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NEW AZOTOBACTER TRANSFORMANTS TO IMPROVE GROWTH OF SQUASH PLANTS UNDER SALINITY STREESS.

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ABSTRACT

This study was carried out at the laboratory of genetic department and the farm of horticulture department Fac. of Agri. Minia University in two successive seasons on f 2003 and 2004. In this study some transformants of *Azotobacter* showed high tolerance against saline water (0.25, 0.50, 0.75, 1.oo, 1.25, 1.50, 1.75, and 2.00 g/ 100 ml w/v). *Azotobacter* transformants were obtained by incubating wild isolate of *Azotobacter* with an isolate of *Bacillus spp.,* tolerant to 30% NaCl, for 2 or 24 hrs. These resulted transformants were tested in pot experiments under four levels of salinity and presence of squash plants as indicator. The results revealed that:

1- The wild type strain of *Azotobacter* was sensitive to 1.5-2.0% NaCl as recipient comparison to the *Bacillus spp*.which resistant to 30%NaCl as donor.
2- The transformants of *Azotobacter* were obtained after two hours incubation were less in their frequencies than those were obtained after 24 hours incubation with DNA of *Bacillus* sp.
3- Adding transformants of *Azotobacter* to salinity soil led to reduction in the symptoms of salinity on squash plants.
4- Different transformants varied in their effects, regards, plant growth characters under salinity conditions and T2 & T4 showed the best effect.

INTRODUCTION

Squash (*Cucurbita pepa L*.) is a popular vegetable in Egypt. Its fruits are used for local consumption and for exportation (El-Maziny and Mohey El-Din 1985). Many investigations have been carried out to study the effect of fertilizing squash plant with different rates of nitrogen and bio-fertilizing on the plant. The effect of inoculation of seeds or soil by *Azotobactr spp.* on plant growth and yield have been studied by several authors (Hussain and Iqbalkhan, 1973 ; Badawy and Amer, 1974) . Hassan *et al.*, (2000) reported that the isolates under his studies had significantly improved the yield of squash more than the chemical fertilizer.

Azotobactr v*inelandii,* afree-living gram-negative soil bacterium, has the ability to fix nitrogen under aerobic conditions. As noted by others (David et al., 1981: Page and Tigerstrom 1982),the development of an effective transformation system for Azotobactr spp. should be facilitate genetic studies of the nitrogen fixing (nif) genes, help to determine precisely how the nitrogenase is protected from inhibition by oxygen, and aid in the routine characterization and analysis of other genes.

Salinity is considered serious problem in many areas of Egypt. The main component of salinity soil is NaCl (Abd El-Mageed1996).Different plant have wide range of salinity tolerance and most agriculture crops are salt sensitive.

Therefore, salinity may be one of the major factors determining yields. Several reports established that progressive increase in salt concentration adversely affected inoculum viability and the nitrogen fixation rate (Wilson and Norris1970 and Balasubramanian and Sinha 1976).
Zablotowicz and Focht (1981) observed that plants inculcated with salt-tolerant *Rhizobium spp.* Strain 176a28 recorded from drought stress to the same level of nitrogen fixation and nodulation as those that received adequate irrigation however plants inoculated with salt sensitive *Rhizobium* strains failed to achieve the same level of recovery. The result imply that salt tolerant *Rhizobium spp*. may be useful to maximize the nitrogen fixation potential in semi arid regions.

There is a firm evidence that indole 3 acetic acid (IAA), gibberellins and cytokines all are produced by various bacteria which line in association with plants. There is also an evidence that the growth hormones produced by the bacteria can in some instances increase growth rates and improve yields of the host plants (Tien *et al* 1979).

The purpose of this study was to find out on transformats of *Azotobacter*, tolerant to saline condition, this *Azotobacter* transformats can be used as soil inoculate to improve squash growth and yield in saline area.

MATERIALS AND METHODS

This investigation was carried out at the Genetics Dept., and Hort. Dept. Fac. Agriculture, Minia University during two successive nili seasons of 2003 and 2004..

