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*Full Length Research Paper*

# Preliminary characterization of *Rhizobium* strains isolated from chickpea nodules

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**A total of 28 *Rhizobium* strains from chickpea nodules were characterized on the basis of morphological, cultural and biochemical characteristics. Most strains produced abundant extracellular polysaccharides, were tolerant to 0.5 M NaCl (53%) and a temperature of 40°C (75%). The majority of the strains showed an intrinsic resistance to the antibiotics (µg ml$^{-1}$) streptomycin (100), kanamycin (50), erytromycin (30), chloramphenicol (200) and penicilin (25). *In vitro* antibiosis assays indicated that *Rhizobium* strains from chickpea nodules exercised an antagonism against *Ascocyhta rabiei* the agent of ascocyhta blight disease of chickpea.**

**Key words:** *Rhizobium*, chickpea (*Cicer arietinum* L.), preliminary characterization.

## INTRODUCTION

Chickpea (*Cicer arietinum* L.) is a major source of protein for human consumption, it provides high quality crop residues for animal feed and helps to maintain soil fertility through biological nitrogen fixation (Herridge et al., 1995; Siddiqi and Mahmood, 2001; Kantar et al., 2007). Chickpea is an important legume crop in Turkey. In Turkey, the crop occupies 650.000 ha of area and contributes to about 18% of the population's protein consumption (Anonymous, 2004). Since most Turkey soils are nitrogen deficient, $N_2$ fixing *Rhizobium* bacteria could increase yield at a low cost and preserve water resource from pollution by nitrates (Haktanır, 1999). However, poor nodulation and the lack of inoculation in field experiments has frequently been reported worldwide, raising doubts about the efficiency of crop inoculation (Graham, 1981; Hardarson, 1993; Somasegara and Hoben, 1994). The lack of response to inoculation can be attributed to instrinsic characteristics of both the host plant and the bacteria as well as the great sensitivity of the symbiosis to environmental stresses such as high temperatures soil dryness and low soil fertility (Freiberg et al., 1997; Soussi et al., 1998; Hungria and Vargas, 2000).

Chickpea in symbiosis with an efficient strain of *Rhizobium ciceri* constitutes an important compenent of crop systems (Freiberg et al., 1997), being capable of supplying between 80 and 120 kg N ha$^{-1}$ to the soil (Herridge et al., 1995).

In the last two years, while isolating bacteria from chickpea nodules collected in different Eskişehir areas in Turkey, our objectives were to study the diversity of these bacteria by using several physiological and biochemical tests, and to select isolates adapted to the climatic conditions in Eskişehir regions.

## MATERIALS AND METHODS

### Isolation

Strains of *Rhizobium* sp. were obtained from root nodules of field grown chickpea plants collected in Eskişehir, Turkey. Root nodules of chickpea were located on the roots and a big with a pink color. Root nodules were sterilized in 0.1% (w/v) sodium hypochloride for 5 min immersed in 95% (v/v) ethanol for 10 s, and then washed six times with sterile rods and streaked onto yeast extract mannitol agar (YEM) agar containing 0.0025% (w/v) congo red (Vincent, 1970). After an inoculation of 3 day at 30°C, single colonies were selected and restreaked on YEM for purify.

### Colony morphology

An inital inoculum of $10^8$ cell ml$^{-1}$ was prepared in YEM broth with a pH initially adjusted at 6.8. Colony morphology (colour, mucoidly,

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transparency, borders, from) was evaluated by streaking a loop of the initial inoculum on YEM agar and allowing the bacteria to grow in the dark at 30°C for 3, 5 and 7 days (Vincent, 1970; Sinclair and Eaglesham, 1984).

#### Phenotypic features

All tests were carried out in triplicate. Before inoculation, strains were grown YEM broth to log phase ($10^8$ cell $ml^{-1}$). When test plates were used, inoculation was performed with 30 µl of these cultures. The results were scored after 5 d incubation at 30°C ( Hungria et al., 2001).

