# Phenotypic characterization of indigenous Egyptian Rhizobial strains for abiotic stresses performance

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**Abstract:** Twenty one *Rhizobium leguminosarum* biovar *trifolii* strains and seven *Rhizobium meliloti* strains were characterized for their nodulation efficiencies and their growth performance against salinity, drought and heavy metals. About 67% of *R. l.* bv. *trifolii* strains and all *R. meliloti* strains were halotolerants. *R. l.* bv. *trifolii* strains were more drought-tolerant than *R. meliloti* strains. About 86% of *R. l.* bv. *trifolii* were multiple-metal resistant, iron resistance was the most abundant (~ 95%) followed by nickel resistant (~ 86%). Six *R. meliloti* strains were multiple-metal resistant, nickel resistance was the most apparent (~ 71%). The salt-and drought-tolerant Rhizobium strains are excellent models to study the resistance mechanism(s), and to elucidate the role of genetics of NaCl and drought tolerance. The characterized rhizobia had different applications. The salt tolerant, drought tolerant, and heavy metals resistance patterns found among the indigenous rhizobial strains are reflecting the environmental stresses pressure predominant in their locations and are very good examples of the importance of using efficient – indigenous rhizbial strain for plant successful inoculation. [Journal of American Science. 2010;6(9):498-503]. (ISSN: 1545-1003).

**Key words**: *Rhizobium leguminosarum* biovar *trifolii*, *Rhizobium meliloti*, nodulation, salinity, drought and heavy metals.

## 1. Introduction

Leguminous plants can obtain most of nitrogen they need from the vast supply of gaseous nitrogen in the air by working symbiotically with special bacteria (rhizobia) in nodules on their roots. The behavior of some nitrogen-fixing systems under severe environmental conditions such as salt stress, drought stress, acidity, alkalinity, nutrient deficiency, fertilizers, heavy metals, and pesticides was reviewed (Zahran, 1999). These major stress factors suppress the growth and symbiotic characteristics of most rhizobia (Duzan *et al*., 2004 & Gálvez, 2005). Therefore, isolation of rhizobia strains capable to tolerate these stresses is essential for efficient nitrogen fixation (Abd El-Halim *et al*., (2001); Abdel -Salam *et al*., (2002); Diouf, (2007); Essendoubi *et al*., 2007 and Woldeyohannes, *et al*., (2007).

This study aimed to isolate and collect indigenous *Rhizobium leguminosarum* biovar *trifolii* and *Rhizobium meliloti* strains from different geographical areas in Egypt and to characterize their performance against different abiotic stresses including drought, salinity and heavy metals effects.

## 2. Material and Methods

### 2.1 Bacterial strains and growth conditions

Rhizobial isolates used through this study are present in Table 1**.** Different Rhizobial strains were kindly obtained from Agriculture Research Centre (ARC), Giza, Egypt, they include *R. l.* bv. *trifolii* ARC 101 (Rt 101), BUG nitragen 142524 (Rt Nit) and ARC 100 strains, and *R. meliloti* A-2 (Rm A-2) and L 530 (Rm L530) strains. Rhizobia growth was measured by turbidity at 420 nm after incubation at $30^0$C with shaking at 250rpm for four days, using Shimadzu spectrophotometer.

**Table(1): Location and nomination of *Rhizobium leguminosarum* biovar *trifolii* and *Rhizobium meliloti* used in this study**

| Strain name | Geographical origin | | Strain name | Geographical origin |
|---|---|---|---|---|
| *Rhizobium leguminosarum* biovar *trifolii* | | | | |
| Rt1 | Kafr el Sheikh, Egypt | | Rt 14 | El Beheira, Egypt |
| Rt 2 | Kafr el Sheikh, Egypt | | Rt 15 | El Beheira, Egypt |
| Rt 4 | Gharbiya, Egypt | | Rt 16 | El Beheira, Egypt |
| Rt 5 | Giza, Egypt | | Rt 17 | Kafr el Sheikh, Egypt |
| Rt 7 | Gharbiya, Egypt | | Rt 18 | Kafr el Sheikh, Egypt |
| Rt 10 | Qena, Egypt | | Rt M1 | Kafr el Sheikh, Egypt |
| Rt 11b | Qena, Egypt | | Rt M2 | Kafr el Sheikh, Egypt |
| Rt 12 | Sohag, Egypt | | Rt H1 | Kafr el Sheikh, Egypt |

| | | | | |
|---|---|---|---|---|
| Rt 13 | Monofiya, Egypt | | Rt H2 | Kafr el Sheikh, Egypt |
| *Rhizobium meliloti* | | | | |
| Rm 1 | Kafr el Sheikh, Egypt | | Rm 4 | Al-Azhar Univ., Cairo, Egypt |
| Rm 2 | Kafr el Sheikh, Egypt | | Rm 5 | Al-Azhar Univ., Cairo, Egypt |
| Rm 3 | Al-Azhar Univ., Cairo, Egypt | | | |

**2.2 Plant material**

Egyptian clover, *Trifolium alexandrinum* (Meskawy) and Alfalfa, *Medicago alfalfa* (local variety), were used in the inoculation experiments with *R. l.* bv. *trifolii* and *R. meliloti* , respectively.

