Molecular, Biochemical and Physiological characterization of symbiotic bacteria isolated from saline soil in Saudi Arabia

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Abstract: Three rhizobial isolates were recovered from root nodules of Medicago sativa, Phaseolus vulgaris and Vicia faba and originating from various locations in Taif region. The isolates were characterized and identified as Rhizobium meliloti-n11, Rhizobium phaseoli-n12 and Rhizobium leguminosarum bv viciæ-n13 respectively. To study the physiological and metabolic responses of the Rhizobium isolates to salt stress, they were grown with different NaCl concentrations. Protein patterns were determined by electrophoresis, as well as determining the accumulation of amino acids. Glutamate and proline were found to increase rapidly in response to osmotic stress by NaCl. RAPD-PCR was used to fingerprint the isolates; a high degree of genetic diversity (95.45% of polymorphism) was observed between Rhizobium isolates. Similarity matrix and dendrogram classified the three isolates into two main clusters, Rhizobium leguminosarum-n13 was related to Rhizobium meliloti with similarity matrix 0.3655, whereas Rhizobium phaseoli-n12 was more distal from other strains (0.2 with Rhizobium meliloti-n11) and (0.297 with Rhizobium leguminosarum-n13). The three strains explained difference in their tolerance to salinity, Rhizobium meliloti-n11 tolerated up to 300 mM NaCl, Rhizobium phaseoli-n12 has moderate tolerance to NaCl, it could tolerate up to 200 mM NaCl, whereas the strain Rhizobium leguminosarum-n13 was the most sensitive strain, this strain could not tolerate up to 100 mM NaCl in the YEM medium and this dosage limited its growth.

Key words: Rhizobium leguminosarum, Rhizobium meliloti, Rhizobium phaseoli, salt stress, RAPD PCR, Amino acids, dendrogram.

INTRODUCTION

An environmental factor that limits crop productivity or destroys biomass is referred to as a stress or disturbance. Salinity in soil or water is one of the major stresses and, especially in arid and semi-arid regions, can severely limit crop production (Shannon1998; Howieson and Ballard, 2004). Nearly 40% of the world's land surface can be categorized as having potential salinity problems (Cordovilla et al., 1994).

Inoculating plants with non-pathogenic bacteria can provide "bioprotection" against biotic stresses, and some root-colonizing bacteria increase tolerance against abiotic stresses such as drought, salinity and metal toxicity (Dimkpa, 2009). Salinity has become an ever-increasing problem in irrigated agriculture (Parker et al., 1977). Legumes establish several mutual, antagonistic, and beneficial interactions with microbes, which are occasionally subject to unfavorable (stressed) environmental conditions. Stressed terrestrial environments include, deserts with arid climate (warm and dry), salt-affected soils, alkaline and acidic soils, soils contaminated with toxic metals, and nutrient deficiency (Zahran, 2010). Most Rhizobia strains which nodulate important crops such as soybean, pea, and clover are very sensitive to salt (Soussi et al., 2001). Several reports have established that progressive increases in salt concentration adversely affect inoculum viability and the symbiotic nitrogen fixation rate (Balasubramanian, 1976). It has been well documented that nitrogen accumulation by the symbiotic systems of soybean, alfalfa, and Glycine javanica is reduced by salinity (Bernstein and Ogata, 1966) and (Wilson, 1970). Saline conditions may limit the symbiosis by (i) affecting survival and proliferation of Rhizobium spp. In the soil and rhizosphere, (ii) inhibiting the infection process, (iii) directly affecting root nodule function, or (iv) reducing plant growth, photosynthesis, and demand for nitrogen.

Salt tolerant rhizobia may have the potential to improve yield of legumes under salinity stress (El-Mokadem, 1991). Rhizobial inoculation increases nodule biomass thus encourages sustainable environmental friendly agriculture by responding perfectly in biological nitrogen fixation (Adewusi et al., 2008). One approach to understanding the ability of Rhizobium to tolerate salt stress has been to identify stress-induced changes of individual proteins under the assumption that stress adaptation results from alterations in gene expression (Natarajan et al., 1996). Salt tolerance of symbiotic nitrogen fixation reportedly depends both on the plant and Rhizobium genotypes (Pessarakli and Zhou 1990; Cordovilla et al., 1995).

