

The pH tolerance capability of mutants provides these strains with an advantage *in vivo* (**Hamedi *et al.*, 2013)**. **Saleki *et al.* (1997)** reported that mutants could be sensitive to low or high levels of pH.

Mutations may or may not produce discernible changes in the observable characteristics (phenotype) of an organism (**Al- Aradi *et al.*, 2017)**. **Bertram (2000)** reported that mutations were derived from DNA or RNA damage which is not repaired.

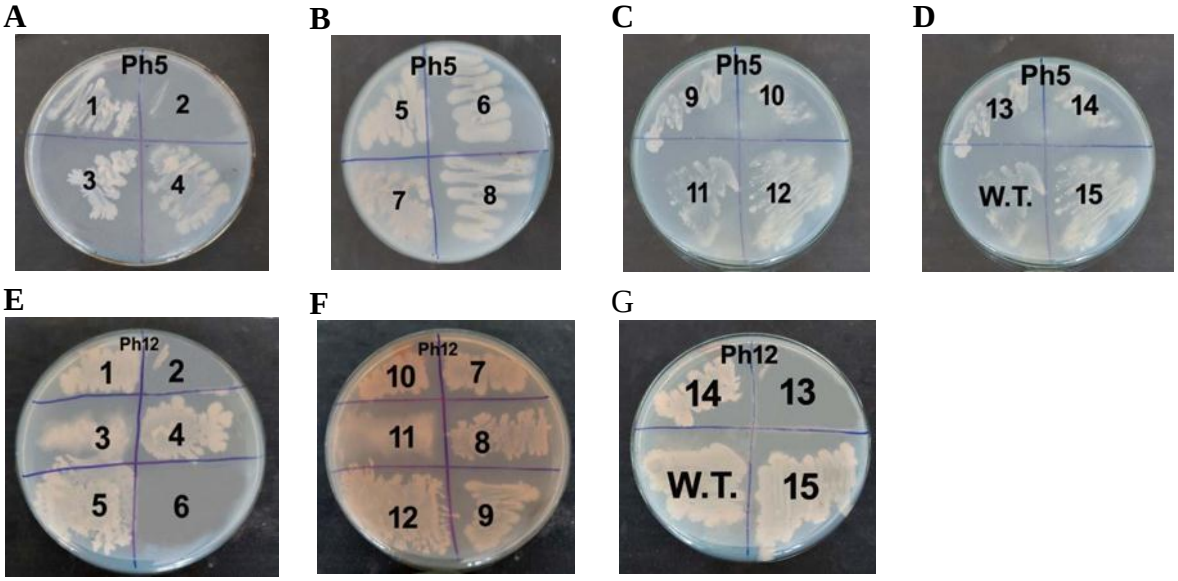

Figure (3). Growth of the *Azotobacter chroococcum* wild type and their mutant strains on medium with pH5 (A to D) and pH12 (E to G).

Table (5). Growth of *Azotobacter chroococcum* wild type and their mutants under different pH levels

Strains	pH							
	5	**6**	**7**	**8**	**9**	**10**	**11**	**12**
W.T.	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
FM1	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
FM2	+/-	+/+	+/+	+/+	+/+	+/+	**+/-**	**+/-**
FM3	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
FM4	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
FM5	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
FM6	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-
FM7	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
FM8	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
FM9	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
FM10	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
FM11	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
FM12	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
FM13	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/-
FM14	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
FM15	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+

*Abbreviations: Growth (+/+); No growth (-/-) and Weak growth (+/-).

5- Salinity tolerance of *Azotobacter chroococcum* wild type and their mutant strains

Salinity test of different *Azotobacter chroococcum* wild type and their mutants were done at different salt concentration (5 to 10%) of NaCl add to Ashby-Sucrose agar medium were shown in **Figure (4).** It was found that all wild type and their mutants grown well at concentrations of NaCl ranged from 5 to 7%, whereas, after increasing the concentration it was observed that wild type strain didn't grow well. On contrast all tested mutants were grown and survived well at 10%NaCl concentration. Random mutagenesis is considered as a powerful tool to adapt stains to growth conditions such as the induction of pH and salt tolerances (**Tillich *et al.*, 2012)**. Induction of mutation in number of halophilic archaea and moderate halophilic bacteria were successfully done by several researchers (**Fitt *et al.*, 1989; Fernandez-Castillo *et al.*, 1990** and **Canovas *et al.*, 1997**). They induced both auxotrophic and salt sensitive mutants by using three chemical mutagens (N-methyl N'-nitro-N-nitrsoguanidine, hydroxylamine, ethyl methanesulfonate).

