

Isolation and Characterization Salt Tolerant Strains Of *Rhizobium leguminosarum* bv. *viciae*

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Abstract

Seven strains of *Rhizobium leguminosarum* bv. *viciae* were isolated and purified from the root nodules of faba bean (*Viciae faba*) plants collected from different agroclimatic locations in Baghdad and Diyala provinces of Iraq. Screening of these strains gives four salinity tolerant strains which tolerate 1-5% sodium chloride supplement to mannitol salt yeast extract (MSY) medium. Colony forming units for the four tolerant strains under stress conditions were significantly reduced in comparison with those under normal conditions. Different tests were used to study the surface properties of stress tolerant strains under normal and stress conditions. No differences were found in the production of lipopolysaccharides (LPS), β (1 \rightarrow 3) glucan and cellulose fibriles under stress and normal conditions. Symbiotic properties of stress tolerant strains were studied by inoculating the respective host plants with these strains. Strain NIMA1 was found to be most efficient in symbiotic nitrogen fixation. No nodulation was observed under stress condition.

Introduction

Rhizobial bacteria fix atmospheric nitrogen in symbiotic association with leguminous plants. Salinity is considered to be the significant environmental factor which determines the survival of these bacteria in soil (1). Genetic variation among rhizobial strains has been reported for tolerating various levels of salinity stress. The mechanisms of tolerance have not been worked out in these bacteria.

Rhizobium leguminosarum bv. *viciae*, which makes symbiotic association with *Viciae faba* plants, is one of those rhizobial strains which have been extensively studied at molecular level for symbiotic characteristics.

The symbiotic relationship between rhizobial bacteria with the host plant is not clear especially under salinity stress conditions. Although the mechanisms of tolerance of different stresses are not very clear, there is no doubt that the soil population of rhizobia is usually a mixture of strains which may differ in their competitive ability (2), tolerance to different stresses (3, 4, 5, 6 and 7), and efficiency in nitrogen fixation (8). There is general agreement that the rhizobia are more tolerant to salt stress than the host plant, but they show considerable strain variability in growth and survival under saline (9). Nitrogen fixing ability of leguminous plants that grow under stress environmental conditions can be improved by inoculating these plants with stress tolerant rhizobia (10). Salinity-tolerant rhizobia showed better survival, nodulation and nitrogen fixation than salinity-sensitive rhizobia under saline condition (11). Selection of stress tolerant and efficient strains is very useful as the selected strains can be introduced into soils of respective stress where competition from naturally occurring rhizobia is lacking. The genetic and biochemical studies on stress tolerance in rhizobia are likely to help in the selection of suitable *Rhizobium*-legume combinations, which are efficient in nitrogen fixation and can tolerate environmental stresses.

Materials and Methods

Bacterial strains : Seven strains of *Rhizobium leguminosarum* bv. *Viciae* were isolated and purified from root nodules of faba bean (*Vicia faba*) plants collected from different agroclimatic locations in Baghdad and Diyala provinces. Vincent (12 and 13) procedure was followed for isolating rhizobial strains from the root nodules.

Growth media: Mannitol salt yeast extract (MSY) medium (per liter: 10g mannitol; 0.2g K_2HPO_4 ; 0.2g $K_2H_2PO_4$; 0.2g yeast extract; 0.1g $MgSO_4 \cdot 7H_2O$; 0.05g $CaCl_2 \cdot 2H_2O$) was used for growing bacterial strains (14).

Screening for salt stress tolerance : The growth of all isolated strains of *R. leguminosarum* bv. *viciae* were tested by streaking these strains on mannitol salt yeast extract (MSY) solid medium supplemented with different concentrations (0.5% to 6.0% with intervals of 0.5%) of sodium chloride (NaCl). Incubation was done at $28\pm 2^{\circ}\text{C}$ for 4-6 days (15 and 16). Six salinity tolerant strains of *R. leguminosarum* bv. *viciae* tolerate upto 1% NaCl, one strain tolerates upto 2.5% NaCl, another tolerates upto 5% NaCl, were selected for further studies.

