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Induction of Salt Tolerant Mutants in Rhizobia Infected *Pisum Sativum* l.

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Abstract: Six *Rhizobium* strains were used in this study. These strains were treated with different concentrations of sea water to be induce salt tolerant mutants. Five isolates were isolated from each concentration and then they were evaluated for IAA production. Six *Rhizobium* strains appeared high level of resistance against erythromycin-ethlsuccinate (*Eryth*) than the other antibiotics. Whereas, RL-2074 and RL-3841 showed resistance to all antibiotics used in this study. On the other hand, the growth of RL-4406 strain was inhibited by all the antibiotics, except for, erythromycin-ethlsuccinate (*Eryth*). *Rhizobium* strains; RL- 2070, RL4406, RL-207 (except for *Eryth* resistance) and RL-3841 (except for *Cm* resistance) were eliminated all antibiotic resistance genes at 37°C. On the other hand, When grown in saline medium all cured strains lost their ability to grow on saline stress, except for, the cured strains RL-4406 and RL-207 which lost their resistance to *Erth* , as well as, RL-3841 which didn't lost the resistance to *Cm*. However, RL-4044 strain harboring resistance genes to antibiotics appeared high stable at elevated temperature and salt stress. All *Rhizobium* mutants of RL-2074, RL-3841, RL-4406, RL-4404 and RL-2070 (except for SWT_{12}, SWT_{17}) showed significant increase in IAA produced in complete medium above the parental strains. On the other hand, all isolates resulted from RL-2070 appeared significant increase in IAA produced in complete and minimal media above the parental strains. All biofertilizer inoculants appeared significant increase in nodulation parameters above uninoculated plants. All *Rhizobium* inoculants induced significant increase in nodule dry weight and nodule number above uninoculated plants grown in normal and salinity soil. All biofertilizer inoculants induced significant increase in root dry weight, shoot dry weight at 45 and 90 days plant-old over uninoculated plants. Soil type was significantly affected on shoot dry weight at 45 and 90 days plant-old. Furthermore, biofertilization appeared the same effect on all growth parameters (shoot dry weight at 45 and 90 days plant-old and root dry weight). Although, the interaction between soil type and biofertilization revealed significant effect on root and shoot dry weight at 45 and 90 days plant-old. Significant increase was obtained in seeds dry weight / pod in salinity soil above that in normal soil. This indicated that salt tolerant mutants reduced the effect of salinity on yield components. Some of *Rhizobium* inoculants (RL-2074, SWT_1, RL-3841, SWT_6, RL-4404, SWT11, RL-2070 and SWT_{21}) induced significant increase in seeds number, number of pods /plant and seeds dry weight per plant above uninoculated ones among two soil types. All biofertilizers induced significant increase in Chl.a, Chl b and total Chl above uninoculated plants in both soil types above uninoculated plants. The interaction between the type of soil and biofertilization, as well as, biofertilizers was significantly affected on pigment concentrations (chlorophyll a,chlorophyll b and total chlorophyll). The results indicated that biofertilization reduced the severe effects of salinity .

Key words: Antibiotic resistance, IAA production, *Pisum sativum*, plasmid curing, *Rhizobium*, salt tolerant mutants.

INTRODUCTION

Pea (*Pisum sativm* L.) is one of the widely spread, early maturing legume crops grown during the winter seasons. The green pods and mature seeds of pea are rich in protein and vitamins. This crop like many other legumes is capable of fixing and utilizing atmospheric nitrogen through symbiotic relationship with *Rhizobium*. [1].

Nearly 40% of the world land surface can be categorized as having potential salinity problem [14]. Moreover, salinity of soil in cultivated area was expected to rise as a result from local salt accumulation due to irrigations and applications of chemical fertilizers [34]. Soil salinity adversely affects the nodulation and nitrogen fixation capacities of rhizobia,

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resulting in lower productivity of legumes [34]. Growth of most rhizobial strains is inhibited by 100 mM NaCl, while some strains can tolerate more than 300 mM concentrations of NaCl [35].

In general, legumes are more sensitive to salinity than their rhizobial counterparts and, consequently, the symbiosis is more sensitive to salt stress than free-living rhizobia. Salt stress may inhibit the initial steps of the symbiosis (nodule initiation, nodule infection, and development) but it also has a depressive effect on nitrogen fixation [55]. Genetic evidence suggests that some of the genes involved in *Rhizobium-legume* symbiosis may be located on plasmids [25]. Rhizobia may use distinct mechanisms for osmotic adaptation upon salt stress, such as the intracellular accumulation of low-molecular-weight organic solutes (osmolytes), including amino acids, sugars, and polyamines, or the accumulation of ions (i.e., K^+) [34]. Response and adaptation to environmental stresses is probably a complex phenomenon involving many physiological and biochemical processes that likely reflect changes in gene expression. The present study aimed to induce salt tolerance mutants in *Rhizobium* strains to be used for better nitrogen fixation in *Pisum sativum* grown in salinity soil to be enhance the efficiency of symbiotic nitrogen fixation under abiotic stress .

MATERIALS AND METHODS

I. Genetic Material :

A- Bacterial Strains and Growth Conditions: Bacterial strains used in this study are listed in Table 1.

Media: Bacterial strains were grown at 28ºC in yeast extract-mannitol medium (YEM) according to Vincent [54].

Minimal medium: This medium was used for IAA assays according to Balasa[8].

Plants: Pea (*Pisum sativum* L.) seeds variety Master B were kindly supplied from Vegetable Research Institute, Agri. Res. Center, Dokki, Giza, Egypt.

b- Antibiotics used: Resistance to antibiotics was tested on YMA agar supplemented with one of the antibiotics listed in Table 2 with the concentration of 100µg/ml for genetically marking bacterial strains used in this work.