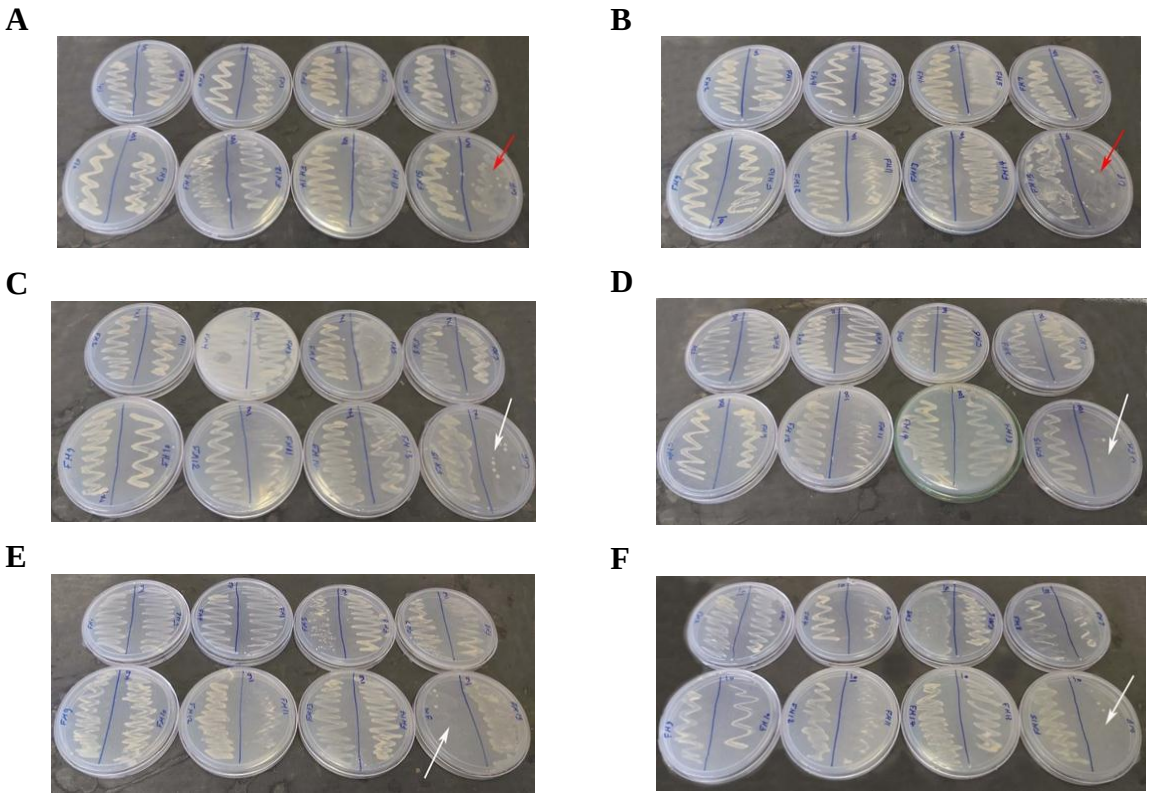

Figure (4). Growth of the *Azotobacter chroococcum* wild type and their mutants on medium supplemented with 5, 6, 7, 8, 9 and 10% NaCl concentrations (A to F, respectively). Arrows referred to wild type growth.

6. Effect of *Azotobacter chroococcum* wild type and their mutants on some vegetative traits of sweet melon (cv. **Kahera 6) seedling under greenhouse condition**

Different growth traits i.e., shoot and root length; leaves number, shoots / roots fresh and dry weight were recorded after 30 days of sowing (Figure 3). Data in Table (6) indicated that there was considerable increase in shoot length trait after treatment with all tested *Azotobacter chroococcum* strains except FM1 treatment which gave value (16.67cm) less than wild type (19.33cm) and untreated control (17.00cm) treatments.