Colony forming units of salt stress tolerant strains : Colony forming units (CFUs) were counted as described by Pant and Gangwar (17). Salt stress tolerant strains were cultured in MSY liquid medium for 24 hours under conditions with and without stress. Cultures were serially diluted and spread on MSY solid medium with and without stress. Incubation was done at $28\pm 2^{\circ}\text{C}$ for 72 hours.

Test for production of cell surface molecules of salt stress tolerant strains : The production of cell surface molecules of salt stress tolerant strains was tested under both normal and stress conditions.

- Test for lipopolysaccharides (LPS) production: This test was done by streaking the salt stress tolerant strains on MSY solid medium supplemented with 1 mg/ml sodium deoxycholate (DOC) and then incubating the plates at $28\pm 2^{\circ}\text{C}$ for 48 hours (18). The growth of the rhizobial strain indicated the ability of this strain to produce LPS.

-Test for cyclic β (1 \rightarrow 3) glucans production : Salt stress tolerant strains were streaked on MSY solid medium supplemented with 0.02% aniline blue dye. The plates were incubated at $28\pm 2^{\circ}\text{C}$ for 48 hours (18). The taking up of the dye by the rhizobial cells of a given strain indicated the ability of this strain to produce cyclic β (1 \rightarrow 3) glucans.

-Test for cellulose fibril production: Salt stress tolerant strains were streaked on YEM solid medium supplemented with 0.1mg/ml congo red dye. The plates were incubated at $28\pm 2^{\circ}\text{C}$ for 48 hours (18). The taking up of congo red dye by cells of a given strain indicates the production of cellulose fibrils by this strain.

Symbiotic response of salt stress tolerant strains : Rhizobial strains, which were isolated and purified from the host plants root nodules, were tested for their nodule inducing ability. Vincent method was

followed for growing the seedling (12). Four seedlings were inoculated in each pot containing 1 Kg sterilized soils. Rhizobial strains were grown in MSY broth culture for 24-48 hours. One ml of the culture was transferred to Enylmer flask containing 25 mls of MSY broth medium and incubated for 24 hours. The bacterial growth was centrifuged at 3000 rpm for 3-5 minutes. The pellet was washed twice in saline solution (0.85% NaCl) and suspended in 25 ml saline (0.85% NaCl). The rhizobial cell suspension was dispensed into pots having growing seedlings. Pots were kept in a greenhouse. Fourteen hours light and 10 hours dark cycle was maintained. The air temperature and the relative humidity of the chamber were maintained at $23\pm 2^{\circ}\text{C}$ and 70-80%, respectively.

Results

Isolation of stress tolerating rhizobial strains : The growth of seven strains of *R. leguminosarum* bv. *viciae* were tested on complete medium supplemented with different concentrations (0.5-6%) of NaCl. Five strains (NIM1, NIM2, NIM3, NIM4, NIM5) tolerate up to 1% NaCl, while one strain (NIM6) tolerates up to 2.5% NaCl. NIM8 found to be tolerate up to 5% NaCl. Four salt tolerant strains were selected for further studies.

Colony forming units (CFUs) of NaCl tolerant strains: Colony forming units of NaCl for four tolerant strains were determined at 24, 48 and 72 hours stages during growth under normal and stress conditions. CFUs at each of these stages under stress conditions were less than those under normal conditions, specially NIMA4, NIMA6 and NIMA8 strains Table (1). CFU of NIMA6 strain differ significantly under stress (2.5% NaCl) conditions in comparison with the same parameter under normal conditions after 72 hours from inoculation.

Cell surface molecules of NaCl tolerant strains under normal and stress conditions : No production of lipopolysaccharide (LPS) was found in *R. leguminosarum* bv. *viciae* strain NIMA8 under stress conditions. In the remaining strains (NIMA1, NIMA4 and NIMA6) the amount of LPS production did not appear to change under stress

conditions in comparison with the normal conditions. The ability to produce cyclic β (1 \rightarrow 3) glucans was present in all studied strains under normal and stress conditions. No change in the production of cellulose fibrils was found under stress conditions in any of the studied strains Table (2).