II. Methodology

Induction of Salt Tolerant Mutants: Rhizobium strains were overnight growing at 28°C in YEM medium using rotary shaker at 120 revolutions per min. (rpm) which giving finally 10^8 CFU/ml. Saline tolerance were induced via sub-cultures strains on different sea water concentrations using 0.2% intervals to enhancement the switch on of saline tolerance genes. Five single colonies from that appeared in every concentration were picked up and sub-cultured on YEM slant agar medium. Resistant colonies obtained were retested and purified on the same medium containing the same concentrations of sea water. Salinity tolerant mutants selected were listed in Table 3.

Antibiotic Susceptibility Testing: It was measured by a plate diffusion method, according to Collins and Lyne [16] with cultures grown to logarithmic growth phase in YEM broth for each strain. The plates were incubated overnight at 28°C and the diameter of resulting zones of inhibition was measured according to Toda *et al*. [52].

Plasmid Curing Test: To determine the resistance to antibiotic is encoded by a plasmid or chromosomal genes, elevated temperature treatment (37°C) was applied. Cultures were inoculated into TY broth medium at elevated temperatures for 48 h and then plated on TY medium containing 5% sucrose. Plates incubated at higher temperatures were transferred to 28°C for another three days. Single colonies from each temperature treatment were picked up and rechecked for the same antibiotic resistance pattern to ensure from the growth characteristics obtained before [10].

IAA-detection with the Salkowski Colorimetric Technique: Production of IAA in the supernatant was assayed using the PC method, as described by Pilet and Chollet [42]. This method was shown to be the most sensitive and most specific [23]. Absorbance was measured at 530 nm. However, IAA concentrations were calculated from the following formula of regression as follows:

$$x = \frac{y - a}{b}$$

Where; y = Optical density at 530 nm, x = Concentration of IAA, b = Regression = 0.0385 and a = Absorbance at 530 nm when the concentration of IAA equal zero =0.215.

10-plant Infection:

Pots Experiments: Pots experiment were conducted during the winter seasons of 2007 and 2008. Plants were grown in plastic pots containing the mixture of sterilizes sand and clay (1:1 w/w) with three replicates. Soil was washed with distilled water several times to

diminishing chloride ions, as well as, autoclaved three times at 121°C for one hour among three days. Plants were inoculated with the parental strains and their mutants at the time of sowing using over night culture suspension growing at 28°C on rotary shaker (120 RPM) which contained finally 10^8 FU/ml. Nodules developed on the roots were counted after 45-days-plant old. The accuracy of this method depends on the ability of a single *Rhizobium* cell to form nodule on the host. Plant infection technique is commonly applied in this study to determine the efficiency of different salt tolerant mutants in symbiosis [54]. In 2008-2009 these experiment was repeated using the pots containing non-saline soil (1.3mM) and salinity soil (4.5 mM). This experiment was conducted in Sakha Agriculture Research Station, Agric. Res. Center, Egypt.

Definition and Traits Studied:

Nodulation and Vegetative Traits:

Nodulation Test: After fourty five days of inoculation, three plants from each replicate were removed and washed carefully with tap water. Nodules number were counted, dried and weighted according to Novak *et al*. [36].

Shoot and Root Dry Weight per Plant: Different plant parts (shoots and roots) at 45-days-plant-old were oven dried at 70°C until reached to a constant mass and then turned immediately to weight.

Plant Height: This trait was measured when the plants became to blooming at the harvest time by centimeters from the first leaf to the apex.

Leaf Area/plant: It was determined using leaf fresh weight method according to Beadle[10].

Chemical Traits:

Photosynthetic Pigments: Chlorophyll concentration in pea leaves was extracted in 80% methanol calculated according to Lichtenthaler and Wellburn [31].

Nitrogen Determination

Nessler Reagent Used for Nitrogen Determination: Nitrogen concentration in pea was extracted in sulphuric and perchloric acid [5]. Absorbance was measured at 425 nm. However, nitrogen and protein concentrations were calculated from the following formula of regression as follows:

$$x = \frac{y - a}{b}$$

Where:

y = Optical density at 530 nm, x = Concentration of nitrogen, b = Regression = 0.14, a = Absorbance at 425 nm when the concentration of N equal zero = 0.01, Crude protein in seed (%) = Seed N_2 percentage x 6.25

Experimental Design and Statistical Analysis: Field experiment was designed in split-plot design. The type of soil (saline or salinity soil) was the main plots arranged in a completely random. However, biofertilization was assigned to subplots within each main plot. Data were subjected to the analysis of variance according to Snedecor and Cochran [27]. Least significant difference (L.S.D.) was used to compare between means if the F-test was significant.

Results and Discussion

Testing *Rhizobium* Strains for Resistance And/or Sensitivity to Antibiotics: Microorganisms are able to grow in the presence of antibiotics if they possess antibiotic resistance. Antibiotic resistance can be divided into natural and acquired resistance. The natural or intrinsic form of antibiotic resistance can be regarded as a genetically determined property of a cell, which matches a gap in the spectrum of action of antibiotic agent. Intrinsic antibiotic (IAR) has been portrayed as a quick and simple method for strain identification within the rhizobiaceae in comparison with standard serological methods [15]. The results recorded in Table 4 indicated that the antibiotic resistance patterns used as genetic markers for six *Rhizobium* strains appeared high level of resistance to erythromycin-ethlsuccinate (*Eryth*) than the other antibiotics used herein. This agreed with Küçük and Kıvanç [30], who found that *Rhizobium* isolates appeared high level of resistance against erythromycin, kanamycin, penicilin and chloramphenicol. The growth of RL-4406 strain was inhibited by all antibiotics, except for, erythromycin-ethlsuccinate (*Eryth*). Whereas, RL-2074 and RL-3841 showed resistance to all antibiotics used herein. Bacterial species of soil, water and sewage may have resistance to natural antibiotics or may acquire these characters from other bacteria through genetic exchange [32].