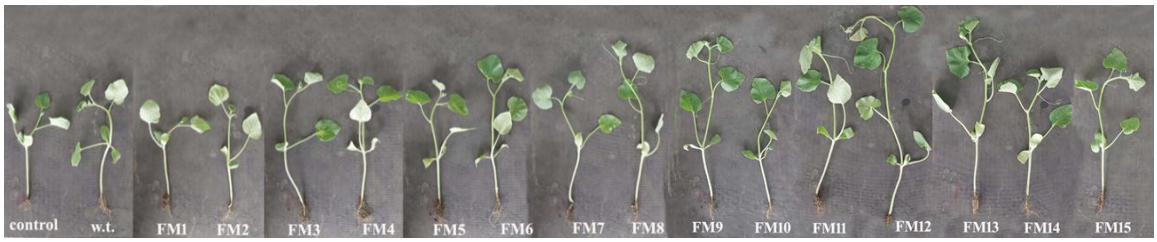

Figure (5). Effect of *Azotobacter chroococcum* wild type and their mutants on different growth traits of sweet melon (cv. Kahera 6) germinated seeds after 30 days of sowing.

The highest value of shoot length were recorded in plants treated with FM12 strain (29.67cm) followed by the two mutants FM13 and FM9 (27.33cm) with a significant increase as compared to wild type and untreated control. Concerning the root length, there was no significant increase in root length trait in plants treated with all tested strain as compared with wild type and untreated control (3.00 and 2.33cm, respectively) treatments except plants treated with FM15 mutant that gave significant value (7.33cm). As shown in Table 6, the highest values of no. of leaves /plant were found in plants treated with the four mutants FM5, FM8, FM11 and FM12 with the same value (6.67) with a significant increase as compared to untreated control (5.33). The highest root fresh weight was observed in plant treated with mutants FM13, FM12, FM8 and FM9 (4.03, 3.67, 3.52 and 3.50g, respectively) which significantly higher than untreated control treatment (1.75g). Data in Table 6 revealed that only plants treated with FM13 strain exhibited significant value as compared to plants treated with wild type strain (2.69g).

Table (6). The vegetative growth traits of sweet melon seedlings recorded after seed covering with the *Azotobacter spp.* wild type and their mutants after 30 days

Treat.	Shoot length (cm)	Root Length (cm)	Leaves no.	Shoot fresh weight (g)	Root fresh weight (g)	Shoot Dry weight (g)	Root Dry weight (g)
Control	17.00	2.33	5.33	1.75	0.07	0.17	0.01
W.T.	19.33	3.00	6.00	2.69	0.10	0.29	0.02
FM1	16.67	5.00	5.33	1.64	0.09	0.19	0.02
FM2	19.17	2.50	5.67	2.17	0.08	0.22	0.02
FM3	21.17	3.83	6.33	1.93	0.06	0.23	0.02
FM4	22.67	3.67	6.33	2.89	0.08	0.29	0.03
FM5	23.00	3.33	6.67	2.84	0.16	0.33	0.02
FM6	23.67	3.00	6.33	2.66	0.10	0.26	0.04
FM7	21.33	5.00	5.67	2.49	0.07	0.24	0.02
FM8	26.33	4.00	6.67	3.52	0.09	0.36	0.02
FM9	27.33	2.67	6.33	3.50	0.08	0.32	0.04
FM10	22.00	3.33	6.00	2.31	0.05	0.25	0.01
FM11	23.67	4.00	6.67	2.75	0.10	0.27	0.04
FM12	**29.67**	3.33	6.67	3.67	0.13	0.41	0.06
FM13	27.33	3.42	6.33	4.03	0.17	0.25	0.03
FM14	21.00	3.33	6.33	2.85	0.11	0.27	0.02
FM15	20.33	**7.33**	6.33	2.16	0.09	0.23	0.03
$LSD_{0.05}$	**8.43**	**2.67**	**1.23**	**1.33**	**0.02**	**0.19**	**0.01**

The highest values of root fresh weight were found after treatment by the three mutants FM13, FM5 and FM12 (0.17, 0.16 and 0.13g, respectively) with a significant differences with those of wild type and untreated control (0.10 and 0.07g, respectively), on the other hand the lowest values were obtained by

treating plants with FM10 and FM3 (0.05 and 0.06g, respectively) as compared to all other treatments. According to data in Table 6, there was observed increment in trait in plants treated with all tested strains as compared to untreated control. The highest values of shoot dry weight were recorded in plants treated with FM12 (0.41g), FM8 (0.36g), FM5 (0.33g) and FM9 (0.32g) with a significant increase as compared to untreated control (0.17g). Finally, the four mutants FM12, FM6, FM9 and FM11 gave a superior plants in root dry weight trait (0.06g FM12) and 0.04g for the other three strains) with a significant increase as compared to both of wild type (0.02g) and untreated control (0.01g). In general, almost all of the tested mutants were able to promote significantly all studied vegetative traits of sweet melon as compare to wild type stain and untreated control.