Various phenotypic and genotypic methodologies are being used to identify and characterize bacteria. Although phenotypic methods play a significant role in identification but the molecular methods are more reliable and authenticated for identification and to study genetic diversity of bacterial isolates. Major molecular techniques include PCR (Polymerase chain reaction), RAPD (randomly amplified polymorphic DNA), RFLP...
(restriction fragment length polymorphism), AFLP (amplified fragment length polymorphism), SSR (single sequence repeats) and 16S-rRNA gene sequencing. RAPD is the most reliable, rapid and practical method (Mehmood et al., 2008). Many authors proposed this method for identification and phylogenetic grouping of Rhizobium isolates (Labes et al., 1996; Sikora et al., 1997; 2002; Bradić et al., 2003).

This research reports the isolation of different Rhizobium strains from the rhizoplane of a legume saline soil from Taif Governorate and fingerprints the isolates at molecular level using RAPD and study the physiological characterization. The principle aim was to isolate tolerant strain for salinity can help their host plant to support adverse ecological conditions.

**MATERIALS AND METHODS**

**Source of cultures:**
In the current work four representative sites were chosen from Taif region at about 50 km northeast down town (40.86° N, 21.16° E), the four sites were Garwa, Almathna, Alwahtand Alwahit which irrigated with groundwater pumped from deep wells. Three strains of Rhizobia (R. meliloti, Rhizobium phaseoli and Rhizobium leguminosarum bv viceae) were isolated from root nodules of Medicago sativa, Phaseolus vulgaris and Vicia faba respectively. The isolates were grown on yeast extract mannitol agar (YMA) medium at 28°C (Vincent, 1970) and incubated for three to ten days depending on the strain and species until colonies appear.

**Rhizobium Strain And Growth Conditions:**
For growth curves, 100 ml volumes of YEM medium (Vincent1970) supplemented with NaCl (0, 100, 200, 300 mM) were prepared in 250 ml Erlenmeyer flasks and inoculated with 1 ml of a late exponential phase cultures of different Rhizobium isolates. Cells were grown at 28°C in an orbital shaker (170 rev min⁻¹). Growth was monitored for 80 h by optical density (O.D.) at 600 nm in a Spectromic 20D spectrophotometer (Spectromic Instruments Inc., NY, USA).

**Salt Tolerance:**
Single colonies of the isolated strains were replica plated on YMA plates supplemented with different concentrations of 50, 100, 200, and 300 mM NaCl.

**Phenotypic Characterization:**
The cell morphology was examined by phase contrast microscopy Gram staining was performed (Doetsch, 1981) and motility verified by the SIM (Hydrogen-Sulfide, Indole, Motility; Mac Faddin, 2000) test. 64 phenotypic features, including utilization of sole carbon and nitrogen sources, resistance to antibiotics, tolerance of dyes and chemicals, temperature and pH ranges for growth and some physiological and biochemical reactions described previously in Bergey’s Manuel (Jordan, 1984 and Alberton et al., 2006) were examined.

**DNA Preparation:**
Bacterial DNA was prepared using the method of Sritharan and Barker (1991). Single colonies were picked and suspended in 100 µl of 10 mM Tris pH 8.0, 1 mM EDTA, 1% Triton X-100 solution and boiled for 5 min. After a single chloroform extraction, 5 ml of the supernatant were used in the amplification reaction.

