# Isolation and molecular characterization of heat and salt tolerance Rhizobia isolated from Saudi Arabia

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**Abstract:** Rhizobia form a very interesting symbiotic relationship with leguminous plants, so that; successful symbiosis between the bacteria and the legumes are not sustained unless the effect of environmental stresses such as salinity and heat are modulated in arid and semiarid regions. Selection of effective, efficient and adapted stress tolerant rhizobial strains could help in ecological studies and increases soil fertility thereby improving the growth of associated plants of these regions. In the present study three indigenous *Rhizobium leguminosarum* bv. *trifolii* isolates were isolated from roots of *Trifolium alexandrinum* plant from Hada El-sham region Saudi Arabia. Isolates were phenotypically and biochemically characterized followed by studying their nodulation efficiency. Screening their tolerance to salt raged from (0.5 to 4% NaCl) and heat with the occurrence of different temperature from (30$^{o}$C to 60$^{o}$C) was evaluated. The isolated strains will be characterized genetically i.e., plasmid profile and DNA fingerprinting by RAPD-PCR also done.

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**Key words:** Rhizobium, Heat tolerance, Salt tolerance, Plasmids, RAPD-PCR

## 1. Introduction

Legumes have been used in agriculture since ancient time and legume seeds or pulses were among the first source of human food and their domestication. Members of the genus *Rhizobium* have been isolated from nodules on leguminous plants and establish symbiosis with nitrogen fixing bacteria of the family Rhizobiaceae (**Wei *et al*., 2003; Peng *et al*., 2008**). The bacteria belonging to the genera *Rhizobium*, *Bradyrhizobium*, *Allorhizobium*, *Rinorhizobium* and *Mesorhizobium* are able to form nodules on their host plants inside of which they fix-nitrogen (**Martinez-Romero, 2003; Willems, 2006**). Each legume species requires a specific species and strain of rhizobia used as a biofertilizers in agriculture by rhizobia-legume symbioses (**Ribeiro *et al*., 2012**). Different ecological factors are dramatically affecting the legume/rhizobia symbiosis; these symbiotic relationships require successful survival ability of rhizobial bacteria under adverse environmental conditions, especially the survival ability of bacteria affected by biotic and a biotic factor such as salinity and temperature (**Zahran, 1999**). Desertification causes disturbance of plant-microbe symbioses, which are a critical ecological factor in helping further plant growth in degraded ecosystems (**Requena *et al*., 2001**). Inoculation of stress tolerant strains of rhizobia may enhance the nodulation and nitrogen fixation ability of plants under stress conditions. The ability of legume hosts to grow and survive in saline conditions is improved when they are inoculated with salt tolerant strains of rhizobia (**Zou *et al*., 1995; Shamseldin and Werner, 2005**). Rhizobial populations vary in their tolerance to major environmental factors (**Sridhar *et al*., 2005; Wei *et al*., 2008; Biswas *et al*., 2008**). Application and extension of inoculation techniques with effective rhizobial inoculants in legumes would result in great economical, environmental and ecological benefits (**Feng-Xian, *et al*., 2009; Belay and Assefa, 2011**). In recent years, research has provided considerable insights into the mechanism of biotic stress tolerance in nitrogen fixing microbes at the molecular level (**Abdel-Salam *et al*., 2010**). Different abiotic stress factors may provoke osmotic stress, which lead to lake adaptive responses. Recently, the authors try to improve biofertilizers treatments asymbiotic nitrogen-fixing bacteria, symbiotic nitrogen-fixing bacteria tolerance to salinity and heat through (**Hamdia and Shaddad, 2010; Abdel-Salam *et al*., 2013**). Total genomic DNA of each strain was extracted as described before (**Kaschuk *et al*., 2006**). Plasmids of different Rhizobia strains were determined in numbers and sizes by the bacterial lysate method (**Hirsch *et al*., 1980; Ried & Collmer, 1987; Abdel-Salam *et al*., 2013**). Several methods have been developed which permit species identification and typing microorganisms, including bacteria. One of these adaptations is DNA