Plasmid Curing and its Relation to Salinity Tolerance: As shown from the results presented in Table 5 that RL- 2070, RL4406, RL-207 (except for *Eryth* resistance) and RL-3841 (except for *Cm* resistance) were eliminated all antibiotic resistance genes at 37°C. On the other hand, when these strains were grown in saline medium all cured ones were lost

their ability to grow on saline stress, except for the

cured strains RL-4406 and RL-207 which didn't lost their resistance to *Erth*, as well as, RL-3841 which didn't lost the resistance to *Cm*. However, RL-4044 strain harboring resistance genes to antibiotics appeared high stable at elevated temperature and salt stress. These results are in agreement with Takeyama *et al*. [50], who reported that the copy number of the plasmid pSY10 (2.7 kb) in *Synechococcus* sp. NKBG 042902 increased five times when NaCl concentration in medium was increased from 0 to 3%, this plasmid, when present in *Salmonella* conferred increased osmotic tolerance imposed by NaCl [13]. On the other hand, Abdel-Salam *et al*. [2] found that all cured isolates lost their antibiotic resistance to all five antibiotics tested, indicated that genes of resistance lost were located on plasmids. Swenson *et al.,*[48] reported that resistant trait to various antibiotics were located in a high copy number of transferable plasmid DNAs and the plasmids of these strains can be used as cloning vector for gram-positive bacteria in recombinant DNA technology. According to these results, in some strains the resistance to some antibiotics and salinity may be under the control of plasmid DNAs; however, the resistance to some other antibiotics may be encoded by chromosomal genes, and different plasmids caused resistance to different antibiotics [6].

Indole Acetic Acid Produced by *Rhizobium* Strains and Their Mutants:- As shown from the results presented in Table 6 all *Rhizobium* mutants (except for SWT_{12} and SWT_{17}) showed significant increase in IAA produced in complete medium above the parental strains, whereas, only two isolates (SWT_{26} and SWT_{29}) of RL-207 appeared the same trend. On the other hand, all isolates resulted from the strain RL-2070 appeared significant increase in IAA produced in complete and minimal media above the parental strains. However, some isolates SWT_6, SWT_7, SWT_9, SWT_{11}, SWT_{12}, SWT_{18}, SWT_{21}, SWT_{24}, SWT_{27} and SWT_{29} appeared significant increase in IAA produced in minimal medium in relation to the parental strains. This agreed with Ghosh and Basu [21], who found that *Rhizobium* sp. isolated from root nodules of *Dalbergia lanceolaria* produced high amount of IAA at 2.5 mg/ml of L-tryptophan concentration. Rhizobia are able to convert tryptophan to IAA, which is the primary naturally occurring auxin in plants [27].Whereas, Theunis *et al*.[50] found that rhizobia are able to synthesise IAA using indole-3-acetamide (IAM) as an intermediate. Bacterial indoleacetic acid (IAA) production has been proposed to play a role in the *Rhizobium*-legume symbiosis.

Evaluate Salinity Tolerant Mutants in Pots Experiment: Environmental factors such as low or high pH, high temperature, low soil moisture, nutrient deficiency, mineral toxicity, and soil salinity can reduce survival and growth of rhizobia in the soil and inhibit rhizobia-legume symbiosis [54]. Adverse environmental conditions like soil salinity, water stress, and heavy metals pollutions, severely affect N assimilation in plants [29]. About 7% of the world's total land area is affected by salt, as is a similar percentage of its arable land [20]. Salinity of soil in cultivated area is expected to rise as a result from local salt accumulation due to irrigations and applications of chemical fertilizers [34].

Data presented in Table 7 showed significant differences in nodulation parameters (number of nodules per plant and nodules dry weight at 45 days plant-old) under the effect of biofertilization. The results revealed significant increase in the number nodules and nodules dry weight in salinity soil above that in normal soil. This indicated that salinity tolerant mutants could tolerate soil salinity and increased nodulation parameters. This result agreed with Miller and Wood, [34], who reported that soil salinity adversely affects on nodulation and nitrogen fixation capacities of rhizobia, resulting in lower productivity of legumes. However, Payakapong *et al*., [38] reported that one particular wild leguminous species *Phaseolus lathyroides* , growth successfully forming effective root nodule in saline soils. In addition, Bhardwaj [11] reported that no nodulation was observed in pea in salinity soil. Growth of most rhizobial strains was inhibited by 100 mM NaCl, while some strains could tolerate more than 300 mM concentrations of NaCl [35].

Data summarized in Table 8 illustrated that all biofertilizer inoculants appeared significant increase in nodulation parameters above uninoculated plants. This agreed with Dadarwal and Sen, [17], who found that both nodulation and yield increased significantly due to inoculation. Inoculation of stress tolerant rhizobia may enhance the nodulation and nitrogen fixation ability of plants under stress condition [4]. The ability of legume hosts to grow and survive in saline condition is improved when they are inoculated with salt tolerant strains of Rhizobia [42]. However, Elsheikh and Osman [18], found that inoculation with strains SVFO1 and strain tal1397 increased shoot dry weight after eight weeks by 21.4% and 29%, respectively, compared to uninoculated plants under salt-stress.