**RAPD-PCR Analysis:**
PCR amplification was performed using eleven random 10-mer arbitrary primers synthesized by Operon biotechnologies, Inc. (Alameda, CA, USA) were used to screen for the RAPD polymorphism. These primers and their nucleotides sequences listed in Table (1).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>OP-B08</td>
<td>GTCCACACCGG</td>
<td>OP-G04</td>
<td>AGCGTGTCTG</td>
</tr>
<tr>
<td>OP-B09</td>
<td>TGGGGAGTCTC</td>
<td>OP-G07</td>
<td>GAACTGCCC</td>
</tr>
<tr>
<td>OPB-19</td>
<td>ACACCCGAGAGR</td>
<td>RUF-213</td>
<td>TTCGGGCGGT</td>
</tr>
<tr>
<td>OP-E04</td>
<td>GTGACATGCC</td>
<td>RUF-216</td>
<td>CAGCAGAACAT</td>
</tr>
<tr>
<td>OP-E05</td>
<td>TCAGGGAGGT</td>
<td>W-18</td>
<td>(CA)₆</td>
</tr>
<tr>
<td>OP-F06</td>
<td>GGGAAATCC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Amplification was conducted in 25µl reaction volume containing the following reagents: 2.5µl of dNTPs (2.5mM), 2.5µl of MgCl₂ (2.5mM), and 2.5µl of 10x Taq buffer, 3.0µl of primer (10 pmol), 3.0µl of DNA template (25ng/µl), 1µl of Taq polymerase (1U/µl) and 10.5 µl of sterile ddH₂O. The DNA amplifications were performed in an automated thermal cycle (model Techno 512) programmed for one cycle at 94°C for 4 min followed by 45 cycles of 1 min at 94°C, 1 min at 36°C, and 2 min at 72°C. The reaction was finally stored at
72°C for 10 min. Amplified products were size-fractioned using 100 bp plus ladder marker (Axygen Biosciences) by electrophoresis in 1.5% agarose gels in TBE buffer at 120 V for 1 h. The bands were visualized by ethidium bromide under UV fluorescence and photographed.

**Data analysis:**

Each analysis was a consensus of at least two replicates runs. The DNA bands were detected by totallab120 advanced analysis of 1D electrophoresis gel images software (nonlinear Dynamics, USA) and scored as + (presence) or - (absence) and all intense and reproducible bands were considered. These data were used to determine the genetic distance between strains. Average linkage (UPGMA-unweighted pair group method with averages) dendrogram among the three Rhizobium isolates was produced using the SPSS v15.0 program for windows Evaluation version.

**Amino acid determination:**

The intracellular amino acid pools of tolerant *Rhizobium* strains grown in high salt concentration (300 mM NaCl) were determined by the following procedure. The bacteria were grown in YEM medium containing 300 mM NaCl until late-logarithmic phase. For each culture, 100 ml of cells was centrifuged at 17,000 xg for 15 min. The pellets were washed and suspended in isotonic salt solutions and then re-centrifuged. The amino acids from the resulting pellets were extracted by suspending in 1 ml of 5% trichloroacetic acid (TCA) at 0°C for 2 h or overnight with occasional blending in a Vortex mixer. The suspensions were then centrifuged at 27,000 xg for 30 min. The supernatants obtained were used for amino acid analysis with a Durrum amino acid analyzer, model D-500, with a single column (1.75 mm by 48 cm) packed with Durrum DC-4A resin. Citrate buffers of pH 3.25, 4.25, and 7.90 were used.

**Protein patterns:**

Cells grown in liquid YEM medium containing 300 mM NaCl to the late logarithmic phase were harvested by centrifugation at 15,000 xg for 5 min at 4°C, washed in 50 mmol l⁻¹ Tris-HCl (pH 7.5) and resuspended in 200 µl of the same buffer. The cells were sonicated for 5 min at 15 s intervals in a refrigerated circuit at 4°C using a sonicator Vibra-Cell 100 Watt model (Sonic & Materials Inc., Danbury, CT, USA). Lysates were centrifuged at 30,000 xg for 20 min to remove cell debris. The total protein content of the extracts was determined by the Lowry method modified by Markwell et al. (1978). Proteins were subjected to SDS-PAGE according to Laemmli (1970), using 30 µg of protein per lane. Protein electrophoresis was carried out in 15% SDS-polyacrylamide gels. Gels were fixed and stained with silver nitrate as described by Blum et al. (1987).