amplification by PCR with low specificity conditions. (Random amplified polymorphic DNA, RAPD-PCR). It uses low specificity primers that are aligned at random with sequences in the bacterial genome. The standardized RAPD-PCR yields reproducible amplification patterns that are characteristic of the strains or clones (**Andrighetto *et al*., 2001; Ortiz-Herrera *et al*., 2004; Awad *et al*., 2011**). Rhizobia isolated from different field locations were subjected to RAPD analysis to study the diversity. The results indicated that RAPD is a very discriminative and efficient method for differentiating and studying genetic diversity of Rhizobium strains (**Rajasundari *et al*., 2009; Berrada *et al*., 2012**).

This work aims at isolation, identification and characterization of different indigenous *Rhizobium* strains from different geographical areas. These strains are usually more adapted than the added inoculants for environmental selection. They will be selected according to some characters according to some ecological factors e.g., salt, heat tolerant to select the best strains for each ecological area. The selected strains will be characterized genetically i.e., plasmid profile and DNA fingerprinting by RAPD-PCR.

## 2. Material and methods

**_Rhizobium_ isolation:**

*Rhizobium* isolates were isolated from Hada Elsham aride region, Saudi Arabia. Clover plants were collected from healthy plants, large sized pink colored nodules were picked from plant roots and surface sterilized by 70% ethanol for 5 min., washed several times with sterilized distilled water to remove any ethanol residues, nodules were crushed over YMA media (**Abdel-Salam *et al*., 2010**). Rhizobium like colonies picked and re-streaked over YMA plates. Three different *Rhizobium leguminosarum* bv. *trifolii* isolated and named Rhi 1, Rhi 2 and Rhi 3.

**Identification of *Rhizobium leguminosarum* bv. *trifolii*:**

The isolated bacterial strains from nodules were identified as *Rhizobium leguminosarum* bv. *trifolii* on the basis of its cultural , biochemical characters (**Hahn, 1966; Kuykendall, 2005**) and their ability to nodulate clover roots (**Jensen, 1942; Stowers and Elkan, 1980; Abdel-Salam, *et al*., 2010**).

**Salt tolerance studies:**

Flasks of YEM (Yeast Extract Mannitol) broth having either variable concentration ((0.5, 1.0, 1.5, 2.5, 3.0, 3.5 and 4.0%) of salt (sodium chloride) inoculated with pure single colony of *Rhizobium* strains grown and incubated at 28±1$^{o}$C for 48h. Optical density OD observation was recorded at 540 nm using spectrophotometer (**Ali *et al*., 2009**).

**Heat tolerance studies:**

In this experiment flasks of YEM, were inoculated with pure rhizobial culture suspensions and incubated at different temperatures (30, 35, 37, 40, 45, 50, 55, and 60ºC) OD readings were performed at the end of 48 h at OD 540 nm under each temperature condition (**Alexandre and Oliveira, 2011**).

**Molecular genetics analysis:**

**Plasmid patterns:**

For determination of plasmids numbers and sizes in *Rhizobia* strains, the bacterial lysate method described by (**Hirsch *et al*., 1980; Abdel-Salam *et al*., 2010**) was used.

**Total DNA Extraction:**

The cell pellets form all isolates was used to extract genomic DNA using (Jena Bioscience, Germany) extraction kit were done according to manufacturer's instructions.