Preparation of treatments:

a- strains:-

1- Nitrogen fixing *Azotobacter chroococum* was isolated in Genetics department by Abed El- Rahem *et al.* (1995)
2- Thermophilic and halophilic isolates of *Bacillus spp.* parent were isolated at 50°C and 30% NaCl from salted fish (Dakhly, 1993) were used as donor for tolerance against saline water.

b- Media:-

Complete medium (Strandberg and Wilson, 1968) was used to grow *Azotobacter* whereas medium deloped by Ali and Hafez 1991 was used to grow the isolate of Halobacterium (*Bacillus sp*.).

c- Isolation of microorganisms :-

1. NaCl Resistant isolates :

A wild type of Halobacterium was grows on liquid complete medium at 30 ^{o}C for overnight. Culture were then diluted using sterilized distaled water (Ali and Hafez 1991).

Diluted culture was streak on complete agar medium supplemented with 30% NaCl. Five plates were incubated at 30°C for 3 days. The resistant colonies were selected and one of these colonies was used as a donor.

2- wild isolates of *Azotobacter chroococum* were obtained from Genetic Dept. Fac. Agric. Minia Univ. Sample (0.1 ml) from this isolate of suitable dilution were plated on complete media, the plates were incubated at

30°C for 3 days..Single colonies were tested on CM and CM supplemented with different concentrations (0.0, 0.25, 0.50, 0.75,1.00, 1.25,1.50,1.75 and 2.00 g/ 100 ml)of NaCl at 30°C for 3 days and selective colonies to (0.50g/L) and used as a recipient parent. Number and percentage of surviving cells were determined under different concentration of NaCl and tolerant isolates were selected for present study.

d- DNA extraction:

DNA from donor strain (Halophilic bacteria) was isolated and purified according to Ausuble *et al.* (1987).

e- Transformation procedure :-

According to Rifaat *et al.* (1974) DNA of the donor strain was added to the recipient cultures with a final concentrations of 30mg/ml. Lithium acetate was also added (1 ml 0.30% w/v) for each culture (Ali and Hafez, 1991). *Azotobacter* transformats were obtained using method developed by Rifaat *et.al.* (1974).

Pot experiments:-

Soil samples were randomly taken from the experimental field before planting at 0-30 cm depth. The chemical and mechanical analysis of the soil were curried out according to Page *et al.* (1982) data of analysis are presented in Table 1.

Table 1: Physical and chemical properties of farm soil.

Property	Value	Property	Value
C.E.C	32.4 mg/100gm soil	Total N%	0.12
E.C.	1.93 mmhos/cm	P	18 ppm
CaCO3	2.14 %	Sand %	26.12
Organic matter	1.72 %	Silt %	32.12
Exch. K	2.18 meq/100 g soil	Clay %	42.75
pH	7.92	Texture	Silty clay

Pot experiment was conducted to evaluate the effects of different levels of salinity presence of *Azotobacter* transformants on some squash growth parameter. Clay plastic pots 50 cm. in claim each contain 3 kg. of collected soil were used. Pots in each salinity level were divided to five groups, each contain 6pots. Each group was inoculated using one of *Azotobacter* transformants (T1, T2, T3& T4) in addition to control (without inoculate).

Salinity treatment was applied using NaCl as a source for salinization. Four concentrations of salt (i.e. 0.0, 0.1, 0.2 and 0.3%) (w/w) salt : soil were used. Thirty pots were used for each level of soil salinity. Six seeds were seeded in each pot. After fifteen days from sowing, plants were thinned to two plants per pot. All irrigation was done with distilled water that added to adjust it near field capacity twice a week/. The experiment was designed as a two way complete factorial with three replications in a completely randomized block. After 30 days all plants were removed and the following data were mean number of number of leaf/plant, leaf area, fresh & dry weight/plant, nitrogen content & uptake in plant and chlorophyll A&B.