#### Sodium chloride and pH tolerance

This was determined on YEM broth containing 0, 0.1, 0.3 and 0.5 M NaCl (El Sheik and Wood, 1989) or in which the pH was adjusted to 3, 3.5, 4, 4.5, 5, 6, 7, 8 and 9 (Rodriguez-Navarro et al., 2000).

#### Temperature tolerance

Growth on YEM broth at 4, 10, 15, 20, 25, 30, 37, 40 and 42°C was used to determine different temperature tolerance.

#### Carbon and nitrogen assimilation

For carbon and nitrogen assimilation studies, sugars and organic acids were prepared as 10% (w/v) solutions which were sterilized by autoclaving or by 0.22 µm filtration and added to the basal salts broth medium to a final concentration of 1% (w/v). After 5 days incubation growth scores were made visually with reference to control plates with carbohydrate omitted (Hungria et al., 2001).

For the evaluation of carbon sources, the medium was added casein, dulcitol, citrate, D(-) fructose, D(+) galactose, D(+) glucose, D(-) mannitol, sucrose, strach, succinate, αL-rhamnose and malate. For the the evaluation of nitrogen sources were added L-asparagine, L-glutamine, L-tryptophan, thymine and glycine (Hungria et al., 2001).

#### Instrinsic resistance to antibiotics and heavy metals

All strains were tested for heavy metals and instrinsic resistance to antibiotics essentially as described by Küçük et al. (2006). The following antibiotics were tested (at the concentrations in parentheses): streptomycin (str, 100 µg $ml^{-1}$), kanamycin (kan, 50 µg $ml^{-1}$), erythromycin (ery, 30 µg $ml^{-1}$), chloramphenicol (chl, 200 µg $ml^{-1}$), penicilin (pen, 25 µg $ml^{-1}$), $CuCl_2.2H_2O$ (Cu, 100 µg $ml^{-1}$), $HgCl_2$ (Hg, 5 µg $ml^{-1}$), $CdCl_2.2H_2O$ (Cd, 20 µg $ml^{-1}$), $ZnSO_4.2H_2O$ (Zn, 25 µg $ml^{-1}$) (Josey et al., 1979; İçgen et al., 2002).

#### A defined medium used for vitamin nutrition studies

A loopful of agar slopes of the strains assayed for vitamin production was transferred to 250 ml flasks containing Difco Vitamin $B_{12}$ Assay Medium, amended with 2 mmol $l^{-1}$ Ca-pantothenate, biotin and thiamine (Laranjo et al., 2004). Growth responses to the presence of vitamins were determined by the method used for the carbon assimilation study (Josey et al., 1979; Soussi et al., 2001).

#### *In vitro* antibiosis assay

The overlay plate technique (Oresnick et al., 1999) was used to check the in vitro inhibition of *Ascocyhta rabiei* by the *Rhizobium* strains. A satured culture ($10^7$ spore $ml^{-1}$) of the *A. rabiei* (recovered from chickpea) grown in Potato Dextrose Broth (Difco) was diluted in YMA medium. 5 µl of a culture of the *Rhizobium* strains was inoculated onto the YMA medium surface. Inhibition zones were measured after 5 days of incubation at 30°C.

## RESULTS AND DISCUSSION

The 28 new strains from naturally occuring root nodules of chickpea plants growing in Eskişehir, Turkey were characterized as phenotypic features. All retrieved strains were Gram negative, moderately motile. Some physiological and biochemical properties of strains are presented in Table 1. The strains under investigation are attributed to the genus *Rhizobium.*

Differences between strains were verified using some morphological parameters, but a high production of mucus was verified in 85% of the strains, 42% were white and 78% opaque (data not shown).

All strains grew in YEM medium with pH values of 5.0 and 8.0, but differences were detected at pH values of 3.0 and 9.0 (Table 1), as observed for other *R. ciceri* strains (Hungria et al., 2001).