Several studies have revealed that seed inoculation with *Azotobacter chroococcum* improves the vegetative growth of numerous crops like, onion (**Kurrey *et al.*, 2018),** Corn (**Okoh, and Irene, 2020)**), wheat (**Kizilkaya, 2008**), and rice (**Kennedy *et al.*, 2004**). This improvement could be derived from several mechanisms like nitrogen fixation, excretion of ammonia, production of phyto-hormones, antifungal substances, siderophore and phosphate solubilization (**Obele *et al.*, 2019** and **Althaf and Srinivas, 2013**).

7. Effect of *Azotobacter chroococcum* wild type and their mutants on germination% and growth indicators of sweet melon (cv. Kahera 6) seedling

After 30 days of planting seeds in pots under greenhouse conditions germination percentage, seedling length and vigor index were recorded as shown in Table 7. Data reveal that, from the sixteen tested *Azotobacter chroococcum* strains only nine mutants increased the percentage of germination of sweet melon seeds significantly compared to the wild type and untreated control.

Seed covered with the nine mutants FM7 to FM15 showed the highest percentage of seed germination (100%). While, the four mutants FM3 to FM6 gave similar results to wild type and untreated control (83.33%). The lowest values of germination percentages were found in seeds treated with the two mutants FM1 and FM2 with the same value (66.67%).

Table (7). Effect of *Azotobacter chroococcum* wild type strain and their mutants on the germination percentage, seedling length and *vigor index* of sweet melon seedling

Treatments	% Germination	Seedling length	*Vigour index*
Control	83.33	20.33	1694.10
W.T.	83.33	22.33	1860.76
FM1	66.67	21.67	1444.74
FM2	66.67	21.67	1444.74
FM3	83.33	25.00	2083.25
FM4	83.33	26.34	2194.91
FM5	83.33	26.33	2194.08
FM6	83.33	26.67	2222.41
FM7	100.00	26.33	2633.00
FM8	100.00	30.33	3033.00
FM9	100.00	30.00	3000.00
FM10	100.00	25.33	2533.00
FM11	100.00	27.67	2767.00
FM12	100.00	33.00	3300.00
FM13	100.00	30.75	3075.00
FM14	100.00	24.33	2433.00
FM15	100.00	27.66	2766.00
$LSD_{0.05}$	**16.17**	**5.11**	**526.55**

Regarding to seedling length, seeds treated with all tested strain s exhibited considerable increase when compared with untreated control treatment. Data in Table (7) revealed that the four mutants (FM12, FM13, FM8 and FM9) were superior to all other tested strains and untreated control. They significantly increase the seedling length as compared to wild type and untreated control treatments. The highest value of seedling length (33.00cm) was observed when seeds treated with FM12. The vigor index increased significantly in sweet melon seedling treated with almost of tested mutants (FM6 to FM15) as compared with both of wild type and untreated control treatments. Treatment with the four mutants (FM12, FM13, FM8 and FM9 showed the highest value of vigor index (3300.00, 3075.00, 3033.00 and 3000.00, respectively). The lowest vigor index values were found in plants treated with the two mutants FM1 and FM2 with the same value (1444.74) as compare to all other treatments (Table 7). Our results were in accordance with these of (**Singh, 2006)** who found that inoculation by *Azotobacter* enhanced seed germination of several non-legume crops such as soybean, rice and cotton. *Azotobacter spp*. can be produce active substances that can increase seed germination, and plant growth such as vitamin B, and different plant hormones (**Ahmad *et al.*, 2005** and **Husen, 2003**).