RESULTS AND DISCUSSION

1-laboratory experiments:-

a- Effect of salt (NaCl):-

Table 2 shows the means and percentages of survivals *Azotobacter chroococum* after treatments with different concentrations of NaCl (0.0, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50.175 and 2.0 g/100 ml complete media CM).The results indicate clear negative correlation between percentage of surviving wild type of *Azotobacter chroococum* and concentration of NaCl in the medium. Salt induced changes could play a role in bacterial gene regulation (Nibbrioin *et al.,* 1989). It seems important to know whether the topological variations induced in haloarcheae by varying the NaCl or $MgCl_2$ concentrations which may be used by the cell to regulate expression.

Table 2:Effect of different concentrations of NaCl on number and percentage of *Azotobacter chroococum* surviving.

Concentration of NaCl (%)	Mean Number of colorves/dish	Percentage of surviving
0.00	224.75	100.0
0.25	224.0	99.67
0.50	216.0	96.11
0.75	188.25	83.76
1.00	120.75	53.73
1.25	55.25	24.58
1.50	11.25	5.01
1.75	1.50	0.70
2.00	0.0	0.0

b- Transformation:

The transformation experiment included crossing, halophilic bacteria with *Azotobacter chroococum* recipient. Table 3 shows frequencies of transformants resulted from interspecific crosses between the donor wild type of halophilic bacteria and *Azotobacter chroococum* as a recipient. In general data in Table 3 show that there was variation in transformants frequencies resulted from different incubation periods. In addition, it can be observed that frequencies of transformants of different period, in one hour (20.08×10^{-6} and 69.4×10^{-6}) two hours (250×10^{-6} and 125×10^{-6}) on 5% and 10% NaCl, respectively.

Data in Table 3 also indicated that frequencies of transformants resulted from the long; incubation period (24 hours), showed higher tolerance to NaCl compare with frequencies resulted from short period of incubation. The higher frequency of transformants was obtained from 24 hours incubation (347×10^{-6}). These results indicated that the frequencies of transformants (13.8×10^{-6}) were obtained at higher concentration of NaCl (15% NaCl). These results may be attributed to the compectence (ability of a cell to be transformed) of the recipient cells. Beattie and Setlow (1970) found that heterospecific transformation between different species of Haemophilus

was lower 1-6 times than homospeciffic transformation. Rifaat *et al*. (1974) studied resistance to streptomycin and However, double transformation of the two markers failed to occur. The level of resistance in the transformants was similar to that in the donor strain. Chloramphenicol transformants were obtained after 12 hours incubation of DNA- recipient cultures, whilst with streptomycin resistance, the competence was observed after 24 hours. They also found that the majority of the chloramphenicol or streptomycin transformants were more or less of similar efficiency of nodule formation and nitrogen fixation to the donor strain. Some transformants were intermediate in their efficiency between donor and recipient strains and none reached the level of the recipient.

Table 3: Number and percentage of *Azotobacter chroococcum* transformants tolerate various NaCl concentrations.

Isolates	Time	Survival Percent.	Concentration of salt (NaCl)						
			0%	5%	10%	15%	20%	25%	30%
Bacillus spp. (donor)		No.	120	120	120	120	120	120	120
		%	100	100	100	100	100	100	100
Azotobacter chroococum (recipient)		No.	150	0.0	0.0	0.0	0.0	0.0	0.0
		%	100	0.0	0.0	0.0	0.0	0.0	0.0
Azotobacter Transformants	1 hr	No.	$72x10^5$	15.0	5.0	0.0	0.0	0.0	0.0
		%	100	$208x\ 10^{-6}$	$69.4x10^{-6}$	0.0	0.0	0.0	0.0
	2 hr.	No.	$72x10^5$	18.0	9.0	0.0	0.0	0.0	0.0
		%	100	$250X10^{-6}$	$125X10^{-6}$	0.0	0.0	0.0	0.0
	24 hr.	No.	$72x10^5$	25.0	14.0	3.0	1.0	0.0	0.0
		%	100	$347x10^{-6}$	$194x10^{-6}$	$13.8x10^{-6}$	$1.38x10^{-6}$	0.0	0.0