The growth responses of 28 strains to NaCl at 0.1, 0.3, 0.5 M are shown in Table 1. Although salitinity is usually not a problem in Eskişehir soils in Turkey, some of the strains (N1, N2, N4, N5, N7, N8, N9, N13, N18, N19, N21, N22, N26, N27 and N28) were tolerant to 0.5 M NaCl. The 14 strains which grew as well with 0.1 and 0.3 M NaCl as on control plates all had a wet colony. All strains grew in YEM medium of 20, 25, 30 and 37°C .The majority of the strains (N1, N2, N5, N6, N8, N11, N18, N19, N21, N24, N28) tolerated high temperatures, growing at 40°C of the other halotolerant *Rhizobium* strains which have been isolated, from leguminous plant (Somasegaran and Hoben, 1994; Hungria et al., 2001; Soussi et al., 2001). A clear example is temperature tolerance in Eskişehir the climate is drier. The 12 strains were examined for the ability to grow at 42°C on YEM broth (Table 1).

Strains were produced copious exopolysaccharide slime on YEM medium. These strains, utilize a wide range of carbohydrates and salts of organic acids as carbon sources which are also diagnostic for root nodule bacteria (Sa et al., 1993; Rodriguez-Navarro et al., 2000; Zerhari et al., 2000). Utilizasation of different compounds by strains, as sole carbon and nitrogen sources is one of the most useful traits for their differentiation (Mpepereki et al., 1997; Hungria et al., 2001). In our experiments, carbon sources were tested and the following starch, casein and L-lysine were not utilized by any of the *R. cicer* strains. Bacteria were not able to use citrate and had a weak growth with dulcitol, as observed before for one or both carbon sources, with other *R. cicer* strains (Vincent, 1970).

All these strains used as sole carbon sources such substances as D (-) fructose, D (+) galactose, D (+) glo-

**Table 1.** pH, NaCl and temperature tolerance in chickpea rhizobia strains.

| *Rhizobium* strain | pH | | NaCl (M) | | | Temperature (°C) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 4 pH | 9 pH | 0.1 | 0.3 | 0.5 | 10 | 15 | 40 | 42 |
| N1 | - | + | + | + | + | + | + | + | + |
| N2 | - | + | + | + | + | + | + | + | + |
| N3 | + | + | + | - | - | + | + | + | - |
| N4 | + | + | + | + | + | + (w) | + | + (w) | + (w) |
| N5 | + | - | + | + | + | + | + | + | + |
| N6 | - | - | + | + | + | + | + | + | + |
| N7 | - | + | - | - | - | + (w) | + | + (w) | + (w) |
| N8 | - | + | + | + | + | + | + | + | + |
| N9 | - | + | + | + | + | - | + | + | - |
| N10 | - | + | + | - | + | + | + | + | + |
| N11 | - | - | + | - | - | + | + | + | + |
| N12 | + | + | + | - | - | + | + (w) | + (w) | - |
| N13 | + | + | + | + | - | + | + | + (w) | - |
| N14 | + | + | + | - | + | + | + | + (w) | - |
| N15 | - | - | + | + | - | + | + | + (w) | - |
| N16 | - | - | + | + | - | + | + (w) | + | - |
| N17 | + | - | + | + | - | + | + | + | + (w) |
| N18 | + | - | + | + | + | + | + | + | + |
| N19 | + | + | + | + | + | + | + | + | + |
| N20 | - | + | + | - | - | + (w) | + | + | - |
| N21 | + | + | + | + | + | + (w) | + | + | + |
| N22 | + | + | + | + | + | + | + | + | + (w) |
| N23 | - | + | + | - | - | - | + | + (w) | + (w) |
| N24 | - | + | + | - | - | + | + | + | + |
| N25 | - | + | + | - | - | + | + | + | - |
| N26 | - | + | + | + | + | + | + | + | + |
| N27 | + | + | + | + | + | + | + | + | - |
| N28 | + | + | + | - | + | + | + | + | + |

w: Weak growth; +: good growth; -: no growth.

glucose, D (-) mannitol, sucrose, succinate, rhamnose and malate. We also found that glycine was a beter nitrogen source for our strains than the other aminoacids studied; however all of them were assimilated.

