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Evaluation of *Rhizobium* isolates from *Melilotus officinalis* nodules at various stress conditions

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Abstract. Pasture are an important part of agricultural systems and to increase their productivity and quality persistent forage legumes are needed. *Melilotus officinalis* is an important forage legume. *Rhizobium* bacteria can form nitrogen fixing nodules on legume roots. In this study, we phenotypically characterized 17 *Rhizobium* isolates from *Melilotus officinalis* nodules. About 47% of isolates were resistant to pH 8, 27% were resistant to 40°C temperature and 17.6 % were resistant to 2-2.5 % NaCl concentrations.

Key words. *Melilotus officinalis* – *Rhizobium* – Isolate.

Résumé. *Les pâturages sont une partie importante des systèmes agricoles, et pour accroître leur productivité et leur qualité, des légumineuses fourragères persistants sont nécessaires.* Melilotus officinalis *est une espèce fourragère légumineuse. Les bactéries* Rhizobium *fixatrices d'azote forment des nodules sur les racines légumineuses. Dans cette étude, nous avons caractérisé phénotypiquement 17* Rhizobium *isolés de nodules de* Melilotus officinalis. *Environ 47% des isolats étaient résistants à un pH de 8, 27% étaient résistants à températures de 40°C et 17,6% étaient résistants à des concentrations de 2-2,5% de NaCl.*

Mots-clés. Melilotus officinalis – Rhizobium – *Isolat.*

I – Introduction

Rhizobium species are soil bacteria, which display a symbiotic interaction with specific legume hosts and most of these are sensitive to fluctuations of environment factors in the rhizosphere and effect the growth and productivity of the whole plant (Hungria and Vargas, 2000). Among several environmental conditions which are limiting factor such as salinity, temperature and pH are probably the most problematic (Hungria and Vargas, 2000).

The effects of varying pH levels on the growth of *Rhizobium* bacteria have been recorded by some authors (Ali *et al*., 2009; Graham *et al.;* 1994; Zerhari *et al*.; 2000). Hashem *et al*. (1998) and Lloret *et al.* (1995) have shown that changes in osmotic potential exerted by salt concentration alter the structure rhizobial cell in response to salt stress. Rhizobial isolates vary in their tolerance to major environmental factors (Rodrigues *et al.,* 2006). This study aimed to isolate and collect *Rhizobium* isolates from *Melilotus officinalis* nodules and to characterize their performance at various stress conditions.

II – Materials and methods

Rhizobium isolates were obtained from the root nodules of *Melilotus officinalis* growing at Şanlıurfa, Turkey. Nodules were disinfected, crushed in a small amount of saline (0.85 % NaCl) suspension and streaked on yeast extract mannitol (YEM) agar plates. The cultures were streaked of plates onto YEM agar for purification and isolation. Cell shape was determined by microscopy (Holt *et al*., 1994).

Stress tolerance. Aliquots of 0.1ml cultures were inoculated into 15 ml YEM broth and incubated at 25, 30, 35, 40 and 45ºC in a shaker incubator (200 rpm) for 48 h. The tolerance to salinity was tested by inoculating the cultures into YEM broth containing NaCl (w/v) (0.1, 0.5, 1, 1.5, 2, 2.5 and 3%). To determine the pH tolerance, the cultures were grown in YEM broth and pH was adjusted 3.5, 4, 4.5, 5, 8, 9 and 10 and incubated at 28°C (Zerhari *et al.,* 2000; Graham *et al.*, 1994).

Hydrolysis of urea. Isolates were inoculated on a YEM agar containing yeast extract instead of urea and 0.012% phenol red as nitrogen source (Zerhari *et al.,* 2000).

Carbohydrate assimilation. The filter sterilized solutions of the carbon sources (10 % (w/v)) (glucose mannitol, galactose, sucrose, fructose, inositol, xylose) were added to YEM broth (Holt *et al.,* 1994).

The nodulation assays. The nodulation assays were performed in sterilized soil. Seeds of the *Melilotus officinalis* were surface sterilized and inoculated by adding 1 ml of isolate. Plants were cultivated in a controlled growth chamber with 15 h of light at 25 °C. Fifty days after the start of treatment, plants were harvested and separated into roots and shoots and nodulation measured.

III – Results and discussion

Seventeen isolates isolated from root nodule of *Melilotus officinalis*. Seventeen isolates showed highest growth at 35°C, five isolates (Mo1, Mo2, Mo3, Mo6, Mo7) at 40°C and two (Mo6 and Mo7) at 45 °C (Table 1). All of the isolates were catalase and urease positive. Temperatures of semi-arid soils usually exceed 40 °C at 5 cm. Although the high temperature tolerance of *Rhizobium* isolates was not correlated with nodulation capacity (Hungria and Vargas, 2000), in general , the tolerance to high temperatures among the species of *Rhizobium* has been recognized as criteria for the selection of local isolates (Hungria and Vargas, 2000; Rodrigues *et al.,* 2006). On the other hand, Meghvansi (2006) reported that the growth and survival of *Rhizobium* bacteria in soil are adversely affected by high soil temperatures. Isolate adaptation to high temperature has been reported and it was suggested that *Rhizobium* isolates from hot dry areas are more temperature and desiccation tolerant than isolates from cooler, more humid regions and that temperature and desiccation tolerance may be related to geographical origion (Hansen, 1994). Isolates were able to catabolize a great variety of carbon sources (Table 1). All of isolates also utilized a wide variety of carbohydrates such as glucose, mannitol, galactose, fructose, inositol, sucrose, xylose. Growth in carbohydrate media was accompanied by copious extracellular polysaccharide slime. Hansen (1994) has reported the importance of exopolysaccharide in nodulation on the growth of *Phaseolus vulgaris*.