8. Effect of *Azotobacter chroococcum* wild type and their mutants on N, P and K content in leaves of sweet melon seedling

Analysis of macro-nutrients (N, P and K) uptake in shoots of sweet melon is presented in Table (8). The highest percentages of N content were found in plants inoculated with the six mutants FM12, FM13, FM6, FM8, FM4 and FM9 (2.08, 2.03, 2.02, 1.99, 1.98 and 1.93%, respectively) with a significant increase as compared to wild type (1.90%) and untreated control (1.85%). Concerning phosphorus content in shoots, data revealed that all treatments gave higher level P content than untreated control. Plants treated with the six mutants FM12 (0.21%), FM13, FM6, FM8, FM9 (0.20%) and FM4 (0.19%) exhibited the highest values P contents in shoots with a significant increase as compare to both of wild type (0.17%) and untreated control (0.16%). The highest values of K content were found in plants treated with the two mutants FM13 and FM12 (1.31 and 1.30%, respectively) with a significant increase as compared to wild type (1.26%) and untreated control (1.24%).

Table (8). Effect of *Azotobacter chroococcum* wild type and their mutants inoculation on N, P and K contents in sweet melon shoot after 30 days of sowing

Treatments	N%	P%	K%
Control	1.85	0.16	1.24
W.T.	1.90	0.17	1.26
FM1	1.90	0.17	1.24
FM2	1.90	0.17	1.23
FM3	1.97	0.18	1.26
FM4	1.98	0.19	1.28
FM5	1.95	0.16	1.28
FM6	1.99	0.19	1.27
FM7	1.91	0.17	1.25
FM8	2.02	0.20	1.29
FM9	1.93	0.19	1.24
FM10	1.84	0.18	1.26
FM11	1.83	0.19	1.24
FM12	2.08	0.21	1.30
FM13	2.03	0.20	1.31
FM14	1.92	0.17	1.23
FM15	1.89	0.18	1.25
$LSD_{0.05}$	**0.13**	**0.02**	**0.04**

The obtained results revealed that inoculation of sweet melon plants with almost of tested *Azotobacter chroococcum* strains particularly the three mutants (FM12, FM13 and FM8) increased the uptake of leaves N, P and K contents considerably higher than untreated control (Table 8). Similar results were reported by **Rajaee *et al.*, 2007** in wheat. They found that inoculation of seeds with *Azotobacter* helps in uptake of N, P and K in addition to micronutrients like Fe and Zn, in wheat, consequently, improve wheat nutrition process.

9. Effect of *Azotobacter chroococcum* wild type and their mutants inoculation on photosynthetic pigments in leaves of sweet melon seedling

As shown in Table (9), data generally revealed that treatment with almost of all tested *Azotobacter chroococcum* strains considerably increased all photosynthetic pigments (chlorophyll a, b and carotenoids) in the sweet melon shoots as compared to untreated control treatment. Inoculation with the three mutants FM12, FM13 and FM8 gave higher percentages of photosynthetic pigments with a significant increase rather than both of wild type and untreated control treatments.

Table (9). Effect of inoculation with *Azotobacter chroococcum* wild type and their mutants on photosynthetic pigments (chlorophyll a, b and carotenoids) in sweet melon shoots after 30 days of sowing

Treatments	chlorophyll a (mg/g FW)	Chlorophyll b (mg/g FW)	carotenoids (mg/g FW)
Control	0.314	0.198	0.216
W.T.	0.315	0.212	0.218
FM1	0.316	0.214	0.218
FM2	0.313	0.217	0.217
FM3	0.317	0.217	0.221
FM4	0.315	0.216	0.220
FM5	0.316	0.217	0.219
FM6	0.321	0.221	0.223
FM7	0.319	0.219	0.219
FM8	0.321	0.220	0.222
FM9	0.323	0.222	0.223
FM10	0.321	0.221	0.223
FM11	0.320	0.221	0.225
FM12	0.325	0.223	0.226
FM13	0.327	0.225	0.226
FM14	0.319	0.219	0.224
FM15	0.320	0.218	0.223
$LSD_{0.05}$	**0.008**	**0.021**	**0.007**

The enhancement of photosynthetic pigments production seen in this study could be due to a higher rate of chlorophyll synthesis which promotes the plant development. **Ramakrishnan** and **Selvakumar (2012)** found increment in total protein and photosynthetic pigments in tomato plants inoculated with *Azotobacter* as compared to control. This improvement could be due to quick increments of plant growth and number of leaves in treated plants.

Finally, it could be concluded that treatment of *Azotobacter chroococcum* wild type strain with different MNNG concentrations may produce a new genetic parameter (Genotype) which could be used as a desirable bio-inoculates to improve growth and quality of sweet melon seedlings.

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