**2.3 Media**

Yeast extract- Mannitol medium (YM) (Fred *et al*., 1932), was used for routine cultivation of rhizobia. When necessary YM medium was solidified with 1.5% agar agar (YMA).

**2.4 Rhizobium isolation**

After washing the root system, nodules were picked off the roots and surface sterilized as described by Fred *et al.,* (1932). The nodules were crushed directly onto the surface of YMA plate and incubated. *Rhizobium*- like colonies were subjected to different cultural and biochemical testes for identification, e.g., growth behavior on YMA medium Fred *et al.,* (1932); growth behavior on PGA medium (Vincent, 1970); Congo red test (Hahn, 1966). A nodule-isolate was finally identified as *R. l.* bv. *trifolii* or *R. meliloti* on the basis of its ability to nodulate its specific host plant i.e., Egyptian clover or alfalfa, respectively.

**2.5 Plant inoculation**

Pots technique**,** using sterilized soil, was used for plant inoculation with Rhizobia as described before (Abdel-Salam *et al*., 2001). At least five plants were used with each bacterial strain.

**2.6 Determination of salt tolerance**

All *Rhizobium* strains were tested against different NaCl concentrations, in YM medium. After incubation at 30$^{o}$C for four days, growth was measured.

**2.7 Determination of drought tolerant strains**

The effect of drought on rhizobia-growth was studied using polyethylene glycol (PEG) 6000. One hundred microliters of YM overnight culture was transferred to 20ml of the same medium supplemented with 15% or 30% PEG**,** after incubation at 30$^{0}$C with shaking at 250 rpm for four days the bacterial growth was measured.

**2.8 Determination of heavy metals resistance**

Four common heavy metals, i.e., nickel sulphate (Ni); 200 mg/l, cobalt chloride (Co); 100 mg/l, zinc sulphate (Zn); 250 mg/l or ferric chloride (Fe); 250 mg/l were added to YMA medium. Rhizobial strains were inoculated at 30$^{o}$C for four days and their growth were recorded.

**3. Results and Discussion**

Nodulation and nitrogen fixation in legume / *Rhizobium* associations are adversely affected by salinity and drought, which can preclude legume establishment and growth or reduce crop yield **(**Duzan *et al*., 2004 and Gálvez, 2005**)**. It has been documented that nitrogen accumulation by the symbiotic system of soybean, alfalfa and *Glycin javanica* is reduced by salinity (Bernstein & Ogata., 1966 and Wilson, 1970). Commercial strains of *Rhizobium* usually cannot tolerate or function under high levels of osmotic stress caused by salinity or drought (Steinborne & Roughley, 1974, Amarger & Lobreau, 1982 and Green, 1991).

This study aimed to isolate and characterize different indigenous *R. l.* bv. *trifolii* and *R. meliloti* strains from different geographical areas in Egypt, which are usually more adapted than induced inoculants due to the environmental selection. It is also aimed to study the effect of some ecological factors, e.g., salinity, drought or heavy metals, to select the best strain for application in certain ecological area**.**

Under salt stress, plant growth and nitrogen fixation were affected (Jebara *et al.*, 2005; Räsänen & Lindström, 2003 and van Hoorn *et al*., 2001). Nodulation was reduced at salinities above 3 dS $m^{-1}$ (Rao *et al*., 2002). That inhibitory effect of salinity on nodulation was attributed to decrease in rhizobial colonization and shrinkage and lack of root hair (Swaraj & Bishnoi, 1999). Since salinity is often association with drought in semiarid and arid regions, the present study aimed to isolate and identify strains of *R. l.* bv. *trifolii* and *R. meliloti* efficient in salinity and drought tolerance.

**3.1 Collection and isolation of rhizobial strains**

Different indigenous Egyptian *Rhizobium* species and strains were isolated or gathered from research institutes (Table 1).