Symbiotic characteristics of faba bean plants inoculated with NaCl tolerant strains: The symbiotic characteristics of faba bean plants inoculated with different salinity tolerant strains were observed Table (3). Mean number of nodules per plant under normal conditions ranged from 14.4 in strain NIMA4 to 20.6 in strain NIMA8. No nodulation was observed under stress conditions. The mean plant dry weights per plant under normal conditions ranged from 3.0gs in strain NIMA4 to 4.6gs in strain NIMA1. On the basis of plant dry weight, the strain NIMA1 was found to be most efficient in symbiotic nitrogen fixation. The plants inoculated with this strain also showed maximum plant fresh weight, nitrogen and protein percent Fig. (1). Decreasing in the mean of all the parameters above was observed for all the respective strains under stress conditions in comparison with the normal conditions.

Discussion

Efficient nodulation and nitrogen fixation under stress soil conditions lead to the increase of the production of legume crops. The growth of rhizobia is a prerequisite for nodule formation. Hence, screening of rhizobial strains for survival and growth in stress laboratory media has resulted in some success in improving the nodulation of some legumes in stress soils (19, 20, 21 and 22). In this work several strains of *R. l. bv. viciae* have been obtained, which can tolerate salt stress conditions (1-5% NaCl). In order to assess the usefulness of these strains in agriculture, field trials are needed for at least those strains, which showed high symbiotic activity under laboratory conditions. The isolation of rhizobial strains, which can tolerate 5.0% NaCl has also been reported by Surange *et al.* (7).

The decrease in CFUs of stress tolerant strains under the respective stresses as compared to that under normal conditions indicated that the

growth of these strains was adversely affected under salt stress. It is obvious that the doubling time of the cells of a stress tolerant strain increased under stress conditions.

Specific cell surface molecules of rhizobia are required for successful infection of legume plants (23, 24 and 25). The altered surface compounds due to stress may result in the failure of legume infection by rhizobia. In the present work when stress tolerant strains were subjected to the respective stresses before inoculation of plants, no nodulation occurred. The failure to form nodules under stress at least in some of strains may be due to altered surface molecules.

R. l. bv. viciae NIMA1 was found to be most efficient nitrogen fixers under normal conditions in laboratory studies among NaCl tolerant strains. However, the performance of this strain under stress conditions in field remains to be studied. Moreover, suitable faba bean plant varieties, which can tolerate these stress conditions, are needed for such studies. When the salt stress tolerant strains were subjected to their respective stress (1-5% NaCl) before inoculation, nodulation did not occur on faba bean plants. This result refers to the deleterious effect of salt on the nodulation process.

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Table (1) Colony forming units (CFUs) of NaCl tolerant strains in MSY medium under normal and stress conditions

X̄ ± S.D of CFUs (x 10 ⁷ cells/ml) at						
Name of strain	24 hours [#]		48 hours [#]		72 hours [#]	
	Normal	Stress	Normal	Stress	Normal	Stress
NIMA 1	21 ± 7.8	33 ± 3.6	33 ± 6.1	81 ± 5.6 ^x	14 ± 4.7	56 ± 20.8 ^x
NIMA 4	126 ± 13.2	165 ± 1.7 ^x	202 ± 36.1	130.6 ± 26.4 ^x	156.6 ± 7.6	140 ± 23.1
NIMA 6	65 ± 16.5	12.3 ± 3.2 ^x	167.6 ± 2.5	75.3 ± 5.4 ^x	31 ± 13.5	20 ± 1.5 ^x
NIMA 8	18.6 ± 1.5	3.3 ± 0.5	31 ± 23.8	20 ± 12	15.3 ± 1.5	1.5 ± 0.6

X̄ ± S.D = Mean ± Standard deviation, # = Each figure is an average of three replicates, x = Significantly different at 5% level from the value in normal condition

Table (2) Production of cell surface molecules by NaCl tolerant strains under normal and stress conditions on solid MSY medium