**Results:**

**Phenotypic characterization:**

White colored, mucoid, and like a drop of water colonies observed on YEMA with Congo red were the characteristics of *Rhizobium* sp. The representative colony used for further biochemical characterization also reflected the similar biochemical characteristics to that of *Rhizobium* sp. From the results of morphological, cultural and biochemical characters as informed in Bergey’s Manuel (Jordan, 1984 and Alberton et al., 2006) (Table 2) and the host from which it was isolated, the isolates were identified as *Rhizobium meliloti*-n11 from *Medicago sativa*, *Rhizobium phaseoli*-n12 from *Phaseolus vulgaris* and *Rhizobium Leguminosarum*-n13 from *Vicia faba* respectively.

<table>
<thead>
<tr>
<th>CHARACTERISTICS</th>
<th><em>R. meliloti</em>-n11</th>
<th><em>R. phaseoli</em>-n12</th>
<th><em>R. leguminosarum</em>-n13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony shape</td>
<td>Round, transparent, non-mucilaginous</td>
<td>creamy</td>
<td>creamy</td>
</tr>
<tr>
<td>Colony color</td>
<td>creamy</td>
<td>creamy</td>
<td>creamy</td>
</tr>
<tr>
<td>Mucocity</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cell shape</td>
<td>Rods</td>
<td>Rods</td>
<td>Rods</td>
</tr>
<tr>
<td>Cell motility</td>
<td>Non-motile</td>
<td>Non-motile</td>
<td>Non-motile</td>
</tr>
<tr>
<td>Gram staining</td>
<td>-ive</td>
<td>-ive</td>
<td>-ive</td>
</tr>
<tr>
<td>Root nodules produced</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fast growth on YEMA</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth at 39-400°C</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Growth in presence of 2% NaCl</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>H2S production</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Growth at PH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4.5</td>
<td>+</td>
<td>+</td>
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<tr>
<td>5.0</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<tr>
<td>8.0</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9.0</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
The growth responses of *R. Meliloti* -n11, *R. phaseoli* –n12 and *R. Leguminosarum*-n13 varied with different NaCl dosage (Fig. 1). Growth of *R. Meliloti* -n11 gradually decreased as the salt concentration increased. This strain could tolerate up to 300 mM in the YEM medium (Fig. 1a). *R. phaseoli* –n12 could tolerate up to 200 mM NaCl in the YEM medium, whereas the concentration of 300 mM reduced the total amount of bacterial growth, *R. phaseoli* –n12 has moderate tolerance to NaCl (Fig. 1b). *R. Leguminosarum*-n13 was the most sensitive strain, it could not tolerate up to 100 mM NaCl in the YEM medium (Fig.1c).
Fig. 1: Effect of NaCl on growth of (a) *R. Meliloti* -n11, (b) *R. phaseoli* -n12 and (c) *R. Leguminosarum* -n13 grown in YEM medium and incubated at 28°C. Growth was measured by the increase in absorbance at 600 nm.