Data presented in Table 9 showed the interaction between soil types and biofertilizer strains on nodulation parameters. The data presented herein showed that all *Rhizobium* inoculants induced significant increase in nodule dry weight and nodule number above uninoculated plants grown in normal and salinity soils. Whereas, four inoculants (Rl-2074, Rl-3841, Rl-4404 and Rl-2070) induced significant

increase in nodule number and nodule dry weight above the same inoculants grown under salinity soil, but the other inoculants (SWT_1, SWT_6, SWT_{11} and SWT_{21}) induced significant increase in nodule dry weight and nodule number above the same inoculants grown in non-saline soil. These results agreed with Ali *et al*. [4] who found that inoculation with stress tolerant rhizobia may enhance nodulation and nitrogen fixation ability of plants under stress condition . This also agreed with Bolaños *et al*.[12], who found that nodules developed in *Pisum sativum* inoculated with *Rhizobium leguminosarum* bv. *vicae* 3841 and growing under saline conditions (75 mmol/l.nacl) were non functional and had abnormal structure .

Rhizobium inoculants (SWT_1, SWT_6, SWT_{11} and SWT_{21}) induced significant increase in nodule dry weight and nodule number above uninoculated plants among two soil types. These result agreed with Micanovic *et al*. [33], who found that *Rhizobium* inoculated plants in addition to 80 kg N/ha increased the average yield of pea plants over uninoculated control.

As shown from the results summarized in Table 10 that the type of soil was significantly affected on nodule dry weight and nodule number. Insignificant effect of biofertilization and its interaction with soil types on different nodulation parameters was agreed with Singleton and Bohlool [44], who found that salinity does not affect colonization of roots by rhizobia but does retard initiation or growth of new nodules , reduce the efficiency of fully formed nodules which had developed earlier under non-saline condition.

Data presented in Table 11 showed the effect of the interaction between soil types and biofertilizer strains on growth traits. It has been shown that *Rhizobium* inoculants (RL-2074, SWT_{22}, Rl-3841, SWT_{12},Rl-4404, SWT8 ,Rl-2070 and SWT18) induced significant increase in shoot dry weight at 45 and 90 days plant-old, as well as, root dry weight above uninoculated plants grown in non-saline and salinity soils. Whereas, four inoculants (Rl-2074, Rl-3841,Rl-4404 and Rl-2070) induced significant increase in shoot dry weight at 45 and 90 days plant-old, as well as, root dry weight above the same inoculants grown under salinity soil, but the other inoculants (SWT_{22}, SWT_{12}, SWT8 and SWT_{18}) induced significant increase in shoot dry weight at 45 and 90 days plant- old and root dry weight above the same inoculants grown in non-saline soil . These results agreed with Ali *et al*. [3], who found that inoculated plants gave significantly higher nodule number (14.2 plant^{-1}), nodule weight (167 mg plant^{-1}), root weight (0.157 g plant^{-1}), shoot weight (2.08 g plant^{-1}) and higher seed yield (1.03 t ha^{-1}). Some *Rhizobium* inoculants (SWT_{22}, SWT_{12}, SWT_8 and SWT_{18}) induced significant increase in shoot dry weight at 45 and 90 days plant- old and root dry weight above uninoculated plants in two soil types. These result agreed with Kapulnik *et al*. [28], who found that total shoot and root weights were significantly increased by inoculation with plant growth- promoting rhizobacteria (PGPR) that are actively colonize plant roots, increase plant growth and yield. PGPR can also enhance the plant competitiveness and responses to external stress factors, as well as, inhibiting soil-borne plant pathogens through antifungal activity [43].

As shown from the results summarized in Table 12 that the type of soil was significantly affected on shoot dry weight at 45and 90 days plant-old. Furthermore, biofertilization appeared the same effect on all growth parameters (shoot dry weight at 45 and 90 days plant-old and root dry weight). Although , the interaction between soil types and biofertilization revealed significant effect on root and shoot dry weight at 45 and 90 days plant-old. Significant effect of soil types on different growth parameters was agreed with Hussein *et al*. [24], who found that salinity stress resulted in reduction of plant growth and yield of pea. Elsheikh and Osman [18] found that inoculation with *Rhizobium* increased shoot dry weight after eight weeks by 21.4% and 29%, respectively, if compared to uninoculated plants under salt-stress.

Data presented in Table 13 showed the effect of the interaction between soil types and biofertilizer inoculants on yield components. The data revealed that some inoculants (RL-2074, SWT_1, RL-3841, SWT_6, RL-4404, SWT_{11}, RL-2070 and SWT_{21}) induced significant increase in seeds number, number of pods /plant and seeds dry weight above uninoculated plants grown in normal and salinity soil. Whereas, four inoculants (RL-2074, RL-3841,RL-4404 and RL-2070) induced significant increase in seeds number, number of pods /plant and seeds dry weight above the same inoculants grown in salinity soil, but the other inoculants (SWT_1, SWT_6, SWT_{11} and SWT_{21}) induced significant increase in seeds number, number of pods /plant and seeds dry weight above the same inoculants grown in normal soil. These results agreed with Shamseldin and Werner, [42], who found that the ability of legume hosts to grow and survive in saline conditions was improved when they are inoculated with salt tolerant strains of rhizobia. The results also agreed with Ali *et al*. [3], who found that plants inoculated with *Rhizobium* strains gave significantly higher nodule number (14.2 plant^{-1}), nodule weight (167 mg plant^{-1}), root weight (0.157 g plant^{-1}), shoot weight (2.08 g plant^{-1}) and higher seed yield (1.03 t ha^{-1}) at the year of 2005-06. In addition, the seed yield of variety BARI Motorshuti^{-1} were 35% and 31% higher for inoculated plants if compared to uninoculated ones for the years 2005-06 and 2006-07, respectively.