**3.2 Nodulation efficiency**

Nodulation efficiencies of *R. l.* bv. *trifolii* strains were determined (Table 2). Different nodulation efficiencies were found among the twenty *R. l.* bv. *trifolii* strains tested. The best strains were Rt4 and Rt7 which produced 16 nodules/plant, followed by Rt14, Rt16 and RtH2 producing 15 nodules/plant, then Rt2 and RtH1 strains, which produced 14 nodules/plant. All nodules were of big or medium size except Rt101 and Rt15 strains, which produced only small-sized nodules.

**Table (2): Nodulation efficiency of *Rhizobium leguminosarum* biovar *trifolii* strains**

| Strain | Mean No. of nodules/plant* | Nodule size** | | Strain | Mean No. of nodules/plant* | Nodule size** |
|---|---|---|---|---|---|---|
| Rt101 | 6 | S | | Rt 13 | 10 | B&M |
| Rt nit | 10 | M&S | | Rt 14 | 15 | B&M |
| Rt 1 | 12 | M&S | | Rt 15 | 7 | S |
| Rt 2 | 14 | B&M | | Rt 16 | 15 | B&M |
| Rt 4 | 16 | B&M | | Rt 17 | 13 | M |
| Rt 5 | 8 | M&S | | Rt 18 | 8 | B, M&S |
| Rt 7 | 16 | M | | RtM1 | 12 | B&M |
| Rt 10 | 8 | M&S | | RtM2 | 13 | B&M |
| Rt11b | 11 | B | | RtH 1 | 14 | B&M |
| Rt 12 | 12 | B&M | | RtH 2 | 15 | B&M |

* Five plants were inoculated with each bacterial strain.

** S: Small size, M: Medium size, B: Big size

### 3.3 Determination of salt-tolerant efficiency

Rhizobial growth at different NaCl concentrations are present in Table 3. Among the twenty-one *R. l.* bv. *trifolii* strains tested, about 67% were halotolerant strains, i.e., able to tolerate up to 4% NaCl. The best performance strains were Rt 5, H1, Rt10 and RtM2 (OD $_{420}$ = 1.74, 1.40, 1.39, and 1.38, respectively). Table 3 also showed that five *R. l.* bv. *trifolii* strains were very sensitive to salt stress, where they could not tolerate 1% NaCl.

All *R. meliloti* strains showed good salt-tolerant efficiencies, where all of them could tolerate up to 4% NaCl. The best *R. meliloti* was Rm 1 strain, which could grow up to OD $_{420}$ = 1.48.

Among the twenty-one *R. l.* bv. *trifolii* strains, isolated from different geographical regions of Egypt, 14 strains (about 67%) and all *R. meliloti* (seven strains) were halotolerant, i.e., tolerate up to 4% NaCl. Halotolerant rhizobial strains have been isolated by different authors (Nair *et al*., 1993 and Steinborne and Roughly 1975). The later group had screened 45 Rhizobium strains, isolated from 32 species of legumes inhabiting diverse ecological regimes; they revealed a broad genetic base in their salt tolerance. Accumulation of glutamate and mannosucrose were the osmolytes of the halotolerant rhizobial strains studied by Essendoubi *et al.*, (2007).

### 3.4 Determination of drought-tolerant efficiency

In the present study rhizobial strains growth were measured after there exposure to 15% or 30% PEG 6000, for four days (results are present in Table 4).

Table 4 showed that among the twenty one *R. l.* bv. *trifolii* strains tested the best five drought-tolerant strains were, Rt12; RT7; Rt11b; Rt16 and Rt H2 which grow at 30% PEG 6000 to OD $_{420}$ nm, 1.34; 1.30; 1.23; 1.23 and 1.15, respectively.

All *R. meliloti* strains were less drought-tolerant than *R. l.* bv. *trifolii* strains. Only Rml 530 strain could not tolerate 30% PEG. While the reminders showed different drought-tolerant efficiencies. The best *R. meliloti* strain was Rm 3 which could grow up to OD $_{420}$ = 0.86, followed by Rm 4 strain which gave an OD $_{420}$ = 0.69, then Rm A-2 strain (OD $_{420}$ = 0.44 at 30% PEG).

The growth and persistence of rhizobia and bradyrhizobia in soils are negatively impacted by drought conditions (Cytryn *et al*., 2007). The salt-and drought-tolerant Rhizobium strains obtained in this study are excellent models to study the mechanism(s) of such resistance, and to elucidate the role of genetics of NaCl and drought tolerance.

### 3.5 Heavy metal resistance

Heavy metal resistance patterns of rhizobial strains were characterized using four common heavy metals, i.e., cobalt; zinc; iron and nickel (data are present in Table 5).