**RAPD PCR:**

Eleven random primers (OP-B08, OP-B09, OP-B19, OP-E04, OP-E05, OP-F06, OP-G04, OP-G07, RUF-213, RUF-216, and W-18) were used in the present study to screen for the RAPD polymorphism of the tested three *Rhizobium* strains (Fig. 2). Five monomorphic and one hundred five polymorphic distinct fragments were detected (Table 3). The results of RAPD analysis showed that all used primers were polymorphic. OP-B08, OP-B09, OP-E04, OP-E05, OP-G04, OP-G07, RUF-213, and W-18 primers showed the highest polymorphism level among the tested strains (100% of polymorphism), while OP-F06 primer showed the lowest polymorphism level (60% of polymorphism) (Fig. 2). The compiled data for the eleven used primers recorded 95.45% of polymorphism. Some strain(s)-specific markers were detected among the three studied *Rhizobium* isolates as shown in Table (4). OP-B08 primer gave specific markers for each *Rhizobium* isolates with molecular weights of 568.469, 448.523, and 200.000 base pairs for *R. phaseoli*, 2141.336, and 1610.272 base pairs for *R. Leguminosarum*, and 1359.706, 622.97, 518.985, and 389.134 base pairs for *R. Meliloti*. The primer showed a common band between the *R. Leguminosarum* and *R. Meliloti* with molecular weight 1102.157 base pair which was absent in *R. phaseoli*. Primer OP-B09 generated four specific markers for the isolate *R. phaseoli* with molecular weights of 2483.802, 974.941, 822.653 and 367.655 base pairs, respectively. It also gave two specific markers for the isolate *R. Leguminosarum* with molecular weights of 1100.716 and 461.383, and 310.307 base pairs. One common between *R. phaseoli* and *R. Meliloti* with molecular weight of 1219.927 base pair, as well as one band was common between *R. phaseoli* and *R. Leguminosarum* with molecular weight of 578.42 base pair. OP-B19 primer showed six specific markers for *R. phaseoli* with molecular weights of 2246.59, 1369.597, 837.291, 612.644, 432.007, and 346.65 base pairs, two specific markers for *R. Meliloti* with molecular weights 720132, and 648.043. OP-E04 primer showed two specific markers for *R. phaseoli* with molecular weights of 1335.624 and 609.436 base pairs, four specific markers for *R. Leguminosarum* with molecular weights of 3391.304, 3043.478, 1232.027, and 836.03 base pairs, and two specific markers for *R. Meliloti* with molecular weights of 2152.865, and 1572.03 base pairs. The primer also showed two common specific molecular markers between two isolates (*R. phaseoli*, and *R. meliloti*) with molecular weight of 565.159 base pair, and one specific marker for the isolates (*R. Leguminosarum* and *R. meliloti*) with molecular weight of 421.915 base pair, and two specific markers for isolates (*R. phaseoli*, and *R. Leguminosarum*) with molecular weight of 343.372 and 261.356 base pairs. Primer OP-F06 gave only two specific markers for *R. phaseoli* with molecular weights of 138.285, and 295.36, and *R. Leguminosarum* with molecular weights of 421.915 base pair, and two specific markers for isolates (*R. phaseoli*, and *R. Leguminosarum*) with molecular weight of 343.372 and 261.356 base pairs. Primer OP-G04 gave three specific markers for *R. phaseoli* with molecular weights of 3217.391, 572.676 and 428.016 base pairs, three specific markers for *R. Leguminosarum* with molecular weights of 2906.533, 2405.766, and 1893.116 base pairs and four specific markers for *R. meliloti* with molecular weights of 1702.975, 1289.158, 491.374, and 204.823 base pairs. Three common specific markers were detected for each two isolates, two for isolates (*R. phaseoli* and *R. Leguminosarum*) with molecular weights of 813.252 and 229.948, and one for isolates (*R. Leguminosarum* and

Genetic Similarity Analysis:

The dendrogram tree among the studied Rhizobium isolates based on RAPD markers were constructed using SPSS v15.0 program depending on the similarity matrix present in Table (5). The analysis was based on the number of markers that were similar between any given pair of isolates. Concerning the isolates, R. leguminosarum and R. meliloti isolates were related to each other with similarity value of 0.365, while isolate pairs (R. phaseoli and R. leguminosarum) and (R. phaseoli and R. meliloti) showed the lowest similarity value of 0.297 and 0.2 respectively.

Dendrogram tree separated the three isolates into two major clusters as shown in Fig. (3), the first cluster was divided into two groups for the isolates (R. leguminosarum and R. meliloti) were highly related to each others. The second cluster presented only R. phaseoli. However, the relationships among the studied three isolates based on RAPD analyses were in partial agreement with the season of their cultivars, Vicia faba and Medicago sativa are winter cultivars while Phaseolus vulgaris is cultivated in summer.