As shown from the results summarized in Table 14 that the type of soil was significantly affected on seeds dry weight. Furthermore, biofertilization appeared significantly effect on all yield components (seeds dry weight, seeds number and number of pods/plant). However, the interaction between soil types and biofertilization revealed the same effect on pods number, seeds dry weight and number of seeds. This agreed with Badr-Eldin *et al*. [7], who found that inoculation with PDB in combination with *R. japonicum* at the presence of rock phosphate or superphosphate did not show significant increases in dry weight, N and P uptakes or seed yield as compared with the treatments inoculated with rhizobia alone. Ahmed *et al*. [1] showed that inoculation of both seed and soil with BARI RPs-2002 produced the maximum pea green seed yield (1.90 t/ha) which was 58% higher than that of uninoculated control. The same authors also found that the highest green pod yield (3.29 t/ha) was found with inoculation of both seed and soil with BARI RPs-2002 which was 53% higher than that of uninoculated control. There was no significant yield variation between the treatments consisting of soil inoculated with BARI RPs-2001 and seed inoculated with BARI RPs-2002, but all the treatments produced significantly higher pod yield over uninoculated control. These findings have the resemblance with the result of Serivastava *et al*. [46], who reported that inoculation of pea seeds with *Rhizobium Leguminosarum* gave maximum pod yield of pea. However, Kanaujia *et al.* [26] obtained similar results in pea crop that the maximum green pod yield was 30.78 g/plant (111 % increase over uninoculated control).

The results obtained in Table 15 showed that all biofertilizers inoculants induced significant increase in Chl.a, Chl b and total Chl above uninoculated plants in both soil types above uninoculated plants. This agreed with Ahmed, *et al*. [1], who found that co-inoculation of both seed and soil with *Rhizobium* was considered to be an effective treatment for achieving the maximum output through cultivation of pea in shallow-red brow terrace soil in plants treated with 50 and 70Mm NaCl, chlorophyll contents were significantly decreased as compared with control, however decreasing chlorophyll contents in plants grown in 10 and 30 Mm NaCl were not significant.

As shown from the results summarized in Table 16 that the interaction between soil type and biofertilization, as well as, biofertilizers were significantly affected on pigment concentrations (chlorophyll a,chlorophyll b and total chlorophyll). However the soil type revealed significant effect on pigment concentrations, except for chlorophyll a. The result obtained here are in agreement with Parveen *et al*. [37], who found that salinity had significant effect on net assimilation rate. However, significant effect of biofertilizers on chlorophyll concentrations agreed with Tranaviciene *et al*. [52], who found that total photosynthetic pigment contents increased with plant age and were higher at higher fertilization rates, as well as, chlorophyll a, b and carotenoid biosynthesis showed similar responses to N fertilization. Furthermore, chlorophylls are very sensitive to changes in nitrogen contents. However, nitrogen showed the most pronounced effect on photosynthetic pigment synthesis at the flowering stage. The photosynthetic capacity of leaves was related to nitrogen content primarily because of proteins of calvin cycle, and thylacoids represent the majority of leaf nitrogen [22].

AS shown from the results summarized in Table 17 all *Rhizobium* inoculants and their mutants induced significant increase in nitrogen content at 45and 90 days plant-old above the uninoculated plants in both saline and non-saline soils. However, all *Rhizobium* strains appeared significant increase in seeds protein content above uninoculated plants in both two soil types. This agreed with Ahmed , *et al.* [1], who found that the performance of *Rhizobium* strains used as seed or soil inoculation were superior in relation to uninoculated control for all the parameters of pea crop, among the treatments, seed and soil inoculated with *Rhizobium* performed best in recording number of pods/plant, number of seeds/pod, 1000-seed weight, pod and seed yields and protein content in pea seeds . Rhizobia and Bradyrhizobia are of great economic importance and they have the ability to invade and form nodules on the roots of leguminous plants, and improve seed chemical composition and quality [19].

Data presented in Table 18 demonstrated that soil type, as well as, biofertilizer inoculants was significantly affected on nitrogen content at 45 and 90 days plant-old and total protein content in seeds . The same trend was also shown by the interaction between soil and biofertilizers. This result agreed with Rao *et al.* [41], who found that although nitrogen content (quantity)decreased with increasing salinity (significantly so in all genotypes), there was no consistent reduction in shoot nitrogen concentration. Stancheva *et al.* [47] demonstrated that dual inoculation of pea plants increased plant biomass, nodulation parameters, N fixation activity at varying levels compared to plants submitted to single inoculation with *Rhizobium leguminosarum*.

Table1: Bacterial strains used in this study.

Strains	Source or Reference	Designation
Rhizobium leguminosarum (NRRL4406)	National center for Agriculture Utilization Research, USA	RL-4406
Rhizobium leguminosarum (NRRL B-4404)	National center for Agriculture Utilization Research, USA	RL-4404
Rhizobium leguminosarum (3841)	Kindly provided by Prof J P W Young, Department of Biology, University of Yourk , UK.	RL-3841
Rhizobium leguminosarum (USDA2070)	Kindly provided by Dr. Peter van Berkum, Microbiologyist, National *Rhizobium* culture collection, USDA, Baltimore Avenue Beltsville	RL-2070
Rhizobium leguminosarum (USDA2074)	Kindly provided by Dr. Peter van Berkum, Microbiologyist, National *Rhizobium* culture collection, USDA, Baltimore Avenue Beltsville	RL-2074
Rhizobium leguminosarum (ARC 207)	Agric. Res. center, Dept. of Microbiology, Giza, Egypt.	RL -207

Table 2: Antibiotics and their abbreviations used as a genetic markers.