Ali *et al.* (1987) showed transformants with different levels of nitrogen fixation efficiency ranged from 4.0 to 40.0 mg nitrogen/ml MM in both intra and inter-specific transformation using *Azotobacter chroococcum* and *Azotobacter vinelandii.* Great differences in nitrogen fixation were obtained among the single transformants of each recipient strains. Some of them were more efficient to fix nitrogen than their recipient or donor strains and other were less efficient. The absence of iron in the transformation medium is an important condition for successful chromosomal DNA mediated transformation. In this case, a membrane protein is induced (Guilkema and Sherman 1984). Similarly, in the absence of iron, four new proteins are found in the membrane of *Azotobacter vinelandii*(Page and Tigerstrom 1982). These proteins may play a role in the competence inducing regimen devised by Page and Tigerstrom, (1982) and Reusch and Sadoff, (1983) may induce the uptake of DNA in general, but the integration step that is essential foe chromosomal DNA-mediated transformation may only be successfully completed at a specific stage during the growth cycle.

II- Pot experiments:-

Number of leaf/plant:

Data in Table 4 show that leaf number per plant of uninoculated plants was significantly decreased under all salinity levels except with the first level of salinity (1000 mg/kg.) compare with control plants. The greater reduction in

leaf number/plant occurred with the high salinity level. These results are in agreement with those reported with many plant species by Shaheen (1984) and Abd El-mageed (1996).

Inoculation of squash plants with various *Azotobacter* strain increase the leaf numbers per plant under different salinity levels. This may be due to the inoculation process which improved the ability of plants to grow under the adverse effects of salinity. The highest values of leaf per plant was obtained with those plants which inoculated with isolate 4 (9.375) followed by isolate 2 (9.333) with significant differences with the control plants (uninoculated plants).

Table 4: effect of salinity on number of leaf/plant and leaf area in presence and absence of *Azotobacter* isolates transformats.

(A) *mg* NaCl / *kg.soil*	(B)					Mean (A)
	Control	Isolate 1	Isolate 2	Isolate 3	Isolate 4	
	Number of leaf/plant					
0.00	9.000	7.167	9.833	7.167	9.500	8.533
1000.00	11.167	9.500	8.667	7.667	9.167	9.233
2000.00	6.500	9.500	9.333	8.833	9.000	8.633
3000.00	3.333	10.167	9.500	9.667	9.833	8.500
Mean (B)	7.500	9.083	9.333	8.333	9.375	8.725
L.S.D. 5%	**A=2.268**		**B=1.662**		**AB= 3.323**	
	Leaf area (cm^2)					
0.00	7.75	8.05	10.99	7.31	13.43	9.50
1000.00	24.60	23.63	23.69	12.80	25.11	21.86
2000.00	13.94	20.42	22.18	21.25	20.22	19.60
3000.00	5.44	26.31	31.36	18.25	27.12	21.70
Mean (B)	12.80	19.60	22.06	14.90	21.47	18.17
L.S.D. 5%	**A= 4.898**		**B=3.658**		**AB= 7.315**	

Leaf area:

The leaf area was significantly increased after inoculation with different isolate of *Azotobacter* particularly with isolate (2) which showed (22.06 cm^2) compared with the control (12.80 cm^2) as shown in Table 4.

Regarding to salinity level, data show that only the fourth level of salinity (3000 mg/kg.) showed adverse effects on leaf area and decreased the values of leaf area particularly in those plants which uninoculated to 5.44 cm^2. after, inoculation with *Azotobacter* strain; these adverse effects of salinity were disappeared.

Fresh and dry weights per plant:-

Date in Table 5 indicated that both fresh and dry weight per plant was not significantly affected under this level of salinity which used in this experiment. This means that squash plants may be more tolerate to higher level of salinity (300 mg /kg.) under this investigation. This results are in contrast with those obtained by Farah *et al*. (1981) who found that fresh weight of bean plants was significantly decreased with increasing salinity levels. On the other hand, both fresh and dry weights of squash plants were significantly increased after inoculation process with isolate of *Azotobacter*

compared with the control plants as shown in Table 5. The highest values of both fresh and dry weights were obtained after inoculation with isolate 4. These results are in agreement with those obtained by Dakhly and Abd El-Mageed (1997) on potato, carrot and tomato and also Fouly *et al.* (2002) on garlic.