Twenty eight strains with approximately equal numbers were examined for growth responses to thiamine and pantothenate. The nine strains (N1, N4, N5, N9, N11, N16, N17, N20, N27) showed positive responses to pantothenate. Negative effects of biotin have been reported before (Laranjo et al., 2004).

The antibiotic resistance of the isolated strains showed a high a level of resistance against streptomycin, erythromycin, kanamycin, penicilin and chloramphenicol (Table 2). Different autors have reported effect of antibiotics on *Rhizobium* bacteria (Sa et al., 1993; Rodriguez-Navarro et al., 2000; Zerhari et al., 2000; Hungria et al., 2001). All of the strains of *Rhizobium* sp. were tested for their tolerance to the heavy metals (Table 2). 89.2% of the strains isolated showed resistance to three metals (Zn, Cr, Ni). Isolates tested five showed different heavy metals and antibiotics in their different combinations (Table 2). 3.5% of the strains showed resistance to five antibiotics in different combinations whereas 1% of the strains showed resistance to tested antibiotic and metals combinations.

In genetic studies, heavy metal resistance traits should be extremely valuable as positive selection markers. The high levels of Zn, Cu and Cd (Sinclair and Eaglesham, 1984; Hungria and Vargas, 2000; Zerhari et al., 2000) suggest that these metals could be used as selective agents for some *Rhizobium* strains. Characteristics relating, to origin are probably associated with adaptations to specific environmental pressures. *In vitro* antibiosis assays showed that *A.rabiei* was sensitive to the *Rhizobium* strains used (inhibition zone > 10 mm). Previous studies showed that the introduction of antibiotic producing strains into soil led to a change in bacterial diversity (Josey et al., 1979; Freiberg et al., 1997; Oresnick et al., 1999). The introduction of a strain of *Rhizobium etli* producing trifolitoxin into soil strongly re-

**Table 2.** Percent resistance of *Rhizobium* sp. strains against antibiotics and heavy metals.

| Resistantance strains | Pattern of resistance of antibiotics and heavy metals |
|---|---|
| 15 (53.5 %) | Str, Kan |
| 15 (53.5 %) | Str, Ery |
| 19 (67.8 %) | Str, Ery, Kan |
| 22 (78.5 %) | Str, Ery, Kan, Chl |
| 25 (89.2%) | Str, Ery, Kan, Chl, Pen |
| 2 ( 7.1 %) | Cu, Cd, Zn |
| 5 (17.8%) | Zn, Cr, Ni |
| 2 (7.1 %) | Zn, Hg, Cu |
| 4 (14.2 %) | Zn, Cd, Ni |
| 3 (3.5 %) | Cu, Hg, Zn, Cd |
| 2 ( 7.1 %) | Cu, Hg, Cd, Ni, Cr |
| 2 (7.1 %) | Str, Kan, Cu, Cd, Zn |
| 2 (7.1%) | Str, Kan, Cu, Hg, Cd |
| 1 (3.5 %) | Str, Ery, Kan, Hg, Ni |
| 1 (3.5 %) | Str, Kan, Cr, Zn, Ni, Cu |

Total number of strain: 28.
Values in parentheses indicates the percentage of the strain %.

duced the natural diversity of indigenous bacteria, due to the sensitive bacteria being excluded (Freiberg et al., 1997). *In vitro* antibiosis assays were conducted to check whether these *Rhizobium* chickpea strains acted on the disease of the *A.rabiei*. The results showed that the *Rhizobium* strains used exercised a strong inhibitory effect on the *A.rabiei*. Consequently the antagonism exercised by these *Rhizobium* strains would have a enhanced extent. Further investigations need to be carried out on the effect of biocontrol of *A.rabiei* on chickpea.

This paper describes, for the first time the isolation of chickpea strains from chickpea nodules in Eskişehir in Turkey. Carbon and nitrogen source utilization capacity may serve as potential strains for the bioremediation of pollutions in soil habitats. Some strains from this study have proved promising results under field conditions with chickpea varieties.

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