**Random amplified polymorphic DNA (RAPD):**

Eight different primers were used in PCR reaction which consists of 10pmol of each arbitrary 10-mer primers and 25 to 50 *n*g of genomic DNA and 12.5 µl of 2x SuperHot PCR Master Mix (Bioron, Ludwigshafen, Germany). The codes and sequences of these oligoprimers are listed in Table (3). The RAPD-PCR amplification reactions were performed in Eppendorf® thermal cycler using the following PCR program: 1cycle at 94°C, 4 min; 35 additional cycles consisting of 94°C 5s, 37°C 20s and 72°C 20s. After the amplification, the PCR reaction products were electrophoresis with 100 bp ladder marker (Fermentas, Germany) on 10 x 14 cm 1.5% agarose gel (Bioshop, Canada) for 30 min using Tris-borate-EDTA Buffer. The gel was stained with 0.5 µg/ml of ethidium bromide (Bioshop, Canada).

**Analysis of the PCR products:**

After the amplification, the PCR reaction products were electrophorised with 100bp ladder marker (Fermentas, Germany) on 10 x 14 cm 1.5% agarose gel (Bioshop, Canada) for 30 min using Tris-borate- EDTA Buffer. The gels were stained with 0.5ug/ml of ethidium bromide (Bioshop, Canada), visualized under the UV light and documented using a GeneSnap 4.00- Gene Genius Bio Imaging System (Syngene, Frederick, Maryland, USA).

**Gels analysis:**

The agarose digital image files were analyzed using Gene Tools software from Syngene. The densitometry scanning of each based on its three characteristic dimensions was carried out. Each band was recognized by its length, width and intensity. Accordingly, the relative amount of each band was measured and scored.

**Determination of Genetic relationship:**

In order to determine genetic relationship among studied bacteria, RAPD bands were scored for

presence (1) or absence (0). The data were transferred to a statistical software program, Statistical Package for Social Science (SPSS), version 10 (SPSS Inc, Chicago, Illinois, USA) to obtain statistical analysis in the form of Jaccard's similarity coefficient (S) showing the genetic similarity among different examined bacterial isolates based on pair-wise comparison.

## 3. Results

Extreme environmental condition in arid and semiarid climates such as temperature, salinity, heavy metal, pH sress ete. are the main problems to growing root nodulating bacteria and affect their symbiotic nitrogen fixation efficiency. Isolation of certain isolated rhizobial strains which resist these ecological factors under field condition are the main important area in the field of nodulation and nitrogen fixation. Three isolated *Rhizobium* strains were isolated and identified according to their cultural, biochemical characters and nodulation on its target plant in test tubes. Three indigenous *Rhizobium leguminosarum* bv. *trifolii* isolates could be obtained through this study. Their original location in (Hada Al-Sham, Saudi Arabia) and their names from Rhi 1, Rhi 2 and Rhi 3 as shown as shown in Fig (1). The number of nodules per plant and the nodule size ranged from 6 nodules per plant strain Rhi1, 5 nodules per plant strain Rhi2, and 7 nodules per plant strain Rhi3. Results showed the variation in nodulation efficiency between the local *Rhizobium leguminosarum* bv. *trifolii* isolates.

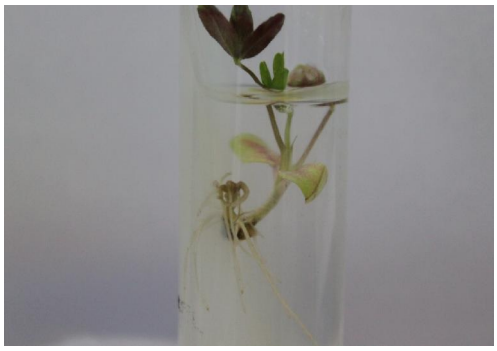


**Fig. (1): Nodulation efficiency of isolated Rhizobial strain.**

**Determination of salt-tolerant efficiency of rhizobial strains:**

Salt-tolerant efficiency of all strains were studied using different concentration of NaCl supplemented in YMA liquid and solid medium ranged from 0.5 to 4% NaCl. Three tested *Rhizobium leguminosarum* biovar *trifolii* (Rhi) were able to tolerate 4% NaCl, all *Rhizobium* showed good salt tolerant Table (1). All of them tolerate 3.5 % NaCl in liquid medium. The best performance of tolerance in all strains were at 3.5% NaCl concentration, spectrophtometric reading at OD $_{540}$ of Rhi 3 was (0.438 at 3.5%), Rhi 1 was (0.136 at 3.5%) and Rhi 2 was (0.102 at 3.5%) respectively. Table (1) also showed that three isolated *R. leguminosarum* biovar. *trifolii* strains moderate sensitive to salt stress at 4% NaCl, especially Strain Rhi 3 tolerate 4% NaCl concentration.