Fig. 2: RAPD PCR of eleven random primers (M) 1kb plus, (1) R. phaseoli, (2) R. Leguminosarum, (3) R. Meliloti.
Table 3: Levels of polymorphism detected with each RAPD primers which tested in the three studied *Rhizobium* strains.

<table>
<thead>
<tr>
<th>RAPD</th>
<th>Primer Name</th>
<th>OP-B08</th>
<th>OP-B09</th>
<th>OP-B10</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomorphic bands</td>
<td></td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Polymorphic bands</td>
<td></td>
<td>10</td>
<td>16</td>
<td>14</td>
<td>105</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>10</td>
<td>16</td>
<td>16</td>
<td>110</td>
</tr>
</tbody>
</table>

Table 4: Rhizobium strain(s)-specific markers were detected among the three studied Rhizobium isolates based on RAPD analyses.

Table 5: Similarity matrix based on the overall marker polymorphism for the studied three *Rhizobium* isolates.

Fig. 3: Dendrogram tree for the *Rhizobium* strains based on RAPD analyses.
Influence of NaCl concentration on the content and composition of amino acid pools. The amino acid pools of *R. Meliloti*-n11, *R. phaseoli*-n12 and *R. Leguminosarum*-n13 grown at 0 mM NaCl were similar to those of other gram-negative bacteria (Brown, 1976; Measures, 1975; Tempest and Meers, 1970). Glutamate and proline were the predominant amino acids, glutamate was accounting for 18.76%, 26.12% and 20.28% while proline was accounting for 14.07, 15.81 and 10.20 of the total amino acid content of *R. Meliloti*-n11, *R. phaseoli*-n12 and *R. Leguminosarum*-n13, respectively (Table 5). Aspartate, alanine and Arginine were the three other amino acids found to be abundant in the pool. The other amino acids were found in relatively small amounts.

**Table 5: Influence of medium NaCl (300mM) on the intracellular free amino acid composition of *Rhizobium* strains**

<table>
<thead>
<tr>
<th>Rhizobium Strains</th>
<th>Amino acid conc. (µM/g of cells [dry wt]a)</th>
<th>Total free amino acids (% of pool)</th>
<th>Proline proportion of pool (%)</th>
<th>Glutamate proportion of pool (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R. Meliloti</em>-n11 (0mMNaCl)</td>
<td>0.49 Arg 3.71 Asp 1.95 Ala 0.88 Isoleu 0.09 Pro 0.9  The 0.92 ND 0.3 1.02 0.56 1.46 0.49 ND 0.61 0.46 21.96 14.07 18.76</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R. Meliloti</em>-n11 (300mMNaCl)</td>
<td>3.91 Arg 6.71 Asp 2.1  Ala 1.3  Isoleu 3.15 Pro 2.3  The 3.12 0.92 ND 0.3 1.02 0.56 1.46 0.49 ND 0.61 0.46 21.96 14.07 18.76</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R. phaseoli</em>-n12 (0mMNaCl)</td>
<td>1.07 Arg 4.1 Asp 1.03 Ala 0.78 Isoleu 3.2  Pro 0.87 The 5.42 0.87 ND 0.44 1.64 0.83 1.35 0.83 ND 0.85 0.89 20.75 15.81 26.12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R. phaseoli</em>-n12 (300mMNaCl)</td>
<td>8.24 Arg 3.3 Asp 11.65 Ala 3.45 Isoleu 27.18 Pro 2.64 The 53.02 2.64 ND 0.44 1.64 0.83 1.35 0.83 ND 0.85 0.89 20.75 15.81 26.12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R. Leguminosarum</em>-n13 (0mMNaCl)</td>
<td>0.94 Arg 3.3 Asp 0.89 Ala 0.9 Isoleu 3.2  Pro 0.87 The 5.42 0.87 ND 0.44 1.64 0.83 1.35 0.83 ND 0.85 0.89 20.75 15.81 26.12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R. Leguminosarum</em>-n13 (300mMNaCl)</td>
<td>7.02 Arg 14.13 Asp 11.65 Ala 6.29 Isoleu 44.78 Pro 2.03 The 41.37 6.29 ND 2.03 7.45 0.39 5.09 5.5 ND 1.13 3.65 61.8 27.6 25.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Free amino acids were extracted by 5% TCA and determined by amino acid analyzer.