Antibiotics	abbreviations
Chloramphenicol	*Cm*
Ampicillin	*Ap*
Neomycinsulphate	*Nm*
Erythromycin-ethlsuccinate	*Eryth*
Duricef	*Dur*

Table 3: Salinity tolerant mutants obtained in this study.

Strains	Final tolerance to sea water concentration	Time needed to appeared salt tolerant isolates on selective medium (days)	Designation
Rhizobium leguminosarum (USDA2074)	10%	3	SWT_1
			SWT_2
			SWT_3
			SWT_4
			SWT_5
Rhizobium leguminosarum (3841)	0.4%	3	SWT 6
			SWT 7
			SWT 8
			SWT 9
			SWT 10
Rhizobium leguminosarum (NRRL B-4404)	0.8%	5	SWT 11
			SWT 12
			SWT 13
			SWT 14
			SWT 15
Rhizobium leguminosarum (NRRL4406)	1.6%	4	SWT 16
			SWT 17
			SWT 18
			SWT 19
			SWT 20

Table 3: Continue

Rhizobium leguminosarum (USDA2070)	10%	3	SWT 21
			SWT22
			SWT 23
			SWT24
			SWT25
Rhizobium leguminosarum (ARC 207)	6%	3	SWT26
			SWT27
			SWT28
			SWT29
			SWT30

Table 4: Genetic markers in different *Rhizobium* strains due to antibiotics

Strains	Antibiotics				
	Cm	*Ap*	*Eryth*	*Rif*	*Nm*
RL-4404	+	+	+	-	+
RL-3841	+	+	+	+	+
RL-4406	-	-	+	-	-
RL-2070	-	-	+	-	+
RL-2074	+	+	+	+	+
R	L -207	+	+	-	+

+, – = Resistance and sensitive to antibiotics, respectively.

Table 5: Effect of high temperature on plasmid curing and its relation to salinity stress.

Bacterial strains	Resistance to antibiotic at 28ºC	Inhibition zones appeared at 37 ºC	Growth of cured strains in YEM media supplemented with 0.2%sea water
RL- 4044	*Cm*	+	+
	Nm	+	+
	Ap	+	+
	Eryth	+	+
RL-207	*Cm*	-	-
	Nm	-	-
	Ap	-	-
	Eryth	+	+
RL-3841	*Cm*	+	+
	Nm	-	-
	Ap	-	-
	Eryth	-	-
	Rif	-	-
RL-4406	*Eryth*	-	+
	Rif	-	+

Table 5: Continue

RL-2070	*Nm*	-	-
	Eryth	-	-
RL-2074	*Cm*	-	-
	Nm	-	-
	Ap	+	-
	Eryth	-	-
	Dur	+	-

-+,- = Growth appeared and disappeared, respectively.

Table 6: Efficiency of *Rhizobium* strains and their mutants in IAA production.

Inoculants	IAA production in complete medium supplemented with tryptophan (mg/ml)	Yield %	IAA production in minimal medium supplemented with tryptophan (mg/ml)	Yield %
Rl-2074	3.37	100	0.06	100
SWT_1	10.35	307.12	1.16	640.48
SWT_2	6.70	198.72	1.05	571.91
SWT_3	6.26	185.66	0.70	384.41
SWT_4	4.87	144.51	0.41	227.44
SWT_5	4.52	133.93	0.64	351.57
Rl-3841	3.78	100	0.24	100
SWT_6	10.13	267.90	2.01	845.80
SWT_7	6.77	179.19	2.01	845.80
SWT_8	6.12	161.85	0.33	140.20
SWT_9	9.46	250.26	1.05	442.02
SWT_{10}	5.73	151.50	0.33	140.20
Rl-4404	1.33	100	0.02	100
SWT_{11}	14.85	1116.79	1.47	6668.18
SWT_{12}	2.16	162.41	0.86	2976.97
SWT_{13}	3.56	267.92	0.19	846.97
SWT_{14}	10.74	807.52	0.02	98.48
SWT_{15}	5.44	409.27	0.25	1122.73
Rl-4406	2.51	100	0.01	100
SWT_{16}	5.44	216.87	0.09	700
SWT_{17}	2.51	99.87	0.07	566.67
SWT_{18}	7.16	285.13	0.45	3430.77
SWT_{19}	6.03	240.33	0.08	633.33
SWT_{20}	3.68	146.75	0.24	1833.33

Table 6: Continue

Rl-2070	1.29	100	0.07	100
SWT_{21}	7.71	597.67	0.97	1315.77
SWT_{22}	3.94	305.17	0.14	193.24
SWT_{23}	9.50	736.59	0.25	333.78
SWT_{24}	7.32	567.49	0.77	1046.85
SWT_{25}	5.28	409.15	0.25	333.78
Rl-207	3.93	100	0.05	100
SWT_{26}	5.03	127.89	0.02	45.14
SWT_{27}	2.03	51.65	0.61	1271.53
SWT_{28}	4.61	117.30	0.01	81.25
SWT_{29}	5.09	129.43	1.05	2190.97
SWT_{30}	3.64	92.71	0.25	514.58
F-tested	**	NS	**	NS
0.05	1.04	43.74	0.26	951.19
0.01	1.38	58.01	0.34	1261.41

NS,** = Insignificant differences , significant at 0.01 probability level, respectively .

Table 7: Means of nodulation parameters affected by salinity.