**Table (1): Levels of optical density (OD) at 540 nm for salt (NaCl) tolerance of *Rhizobium* isolates**

| Strain | NACL concentration levels | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **0.5** | **1** | **1.5** | **2** | **2.5** | **3** | **3.5** | **4** |
| Rhi 1 | 0.303 | 0.31 | 0.336 | 0.315 | 0.424 | 0.455 | 0.136 | 0.001 |
| Rhi 2 | 0.324 | 0.320 | 0.293 | 0.256 | 0.294 | 0.341 | 0.102 | 0.003 |
| Rhi 3 | 0.705 | 0.504 | 0.722 | 0.733 | 0.714 | 0.677 | 0.438 | 0.134 |

**Determination of heat tolerant efficiency of rhizobial strains:**

Heat tolerant efficiency of the three isolated strains under study was done by growing *Rhizoium* strains at different temperatures in liquid medium. The temperatures ranged from 30 as a control to 60$^{o}$C as indicated in table (2). All tested *Rhizoium* strains tolerate 45$^{o}$C, while Rhi 3 strain could tolerate 50$^{o}$C, The best performance of tolerance in all strains were at 45$^{o}$C concentration Rhi 3 (OD $_{540}$ = 0.442), Rhi 2 (OD $_{540}$ = 0.368) and Rhi 1 (OD $_{540}$ = 0.344) respectively. While strain Rhi 3 could tolerate 55$^{o}$C its OD level was (OD $_{540}$ = 0.132) but Rhi 1 and Rhi 2 could not tolerate 55$^{o}$C.

Results also indicated that there are association between isolate Rhi 3 in their tolerance to salt and heat, which indicate best performance at 4% NaCl and at 55$^{o}$C, Tables 1 and 2.

**Plasmid patterns of local *R. leguminosarum* bv. *trifolii* isolates:**

Plasmid patterns of our 3 local *R. leguminosarum* bv. *trifolii* isolates are present in Fig.(2) . All of the local *R. leguminosarum* bv. *trifolii* isolates had different molecular weight indigenous plasmids. Plasmid number was similar among tested strains, all *Rhizobium* strains indicated 4 plasmids per strain one of them large plasmid and 3 small plasmids found in each strain. The similarity in plasmid patterns among local isolates of *R. leguminosarum* bv. *trifolii* is an indication for their genetic similarity.

**Table (2): optical density (OD) levels at 540 nm of heat tolerant *Rhizobium* isolates**

| Strain | Temperature Range | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 30 | 35 | 37 | 40 | 45 | 50 | 55 | 60 |
| Rhi 1 | 0.32 | 0.446 | 0.314 | 0.431 | 0.344 | 0.001 | 0 | 0 |
| Rhi 2 | 0.367 | 0.377 | 0.380 | 0.400 | 0.368 | 0.003 | 0 | 0 |
| Rhi 3 | 0.691 | 0.516 | 0.331 | 0.495 | 0.442 | 0.247 | 0.132 | 0 |