Nitrogen content and uptake in plant:

Data presente in Table 6 show that nitrogen content in the plant increased significantly after inoculation with *Azotobacter* isolate in comparison with the control. The highest values of nitrogen content in plant was obtained after inoculation with isolate 2 which showed the highest values of nitrogen content (10746) in the plant.

Table 5: Effect of salinity on fresh and dry weight (gm)/plant in presence and absence of *Azotobacter* isolates.

(A) *mg* NaCl / *kg.soil*	(B)					Mean (A)
	Control	Isolate 1	Isolate 2	Isolate 3	Isolate 4	
	Fresh weight (gm)					
0.00	8.367	6.423	15.327	7.617	12.190	9.985
1000.00	13.967	13.983	9.693	10.517	12.113	12.055
2000.00	6.173	9.843	13.303	16.287	9.937	11.159
3000.00	4.867	17.853	12.853	15.660	14.963	13.195
Mean (B)	**8.343**	**11.971**	**12.794**	**12.520**	**12.963**	**11.586**
L.S.D. 5%	**A= N.S.**		**B= 2.321**		**AB= 4.643**	
	Dry weight (gm)/plant					
0.00	2.70	1.69	4.54	1.50	3.52	2.79
1000.00	3.66	2.98	2.01	3.48	3.64	3.15
2000.00	2.20	2.53	2.50	3.71	3.25	2.84
3000.00	1.27	4.66	3.78	3.81	3.78	3.46
Mean (B)	**2.46**	**2.96**	**3.21**	**3.13**	**3.54**	**3.06**
L.S.D. 5%	**A= N.S.**		**B=0.665**		**AB=1.331**	

Table 6: Effect of salinity on N-content and up take in plant (mg/gm) in presence and absence of *Azotobacter* isolates.

(A) *mg* NaCl / *kg.soil*	(B)					Mean (A)
	Control	Isolate 1	Isolate 2	Isolate 3	Isolate 4	
	N. content					
0.00	1.273	1.487	1.510	1.407	1.387	1.413
1000.00	1.290	1.640	1.744	1.507	1.527	1.542
2000.00	1.143	1.597	1.787	1.857	1.860	1.649
3000.00	1.260	1.400	1.943	1.720	1.587	1.582
Mean (B)	**1.242**	**1.531**	**1.746**	**1.622**	**1.590**	**1.546**
L.S.D. 5%	**A= 0.07045**		**B=0.09481**		**AB= 0.1896**	
	N. uptake					
0.00	3.507	2.523	6.870	2.110	4.850	3.972
1000.00	4.777	4.903	3.960	5.227	4.927	4.665
2000.00	2.543	3.857	4.500	6.960	6.013	4.775
3000.00	1.613	6.540	7.340	6.487	5.973	5.591
Mean (B)	**3.110**	**4.456**	**5.550**	**5.196**	**5.441**	**4.751**
L.S.D. 5%	**A= 1.598**		**B=1.086**		**AB= 2.173**	

In the meantime, the nitrogen content in plant was incrased with increasing salinity level to 2000 mg/kg. of NaCl, but decreased with the highest level of salinity (3000 mg/kg.).

Nitrogen uptake (mg/g DW):-

Presented Data in Table 6 show that nitrogen uptake in treated plants was significantly increased in those plants which inoculated with different isolate of *Azotobacter* compare with the control plants. While, nitrogen uptake in control plants was 3.11 only it reached 5.550 in plants treated with *Azotobacter* isolate2. This increase may be as result for increasing nitrogen content in soil due to efficacy of this transformants in fixing atmosphere nitrogen and make it available for plants. This increase N uptake in plants.

Chlorophyll contents:-

Data presented in Table 7 show that both chlorophylls A and B were markedly increased (particularly with chlorophyll A) after inoculated the plants with *Azotobacter* isolates compared to the control plants. The highest values of chlorophyll A (22.84) was obtained after inoculated with isolate (2) while, the highest value of Chlorophyll B (11.68) was obtained with those plants which inoculation with isolate (3). This may be after improving which occurred in physiological process with inoculation process as a result for increasing in nitrogen content and uptake in plants as well as growth hormones increasing after inoculation with *Azotobacter.*

Table 7: Effect of salinity on Chlorophyll contents A and B in presence and absence of *Azotobacter* isolates.