**Table (3): Rhizobial strains growth in YM medium supplemented with different NaCl concentrations***

| Strain | NaCl concentration | | | | | |
|---|---|---|---|---|---|---|
| | 0% | 1% | 2% | 3% | 3.5% | 4% |
| Rt 1 | 1.06 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Rt 2 | 1.43 | 1.56 | 1.64 | 1.64 | 0.83 | 0.00 |
| Rt 4 | 2.14 | 0.62 | 0.51 | 0.26 | 0.23 | 0.21 |
| Rt 5 | 1.94 | 2.00 | 2.02 | 1.88 | 1.78 | 1.74 |
| Rt 7 | 1.74 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Rt 10 | 1.90 | 1.89 | 1.05 | 1.01 | 1.22 | 1.39 |
| Rt 11b | 1.94 | 1.97 | 1.96 | 1.79 | 1.59 | 1.32 |
| Rt 12 | 1.55 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Rt 13 | 1.42 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Rt 14 | 1.96 | 1.81 | 0.74 | 0.48 | 0.34 | 0.12 |
| Rt 15 | 1.85 | 1.84 | 1.08 | 0.28 | 0.18 | 0.00 |
| Rt 16 | 1.84 | 1.73 | 1.32 | 1.32 | 1.12 | 1.12 |
| Rt 17 | 1.91 | 1.91 | 1.73 | 1.19 | 1.12 | 1.34 |
| Rt 18 | 1.88 | 0.24 | 0.18 | 0.36 | 0.34 | 0.18 |
| Rt M1 | 1.60 | 1.00 | 1.02 | 1.24 | 1.28 | 1.34 |
| Rt M2 | 1.63 | 1.36 | 1.22 | 1.27 | 1.21 | 1.38 |
| Rt H1 | 1.66 | 1.34 | 1.31 | 1.32 | 1.27 | 1.40 |
| Rt H2 | 1.78 | 0.16 | 0.15 | 0.12 | 0.00 | 0.12 |
| Rt 101 | 1.77 | 1.04 | 1.01 | 1.30 | 1.28 | 1.37 |
| Rt nit | 1.63 | 1.39 | 1.15 | 1.31 | 1.24 | 1.32 |
| ARC100 | 1.54 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Rm1 | 1.36 | 1.69 | 1.82 | 1.72 | 1.62 | 1.48 |
| Rm 2 | 1.84 | 1.69 | 1.63 | 0.70 | 0.64 | 0.41 |
| Rm 3 | 1.57 | 1.81 | 1.63 | 0.76 | 0.74 | 0.44 |
| Rm 4 | 1.55 | 1.70 | 1.71 | 1.66 | 0.87 | 0.58 |
| Rm 5 | 1.86 | 1.77 | 1.75 | 1.45 | 0.80 | 0.54 |
| Rm A-2 | 1.24 | 1.85 | 1.68 | 1.68 | 0.55 | 0.27 |
| Rm L530 | 1.77 | 1.18 | 1.27 | 1.20 | 0.68 | 0.43 |

Rhizobial growth was measured spectrophotometrically at OD $_{420}$ nm, after incubation at 30$^{o}$C with shaking for 4 days.

**Table (4): Rhizobial strains growth in YM medium under different drought stress***

| Strain | YM liquid medium supplemented with PEG 6000 | | |
|---|---|---|---|
| | 0 % | 15 % | 30 % |
| Rt 1 | 1.06 | 0.48 | 0.17 |
| Rt 2 | 1.43 | 1.09 | 0.37 |
| Rt 4 | 2.14 | 0.34 | 0.23 |
| Rt 5 | 1.94 | 1.43 | 0.83 |
| Rt 7 | 1.74 | 1.48 | 1.30 |
| Rt 10 | 1.90 | 0.29 | 0.52 |
| Rt 11b | 1.94 | 1.43 | 1.23 |
| Rt 12 | 1.55 | 1.37 | 1.34 |
| Rt 13 | 1.42 | 0.12 | 0.33 |
| Rt 14 | 1.96 | 1.58 | 0.82 |
| Rt 15 | 1.85 | 1.45 | 0.93 |
| Rt 16 | 1.84 | 1.34 | 1.23 |
| Rt 17 | 1.91 | 0.39 | 0.15 |
| Rt 18 | 1.88 | 0.57 | 0.23 |
| Rt M1 | 1.60 | 0.36 | 0.18 |
| Rt M2 | 1.63 | 0.28 | 0.16 |
| Rt H1 | 1.65 | 0.36 | 0.16 |
| Rt H2 | 1.78 | 1.25 | 1.15 |
| Rt 101 | 1.77 | 0.34 | 0.21 |
| Rt nit | 1.63 | 0.30 | 0.12 |
| ARC 100 | 1.54 | 0.58 | 0.13 |
| Rm1 | 1.36 | 1.05 | 0.22 |
| Rm 2 | 1.14 | 1.25 | 0.41 |
| Rm 3 | 1.57 | 1.41 | 0.86 |
| Rm 4 | 1.55 | 1.38 | 0.69 |
| Rm 5 | 1.86 | 0.83 | 0.39 |
| Rm A-2 | 1.24 | 1.01 | 0.44 |
| Rm L 530 | 1.77 | 0.46 | 0.10 |