Strains	LPS		β (1→3) glucans		Cellulose fibrils	
	Normal	Stress	Normal	Stress	Normal	Stress
NIMA1	++	++	++	++	++	++
NIMA4	++	++	++	++	++	++
NIMA6	++	++	++	++	++	++
NIMA8	++	-	++	++	±	++

LPS= Lipopolysaccharides, ++ = High production, ± = Less production, - = No production

Table (3) Symbiotic characteristics of faba bean plants inoculated with NaCl tolerant strains of *R. leguminosarum* bv. *viciae*

Strain	Condition	Mean Fresh weight (g/plant)	Mean Dry weight (g/plant)	Mean no. of nodules (nodule/plant)	Nitrogen percentage (%)	Protein percentage (%)
NIMA 1	Normal	28.7 ± 0.9	4.6 ± 0.8	20.4 ± 0.8	8.5 ± 0.9	53.1 ± 1.1
	Stress	21.3 ± 1.04	2.1 ± 0.9	0.0 ± 0.0 ^{xx}	2.4 ± 1.8 ^x	15.0 ± 0.7 ^x
NIMA 4	Normal	24.2 ± 2.7	3.0 ± 0.5	14.4 ± 0.9	5.4 ± 1.8	33.7 ± 0.9
	Stress	20.2 ± 0.9	2.0 ± 1.6	0.0 ± 0.0 ^{xx}	2.3 ± 1.8 ^x	14.3 ± 1.2 ^x
NIMA 6	Normal	25.2 ± 2.1	3.1 ± 0.4	18.4 ± 2.9	6.1 ± 1.0	28.2 ± 1.8
	Stress	20.3 ± 1.5	2.0 ± 0.6	0.0 ± 0.0 ^{xx}	2.4 ± 0.3 ^x	15.0 ± 0.7 ^x
NIMA 8	Normal	26.6 ± 1.2	3.6 ± 1.1	20.6 ± 2.1	7.0 ± 1.0	43.7 ± 3.0
	Stress	21.4 ± 1.1	2.1 ± 0.7	0.0 ± 0.0 ^{xx}	2.1 ± 1.0 ^x	13.1 ± 0.6 ^x
NIMA 8	Normal	18.5 ± 1.2	1.9 ± 0.1	—	2.0 ± 1.0	12.5 ± 0.5

Control = uninoculated plants, xx=Significant difference (at 1% level) with uninoculated plants, x=Significant difference (at 5% level) with uninoculated plants