Nodulation parameters	N	S	F-test	L.S.D	
				0.05	0.01
No. of nodules	43. 70	51. 37	0	4.91	11.31
Nodule DW (mg/plant)	0.13	0.14	0	0.01	0.02
Average weight of nodule	0.003	0.002	NS	0.0004	0.001

NS and ** = Insignificant , significant at 0.05 probability level, respectively.
N,S = Non-saline and salinity soil, respectively. DW = Dry weight

Table 9: Means of nodulation parameters affected by inoculation and both soil types.

Biofertilizers	No of nodules/plant	Nodule DW(g/plant)	Average weight of nodule
Uninoculated	1.83	0.004	0.002
Rl-2074	22.83	0.08	0.003
SWT_1	91.67	0.20	0.002
Rl-3841	39.00	0.12	0.003
SWT_6	78.17	0.23	0.002
Rl-4404	29.67	0.11	0.003
SWT_{11}	74.50	0.20	0.003
Rl-2070	23.00	0.09	0.003
SWT_{21}	67.17	0.16	0.003
F-Test	**	**	**
LSD 0.05	4.40	0.017	0.001
0.01	5.92	0.023	0.002

** = Significant at 0.01 probability level. DW = dry weight.

Table 9: Means of nodulation parameters affected by two soil types under the effect of biofertilizer inoculants

Biofertilizers	No of nodules /plant		Nodule D W.(g)		Average weight of nodule	
	N	S	N	S	N	S
Uninoculated	2.00	1.67	0.030	0.020	0.00	0.00
Rl-2074	27.00	18.67	0.10	0.06	0.003	0.002
SWT_1	77. 00	106.33	0.20	0.21	0.003	0.002
Rl-3841	44.67	33.33	0.12	0.10	0.003	0.003
SWT_6	51.00	105.33	0.21	0.25	0.002	0.002
Rl-4404	34.00	25.33	0.11	0.11	0.003	0.003
SWT_{11}	68.00	81.00	0.19	0.20	0.003	0.002
Rl-2070	25.33	20.67	0.10	0.09	0.003	0.002
SWT_{21}	64. 33	70.00	0.11	0.21	0.002	0.003
F-Test	**	**	**	**	**	**
LSD 0.05	6.22	6.22	0.02	0.02	0.001	0.001
0.01	8.37	8.37	0.03	0.03	0.002	0.002

** = Significant at 0.01 probability level . DW = Dry weight. N, S= Nonsaline and salinity soils, respectively.

Table 10: Mean squares obtained from split plot analysis for different nodulation parameters affected by biofertilization in two soil types

S.V	D.F	No of nodules	Nodule DW †	Average weight of nodule
Main plot	5			
Rep.	2	23.41	0.0001	0.0000001
Soil types	1	794*	0.01*	0.0000002
Error A	2	17.56	0.0001	0.0000001
Sub plot				
Biofertilizers	8	5768	0.03	0.000004
Soil x Bio.	8	709	0.002	0.000001
Error B	32	14	0.0002	0.0000002

NS and ** = Insignificant , significant at 0.01 probability level . † = Dry weight.

Table 11: Means of growth parameters affected by two soil types under the effect of biofertilizeation.

Biofertilizers	Root DW† (g/plant)		Shoot DW† (at 45days plant -old) (g/plant)		Shoot DW† (at 90 days plant-old) (g/plant)	
	N	S	N	S	N	S
Uninoculated	0.09	0.02	0.54	0.46	0.44	0.36
Rl-2074	0.19	0.13	1.70	0.85	0.67	0.56
SWT_1	0.26	0.32	1.81	1.84	0.88	0.91
Rl-3841	0.13	0.13	2.60	1.56	0.76	0.67
SWT_6	0.23	0.29	1.71	2.31	0.89	0.92
Rl-4404	0.17	0.11	1.96	1.21	0.65	0.54
SWT_{11}	0.11	0.24	2.23	2.25	0.95	1.01

Table 11: Continue

Rl-2070	0.17	0.13	2.11	1.52	0.68	0.49

SWT_{21}		0.29	0.28	2.59	2.97	0.96	0.97
F-Test		**	**	**	**	**	**
LSD	0.05	0.03	0.03	0.08	0.08	0.011	0.011
	0.01	0.04	0.04	0.11	0.11	0.014	0.014

** = Significant at 0.01 probability level. † = Dry weight.
N, S= Non-saline and salinity soils, respectively.

Table 12: Mean squares obtained from split plot analysis for different growth parameters affected by biofertilization in two soil types

S.V	D.F	Root DW† (g/plant)	Shoot DW (at 45 days plant-old) (g/plant)	Shoot DW (at 90 days plant-old) (g/plant)
Main plot	5			
Rep.	2	0.0001	0.002	0.00002
Soil types	1	0.001	1**	0.04**
Error B	2	0.0002	0.001	0.00003
Sub plot				
Biofertilizers	8	0.04**	2**	0.25**
Soil x Bio.	8	0.01**	0.4**	0.01**
Error A	32	0.0003	0.002	0.00002

NS and ** = Insignificant and significant at 0.01 probability level, respectively. DW = Dry weight.

Table 13: Means of yield components affected by two soil types under the effect of biofertilization.

Biofertilizers	No of pods/plant		Seeds D.W		No of seeds/pod	
	N	S	N	S	N	S
Uninoculated	2.67	2.00	0.002	0.001	2.33	2.00
Rl-2074	7.00	4.33	3.13	3.52	5.00	4.67
SWT_1	5.00	6.67	4.43	5.59	5.67	5.67
Rl-3841	7.33	3.67	3.57	3.18	4.67	3.33
SWT_6	5.33	8.33	5.59	6.14	4.67	6.00
Rl-4404	6.00	3.33	3.51	3.80	3.33	3.33
SWT_{11}	5.33	7.33	4.27	5.39	4.67	5.00
Rl-2070	7.67	3.67	3.59	3.29	4.00	3.33
SWT_{21}	6.00	7.67	3.69	6.06	5.00	5.33
F-Test	**	**	**	**	**	**
LSD 0.05	0.86	0.86	0.96	0.96	0.86	0.86
0.01	1.16	1.16	1.29	1.29	1.16	1.16

** = Significant at 0.01 probability level.
N, S= Non-saline and salinity soils, respectively.

Table 14: Mean squares obtained from split plot analysis for yield components affected by biofertilization in two soil types.