(A) ***mg* NaCl / *kg.soil***	**(A)**					**Mean (A)**
	Control	**Isolate 1**	**Isolate 2**	**Isolate 3**	**Isolate 4**	
	Chlorophyll A					
0.00	8.67	22.21	18.50	15.97	20.64	17.20
1000.00	10.19	20.30	24.18	20.71	19.10	18.90
2000.00	9.29	19.74	22.22	24.30	25.97	20.30
3000.00	5.20	18.19	26.44	24.47	21.13	19.09
Mean (B)	**8.34**	**20.11**	**22.84**	**21.36**	**21.71**	**18.87**
L.S.D. 50%		**A= 0.959**		**B= 1.149**		**AB= 2.298**
	Chlorophyll B					
0.00	6.25	11.46	8.27	8.97	10.07	9.01
1000.00	5.34	8.09	9.46	7.37	7.13	7.48
2000.00	3.27	6.80	14.24	16.42	18.54	11.85
3000.00	3.30	8.67	12.05	13.97	10.02	9.60
Mean(B)	**4.54**	**8.76**	**11.00**	**11.68**	**11.43**	**9.48**
L.S.D. 5%	**A= 5.446**		**B=2.339**		**AB=4.679**	

Also data in Table 7 show that there was an increase in chlorophyll A and B contents with increasing salinity level but this increase was unstable from one salinity level to another.

Also, these results show that the interactions effect between inoculation process with *Azotobacter* transformants and salinity levels were significant for all previous characters. This clearly show the major role of inoculation process in define the adverse effects for salinity levels on plant

growth and plant absorption and uptake for soil elements particularly nitrogen element

Therefore, it can be concluded from the present results that the use of new *Azotobacter* transformants as bio- fertilizer for squash production could reduce the chemical nitrogen fertilizer without reducing productivity. This treatment may be will decrease the high cost of chemical fertilizer as well as the pollution of the environment and produce healthy food.

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تحسين النمو لنباتات الكوسة تحت ظروف الملوحة باستخدام متحولات ازوتوباكتر جديدة

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أجريت هذه الدراسة في معامل قسم الوراثة ومزرعة كلية الزراعة جامعة المنيا في موسمين (٢٠٠٣ ، ٢٠٠٤) وتم خلال هذه الدراسة استحداث عزلات متحولة من الازتوباكتر محتملة للملوحة واختبارها في تجربة أصص تحت مستويات من الملوحة (باستخدام محلول ملحي من كلوريد الصوديوم تركيز ٠,٢٥ , ٠,٥٠, ٠,٧٥, ١,٠٠, ١,٢٥, ١,٥٠, ١,٧٥, ٢,٠٠ جم / ١٠٠مليلتر) لاختبار كفاءة العزلات واستخدامها في تجارب الحقل وكانت أهم النتائج كالتالي:-

١- عند اختيار الطراز البرى للأزوتوباكتر والمستخدم كمستقبل لصفة تحمل الملوحة وجد انه حساس عند ١,٥ -٢% ملح كلوريد صوديوم مقارنة بال *Bacillus sp.* الذي يتحمل الملوحة حتى ٣٠%كلوريد صوديوم والمستخدم كمانح لصفة تحمل الملوحة.

٢- أظهرت الدراسة إن الازوتوباكتر المتحولة على ساعتين التحضين اقل من تكرارها من المتحولات على ٢٤ ساعة من التحضين من الـ DNA الخاص بالـ *Bacillus sp.* .

3- تبين من التجربة بالأصص إن إضافة متحولات للأزوتوباكتر بالتربة الملحية يقلل تأثير زيادة الملوحة على نباتات الكوسة.

4- كما وجد اختلافات بين المتحولات وبعضها في التأثير على صفات النمو لنباتات الكوسة تحت مستويات الملوحة المختلفة في التربة وكان أفضل العزلات هي المتحولات أرقام T٢ ، T٤ في معظم الصفات تحت الدراسة .