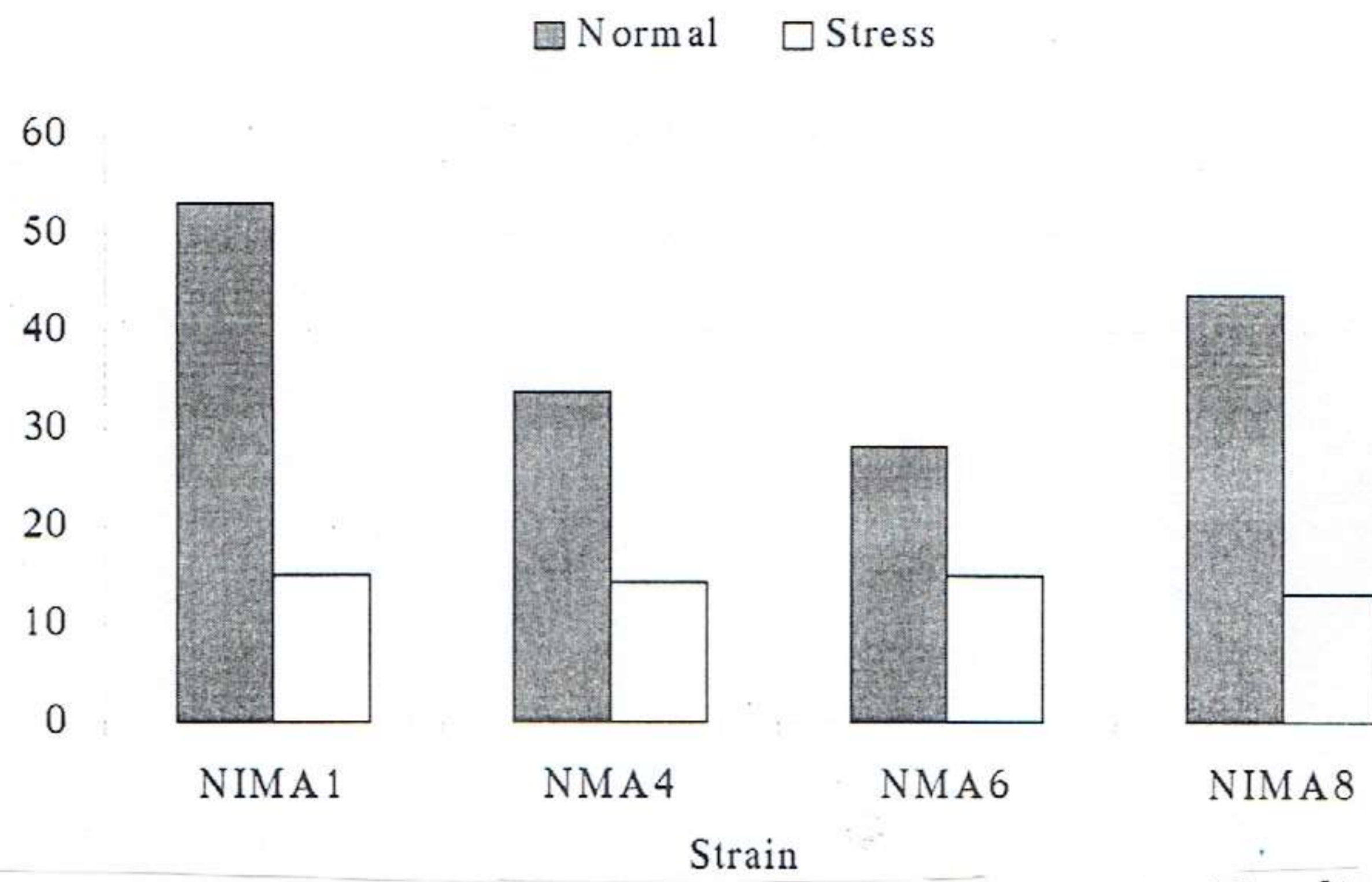


Fig (1) Protein percentage of faba bean plants infected with salt tolerant strains

عزل وتشخيص سلالات متحملة للملوحة من بكتريا *Rhizobium leguminosarum* bv. *Viciae*

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الخلاصة

عزلت ونقيت سبع سلالات من بكتريا *Rhizobium leguminosarum* bv. *Viciae* من العقد الجذرية لنباتات الباقلاء (*Viciae faba*) التي جمعت من مناطق زراعية مختلفة من محافظتي بغداد وديالى. تم عزل أربعة سلالات متحملة للملوحة والتي يمكن أن تتحمل تركيز 1-5% من ملح الطعام (NaCl) في الوسط الغذائي مستخلص الخميرة-المانيتول الملحي (*Mannitol salt- yeast extract medium*). وحدة تكوين المستعمرات (Colony forming units) للسلالات الأربعة المعزولة قد انخفضت تحت ظروف الشد الملحي بالمقارنة مع الظروف الطبيعية. لا توجد اختلافات في إنتاج السكريات المتعددة الدهنية (Lipopolysaccharides) ، بيتا 1 ← 3 كلوكان (β (1 → 3) glucan) ووليفات السيلوز (Cellulose fibriles) تحت ظروف الشد الملحي بالمقارنة مع الظروف الطبيعية. درست الخواص التكافلية للسلالات المتحملة للملوحة بعد زراعتها مع نباتات الباقلاء. السلالة (NIMA1) اعتبرت الأكثر فعالية في تثبيت النيتروجين. لم تلحظ العقد الجذرية تحت ظروف الشد الملحي.