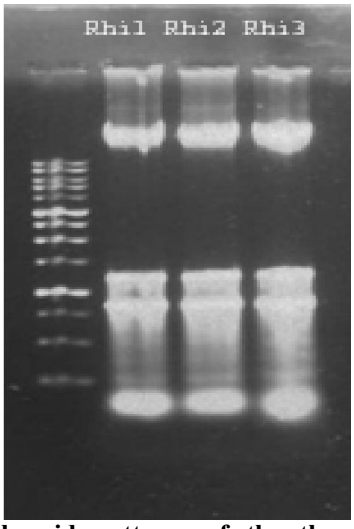
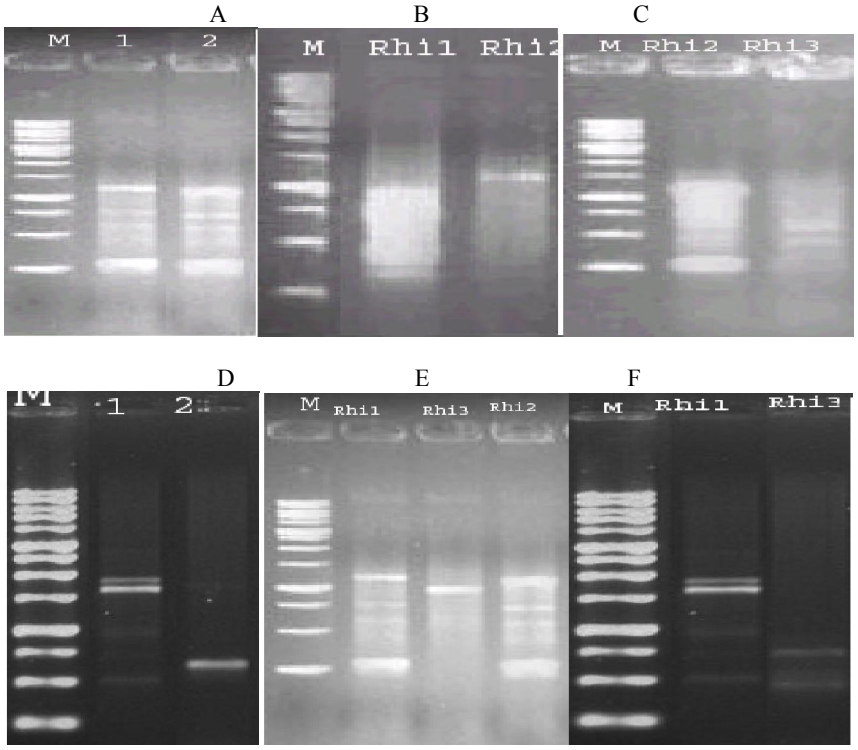



**Fig. 2. plasmid patterns of the three isolated *Rhizobium***

**Genetic fingerprinting with RAPD primers:**

Genetic fingerprinting was carried out via RAPD PCR technique using 8 different RAPD primers to detect the variation between the tested isolates within their genomes. Genomic DNA, extracted from bacterial isolates was used as templates for RAPD-PCR amplification. In this study, 8 random 10- mer primers were used. These primers have amplified 58 PCR product bands, table (3). The 8 primers produced multiple band profiles with a number of amplified DNA fragments ranging from1 to 12 the size and number of amplified fragments also varied with different primers.

The size of amplified fragments ranged from 100 bp approximately to approximately 3500 bp as shown in Figure (3). Maximum of 12 fragments was amplified with primers (OPA-02 and OPB-06), and minimum of 1 fragments were amplified with primer (OPA-04). Arrangement of primers depending on the generated number of fragments of DNA is OPA-04, OPC-02, OPC-05, OP-A15, OP-C11, OP-A8, OPA-02 and OP-B6. Twelve bands are the lowest total number, which was in RHi3. Whereas, 28 bands are the highest total number, which was in RHi2. Arrangement of strains depending on the number of fragments of DNA is RHi3, RHi1, RHi 2.