b ND, Not detectable (concentration less than 0.2 µM/g of cells [dry weight]).

Furthermore, Table 5 shows that glutamate and proline increased to extremely high levels with increasing NaCl added to the medium. Relative to controls grown in the absence of NaCl, the levels of glutamate in the intracellular pools for cells grown at 300 mM increased 12.45, 9.76 and 8.83 fold while proline increased 10.4, 8.28 and 18.89 fold for *R. Meliloti*-n11, *R. phaseoli*-n12 and *R. Leguminosarum*-n13, respectively. Glutamate accounted for 41.57%, 33.73% and 25.60% while proline accounted for 26.04%, 17.29% and 27.60% of the total amino acid pool for *R. Meliloti*-n11, *R. phaseoli*-n12 and *R. Leguminosarum*-n13, respectively when cells were grown at 300 mM NaCl. As medium salt concentration was increased at 300 mM, aspartate increased 2.45, 3.24, and 4.28 fold, alanine increased 3.59, 11.31 and 13.03 fold while Arginine 2.6, 7.7 and 7.4 fold for *R. Meliloti*-n11, *R. phaseoli*-n12 and *R. Leguminosarum*-n13, respectively. Other amino acids showed very little change.

The electrophoretic protein pattern of the tolerant isolated *Rhizobium* strains (*R. Meliloti*-n11 and *R. phaseoli*-n12) under control and salt treatments conditions (300 mM NaCl) are shown in Fig. 3. High salt concentration affected the synthesis of cell proteins in the two tolerant strains. With *R. Meliloti*-n11 Comparative analysis of the lanes (lanes 1, 2) showed production of two new protein bands (48 and 32 kDa) which were not produced by the strain under control condition. Furthermore, protein bands of molecular weights 108, 68, and 53 kDa, were intensified at this salt concentration. The band of molecular weight 93 kDa was reduced compared with the control. The same changes in the production of specific proteins were also noted in *R. phaseoli*-n12, but the response of this strain to salt concentration varied in several aspects from that of *R. Meliloti*-n11. Thus *R. phaseoli*-n12 failed to synthesize proteins of 36, 43, and 70 kDa and reduced the intensity of bands of 54, 66, 68, and 74 kDa (lanes 3, 4), which were observed in the control.

**Discussion:**

In this study, high salt concentration inhibited the growth of the three *Rhizobium* strains. The strain *Rhizobium melliloti*-n11 tolerated up to 300 mM NaCl, *Rhizobium phaseoli*-n12 has moderate tolerance to NaCl, this strain could tolerate up to 200 mM NaCl, whereas the strain *Rhizobium leguminosarum*-n13 was the most sensitive strain, this strain couldn't tolerate up to 100 mM NaCl in the YEM medium. On the other hand, extension in the lag phase of cell multiplication was notified with increasing salt concentration, as indicated for other *Rhizobium* nodulating *Cicer arietinum* (Zurayk et al. 1998).

RAPD PCR technique provides reliable information on the diversity of Rhizobium populations in soils (Laguerre et al., 1996; and Pinto et al., 1998; Oliveira et al., 2000).