S.V	D.F	No of pods/plant	Seeds dry weight	No of seeds/pod

Main plot	5			
Rep.	2	0.52	0.002	0.07
Soil types	1	5	1*	0.03
Error A	2	0.52	0.04	0.30
Sub plot				
Biofertilizers	8	11**	13**	7**
Soil x Bio.	8	11**	5**	1**
Error B	32	0.3	0.02	0.3

NS and ** = Insignificant , significant at 0.01 prpbability level, respectively.

Table 15: Means of biochemical parameters affected by soil types under the effect of biofertilization.

Biofertilizers	Chl.a(mg/g)		Chl.b(mg/g)		Total chl. (mg/g)	
	N	S	N	S	N	S
Uninoculated	0.18	0.13	0.94	0.59	1.12	0.72
RL-2074	0.47	0.12	4.44	4.02	4.90	4.14
SWT_1	0.97	1.05	4.30	2.95	5.27	3.10
RL-3841	0.67	0.53	2.39	2.13	3.06	2.66
SWT_6	1.88	1.83	2.74	3.21	4.56	5.04
RL-4404	0.70	0.60	2.43	1.49	3.13	2.09
SWT_{11}	1.12	1.51	2.99	1.38	4.11	2.89
RL-2070	0.90	0.90	1.61	1.40	2.51	2.30
SWT_{21}	1.08	1.24	2.24	5.04	3.33	6.28
F-Test	**	**	**	**	* *	**
LSD 0.05	0.16	0.16	0.35	0.35	0.32	0.32
0.01	0.21	0.21	0.47	0.47	0.43	0.43

** =Significant at 0.01 probability level .
N, S= Non-saline and salinity soils, respectively.

Table 16: .Mean squares obtained from split plot analysis for biochemical parameters affected by biofertilization in two soil types.

S.V	D.F	Chl. a	Chl. b	Total chl.
Main plot	5			
Rep.	2	0.001	0.09	0.07
Soil types	1	0.00003	1**	1**
Error A	2	0.02	0.004	0.001
Sub plot				
Biofertilizers	8	1.63**	8**	11**
Soil x Bio.	8	0.06**	3**	3**
Error B	32	0.01	0.04	0.2

NS and ** = Insignificant , significant at 0.01 probability level , respectively.

Table 16: Mean squares obtained from split plot analysis for biochemical parameters affected by biofertilization in two soil types.

S.V	D.F	Chl. a	Chl. b	Total chl.

Main plot	5			
Rep.	2	0.001	0.09	0.07
Soil types	1	0.00003	1**	1**
Error A	2	0.02	0.004	0.001
Sub plot Biofertilizers	8	1.63**	8**	11**
Soil x Bio.	8	0.06**	3**	3**
Error B	32	0.01	0.04	0.2

NS and ** = Insignificant , significant at 0.01 probability level , respectively.

Table 17: Means of shoot nitrogen and grains protein contents affected by the interaction between soil types and biofertilizer inoculants.

Biofertilizers	N% in shoot at 45 days plant-old		N% in shoot at 90 days plant-old		Seeds protein%	
	N	S	N	S	N	S
Uninoculated	0.57	0.35	0.50	0.32	5.48	3.93
RL-2074	2.90	1.18	0.88	0.77	17.84	11.21
SWT_1	1.25	2.32	0.86	1.21	16.31	20.35
RL-3841	3.78	1.29	1.15	0.86	20.08	10.46
SWT6	2.81	2.96	1.03	1.06	15.08	16.07
RL-4404	1.66	1.02	0.99	0.67	18.16	11.37
SWT_{11}	3.78	4.44	0.74	0.96	16.10	19.05
RL-2070	4.44	1.20	0.90	0.55	15.65	7.62
SWT_{21}	0.90	2.56	0.90	1.10	15.65	18.95
F-Test	**	**	**	**	**	**
LSD 0.05	0.75	0.75	0.15	0.15	2.75	2.75
0.01	1.02	1.02	0.20	0.20	3.71	3.71

** = Significant at 0.01 probability level, respectively. N, S= Non-saline and salinity soils, respectively.

Table 18: Mean squares from split-plot analysis of variance for nitrogen and protein contents of plants grown in the two soil types under the effect of biofertilization.

S.V	D.F	N% in shoot at 45days plant-old	N% in shoot at 90 days plant-old	Seeds protein%
Main plot	5			
Rep.	2	0.91	0.004	2.85
Soil types	1	4*	0.03*	76**
Error A	2	0.21	0.001	0.71
Sub plot Biofertilizers	8	7**	0.2**	103**
Soil x Bio.	8	4**	0.1**	45**
Error B	32	0.2	0.001	3

NS and ** = insignificant and significant at 0.05 and 0.01 probability levels, respectively

In conclusion, this study investigated that salt tolerant genes are plasmid-borne in most *Rhizobium*

strains. *Rhizobium* salt tolerant mutants were efficient in secrete IAA and significantly increase the growth, as well as, yield components above uninoculated plants and improved biochemical traits such as seed chemical composition and quality, as well as, chlorophyll formation under abiotic stress.

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