* Rhizobial growth was measured spectrophotometrically at $OD_{420}$ nm, after incubation at 30°C with shaking for 4 days.

**Table (5): Heavy metals resistance patterns of rhizobial strains***

| Strains | Rhizobial growth | | | | |
|---|---|---|---|---|---|
| | Co | Zn | Fe | Ni | Control |
| Rt 1 | + | + | + | + | ++ |
| Rt 2 | + | - | + | + | ++ |
| Rt 4 | - | + | + | + | ++ |
| Rt 5 | + | + | + | + | ++ |
| Rt 7 | + | - | + | + | ++ |
| Rt 10 | + | + | + | + | ++ |
| Rt 11b | + | + | + | + | ++ |
| Rt 12 | - | - | + | - | ++ |
| Rt 13 | - | + | + | + | ++ |
| Rt 14 | - | + | ++ | ++ | ++ |
| Rt 15 | - | + | ++ | + | ++ |
| Rt 16 | + | + | + | + | ++ |
| Rt 17 | ++ | ++ | + | + | ++ |
| Rt 18 | - | + | + | + | ++ |
| Rt M1 | + | + | + | + | ++ |
| Rt M2 | + | + | + | + | ++ |
| Rt H1 | - | + | - | - | ++ |
| Rt H2 | - | - | + | + | ++ |
| Rt 101 | - | - | + | - | ++ |
| Rt nit | - | - | + | + | ++ |
| ARC 100 | - | + | + | + | ++ |
| Rm1 | + | + | + | + | ++ |
| Rm 2 | + | + | - | - | ++ |
| Rm 3 | - | + | + | + | ++ |
| Rm 4 | - | - | - | + | ++ |
| Rm 5 | - | - | + | + | ++ |
| Rm A-2 | + | - | - | + | ++ |
| Rm L 530 | + | - | + | - | ++ |

* YMA medium was supplemented with nickel sulphate (Ni); 200 mg/l, cobalt chloride (Co); 100 mg/l, zinc sulphate (Zn); 250 mg/l or ferric chloride (Fe); 250 mg/l. Bacterial growth was detected after four days incubation at 30°C. -: No growth; +: Weak growth; ++: Good growth.

About 86% of *R. l.* bv. *trifolii* had multiple-metal resistant. Eight strains were resistant to all four heavy metal, and eight strains were resistant to three heavy metals and two strains were resistant to two heavy metals. Iron resistance was the most heavy metal resistant (~ 95%) followed by nickel resistant (~ 86%), zinc resistant (~ 71%) and cobalt resistant (~ 48%).

Among the seven *R. meliloti*, Rm1 strain was resistant to the four heavy metals and Rm3 strain was resistance to three heavy metals. The most heavy metal resistance found among *R. meliloti* strains was nickel resistance (~ 71%) followed by iron or cobalt resistance (~ 57% each) then zinc resistance (~ 43%).

Different heavy metals, e.g., cadmium, cobalt, and nickel, have a great influence on nodulation (Fyson *et al.*, 1984 and Ibekwe *et al.*, 1995). The present studies indicated that rhizobial strains can be screened for better salt and drought tolerance and are in agreement with Diouf (2007) and Essendoubi *et al.* (2007), who suggested the adaptability of natural rhizobial populations to major ecological environmental stress and their ability to establish symbiotic associations within these soil environments.

## 4 Conclusions

The salt tolerant, drought tolerant, and heavy metals resistance patterns found among the indigenous rhizobial strains are reflecting the environmental stresses pressure predominant in their locations and are very good examples of the importance of using efficient – indigenous rhizbial strain for plant inoculation in each specific area. *Rhizobium* with the genetic potential for increased tolerance to drought and / or salinity could enhance production of food and forage in legume in semiarid and arid regions of the world.

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