The two clusters established by the analyzed strains indicated the influence of environmental conditions (seasonal cultivation effects) or soil characteristics on the genetic distribution of these Rhizobia (Patricia et al. 2004).
Fig 3: Stained SDS-PAGE of total soluble protein of *R. Meliloti* -n11 grown in YEM medium and incubated, under control conditions (lane 1) and with 300 mM NaCl (lane 2), and *R. phaseoli* –n12 grown in YEM medium, under control conditions (lane 3), and with 300 mM NaCl (lane 4). Lane M, molecular mass standards (Kit MW GF-1000, Sigma). The arrows on the left and on lane 4 indicate the changes observed that are explained in the text.

One of the goals of this study was to analyze the amino acid pools of *R. Meliloti*-n11, *R. phaseoli*-n12 and *R. Leguminosarum*-n13 grown in media 300mM of NaCl. Intracellular glutamate was found to increase rapidly in response to osmotic stress by NaCl. Glutamate has also been found to accumulate in several gram negative bacteria by other investigators (Tempest and Meers, 1970; Measures, 1975; Nakamura, 1979). Changing the osmolarity of the medium with NaCl increased intracellular proline content, although proline accumulation is important in many gram positive bacteria, as well as some gram negative bacteria. In general, bacterial species which accumulate proline are more salt tolerant than those which do not (Measures, 1975).

Salt significantly increased the total amino acid pool *R. Meliloti*-n11 and *R. phaseoli*-n12 (Table 4), as has been reported in other *Rhizobium* species (Yap and Lim 1983). It has been suggested that increases in total amino acids may be a consequence of protein degradation (Botsford and Lewis 1990; Soussi et al., 2001), although the accumulation of high molecular weight Glutamate and proline which were the predominant amino acid.

In media with 300 mM NaCl, prominent qualitative and quantitative differences were detected. Mainly the lack of some low molecular weight polypeptides in the strain of *R. phaseoli*-n12 suggesting that these proteins are not sole mediator of NaCl tolerance and an increase in the relative abundance of several high molecular weight polypeptides in the strain of *R. Meliloti*-n11, the induction of these proteins may correlate to resistance to NaCl treatment. These findings agree with results reported for a Sesbania nodulating *Rhizobium* strain (Natarajan et al., 1996; Soussi et al., 2001). The shift towards high molecular weight polypeptides in protein patterns of *R. Meliloti*-n11 may be due to general protease inhibition (Saxena et al., 1996). The prolonged lag phase in the growth curve (figure 1) may correlate to the time required for production of these proteins.

The dendrogram showed that *R. meliloti* and *R. leguminosarum* are more relevant comparing with the relation to *R. phaseoli*, the reason of this may be referred to the crops (*Medicago sativa* and *Vicia faba*) which are winter cultivars whereas *Phaseolus* vulgaris is a summer cultivar. Final we recommend using the tolerant *R. meliloti* strain as vital fertilizer to help their host plant to support adverse ecological conditions.

**Conclusions:**

In this study, we have discussed the tolerance of *R. Meliloti*-n11, *R. phaseoli*-n12 and *R. Leguminosarum*-n13 for salinity. The strain *Rhizobium meliloti*-n11 tolerated up to 300 mM NaCl, *Rhizobium phaseoli*-n12 has moderate tolerance to NaCl, This strain could tolerate up to 200 mM NaCl, whereas the strain *Rhizobium leguminosarum*-n13 was the most sensitive strain. Intracellular glutamate and proline were found to increase rapidly in response to osmotic stress by NaCl. Mainly the lack of some low molecular weight polypeptides in the strain of *R. phaseoli*-n12 suggesting that these proteins are not sole mediator of NaCl tolerance and an increase in the relative abundance of several high molecular weight polypeptides in the strain of *R. Meliloti*-n11, the induction of these proteins may correlate to resistance to NaCl treatment. The dendrogram showed that *R. meliloti* and *R. leguminosarum* are more relevant comparing with the relation to *R. phaseoli*.
In addition, the results of this study confirm that PCR is a useful tool for phylogenetic and ecological investigations of rhizobial communities and will also permit to conduct competitiveness and persistence studies on these rhizobia strains when inoculated in Saudi Arabia soil as inoculants.

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