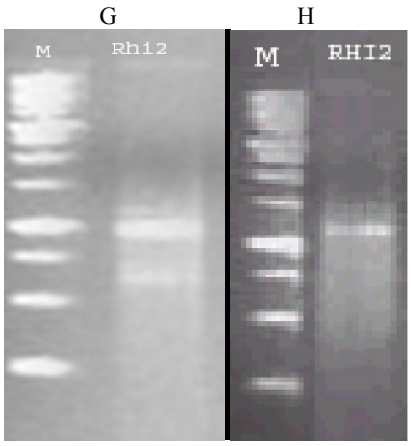



**Figure (3): A: Rhi3 and Rhi1 with OP-A8 primer B: Rhi1and Rhi3 with OP-A15 primer, C: Rhi2 and Rhi3 with OP-B6 primer D: Rhi2 and Rhi1 with OPC-05 primer E: Rhi1, Rhi3, Rhi 2 respectively with OPA-02 primer F: Rhi2 and Rhi1 with OPC-05 primer G: Rhi2 with OPC-02 primer H: Rhi2 with OPA-04 Primer.**

**Table (3): List of primers, their nucleotide sequences and total number of bands for each strain produced by 8 primers separately.**

| Primer code | Primer sequence | isolates | | | Total No. of amplified bands |
|---|---|---|---|---|---|
| | | RHi1 | RHi2 | RHi3 | |
| OP-A8 | GTGACGTAGG | 6 | | 4 | 10 |
| OP-A15 | TTCCGAACCC | 6 | | 1 | 7 |
| OP-B6 | GTAGACCCGT | | 9 | 3 | 12 |
| OP-C11 | GAACGGACTC | | 7 | 1 | 8 |
| OPA-02 | TGCCGAGCTG | 4 | 5 | 3 | 12 |
| OPC-05 | GATGACCGCC | 2 | 4 | | 6 |
| OPC-02 | GTGAGGCGTC | | 2 | | 2 |
| OPA-04 | AATCGGGCTG | | 1 | | 1 |
| Total | | 18 | 28 | 12 | 58 |

## 4. Discussion

The legume-rhizobium symbiotic association plays an important role in agriculture sustainability. The response of Rhizobium to salt and heat stresses of these symbiotic systems can certainly be helpful for the development of more efficient rhizobia inoculants.

Salinity affects agricultural production in arid and semiarid regions of arable lands, where rainfall is limited and is not sufficient to transport salts from the plant root zone (**Tester and Davenport, 2003**). Salt-tolerant efficiency of all strains were done using different concentration of NaCl supplemented in YMA liquid and solid medium ranged from 0.5 to 4% NaCl. Three tested *Rhizobium leguminosarum* biovar *trifolii* (Rhi) were able to tolerate 3.5 % NaCl, These results are in agreement with the work of (**Sharma *et al*., 2013; Nair *et al*., 1993).**

High root temperatures strongly affect bacterial infection and N2 fixation in several legume species. Heat tolerant efficiency of the three isolated strains under study was done by growing *Rhizoium* strains at different temperatures in liquid medium. The temperatures ranged from 30$^{o}$C as a control to 60$^{o}$C. All tested *Rhizoium* strains tolerate 45$^{o}$C, while Rhi 3 strain could tolerate 50$^{o}$C these results are inagreement with (**Laranjo and Oliveira, 2011**) in their work with *Mesorhizobium sp* showed high tolerances to heat shock.

The similarity in plasmid patterns among local isolates of *R. leguminosarum* bv. *trifolii* is an indication for their genetic similarity. The same results have been reported among field populations of *Rhizobium spp* as indicated by (**Ibrahim *et al*., 1998; Abdel-Salam *et al*., 2002 and Abdel-Salam *et al*., 2013**).

Genetic fingerprinting was carried out via RAPD PCR technique using 8 different RAPD primers to detect the variation between tested isolates within their genomes. Results showed variation between the tested isolates in their RAPD patterns. Several investigators (Rajasundari ***et al*., 2009; Berrada *et al*., 2012)** demonstrated that the number and size of amplified DNA fragments are varied with the different primers tested.

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