The growth of isolates at various pH treatments was different (Table 1). The optimum growth temperature is 25 to 35°C (Table 1); most isolates grow at 35°C. Five were tolerant of 40°C. For most *Rhizobium* isolates the temperature of growth in culture ranges from 28°C to31°C, with many unable to grow below 10° C or at 37°C (Hungria and Vargas, 2000; Zerhari *et al.,* 2000). The most of cowpea root nodules isolates isolated from the hot dry environment of the Sahel savannah of west Africa grew at 40°C (Eaglesham and Ayanaba, 1984). None of the isolates grew at pH 3.5, 4.0, 4.5 and 10.0. Mo3, Mo6, Mo8, Mo11, Mo12, Mo13, Mo14, Mo15 and Mo16 isolates did not growth at pH 8. Similar observations were recorded by Rodrigues *et al.* (2006) and Graham *et al.* (1994). It is probable that an organism challenged at extreme pH will be less tolerant to toxic substances than under at pH regime that is close to the optimum conditions. Graham *et al.,* 1994 and Zerhari *et al.,* 2000, who recorded that the optimum pH of the growth of *Rhizobium* isolates is between 6 and 7 pH. Lipopolysaccharide are known to play a key role in the pH tolerance of *Rhizobium* species (Zahran *et al.,* 1994). Eight isolates (Mo1, Mo2, Mo4, Mo5, Mo7, Mo9, Mo10 and Mo17) grew at pH 8.0. The isolate Mo1 growth at pH 9.0. The effects of the salt concentrations were variable depending on the salt concentrations used and the isolates tested (Table 2). Generaly, *Rhizobium* isolates are more tolerant of osmotic stress than their leguminous host (Hashem *et al.*, 1991). The different *Rhizobium* species or isolates vary in their sensitivity to salt (Barnet and Catt, 1991). Although salinity is usually not a problem soils, salt concentration of 2% and 2.5% (w/v) NaCl were tolerated by a few isolates (Mo1, Mo3, Mo4) of *Rhizobium* (Table 2).

Table 1. Utilization of carbohydrate sources, growth at pH and temperature of isolates

Isolate	Utilization of carbohydrate sources†	Growth at pH							Growth at °C				
		3.5	4.0	4.5	5.0	8.0	9.0	10.0	25	30	35	40	45
Mo1	+	-	-	-	-	+	+	-	+	+	+	+	-
Mo2	+	-	-	-	-	+	-	-	+	+	+	+	-
Mo3	+	-	-	-	-	-	-	-	+	+	+	+	-
Mo4	+	-	-	-	-	+	-	-	+	+	+	-	-
Mo5	+	-	-	-	-	+	-	-	+	+	+	-	-
Mo6	+	-	-	-	-	-	-	-	+	+	+	+	+
Mo7	+	-	-	-	-	+	-	-	+	+	+	+	+
Mo8	+	-	-	-	-	-	-	-	+	+	+	-	-
Mo9	+	-	-	-	-	+	-	-	+	+	+	-	-
Mo10	+	-	-	-	-	+	-	-	+	+	+	-	-
Mo11	+	-	-	-	-	-	-	-	+	+	+	-	-
Mo12	+	-	-	-	-	-	-	-	+	+	+	-	-
Mo13	+	-	-	-	-	-	-	-	+	+	+	-	-
Mo14	+	-	-	-	-	-	-	-	+	+	+	-	-
Mo15	+	-	-	-	-	-	-	-	+	+	+	-	-
Mo16	+	-	-	-	-	-	-	-	+	+	+	-	-
Mo17	+	-	-	-	-	+	-	-	+	+	+	-	-

†Glucose, galactose, fructose, xylose, mannitol, sucrose, inositol.

Table 2. Some characteristics of isolates

Isolate	Catalase	NaCl tolerance %							Mobility	Hydrolysis of urea
		0.1	0.5	1	1.5	2	2.5	3		
Mo1	+	+	+	+	+	+	+	-	+	+
Mo2	+	+	+	+	+	-	-	-	+	+
Mo3	+	+	+	+	+	+	+	-	+	+
Mo4	+	+	+	+	+	+	+	-	+	+
Mo5	+	+	+	+	+	-	-	-	+	+
Mo6	+	+	+	+	+	-	-	-	+	+
Mo7	+	+	+	+	+	-	-	-	+	+
Mo8	+	+	+	+	+	-	-	-	+	+
Mo9	+	+	+	+	+	-	-	-	+	+
Mo10	+	+	+	+	+	-	-	-	+	+
Mo11	+	+	+	+	+	-	-	-	+	+
Mo12	+	+	+	+	+	-	-	-	+	+
Mo13	+	+	+	+	+	-	-	-	+	+
Mo14	+	+	+	+	+	-	-	-	+	+
Mo15	+	+	+	+	+	-	-	-	+	+
Mo16	+	+	+	+	+	-	-	-	+	+
Mo17	+	+	+	+	+	-	-	-	+	+

These results can be explained in this way; the growth inactivated by increased NaCl concentration of same isolates and this may be consistent with the life cycle characteristics of the tested isolates. All of the isolates grewth at 0.1%, 0.5, 1.0, 1.5% (w/v) NaCl (Table 2). Isolates were obtained from root nodules of *Melilotus officinalis*, were gram negative, non-spore forming rod-shaped bacteria. After 3 day the colonies formed on YEM agar plates were circular, convex. All of the isolates were motile in agar (0.3 %). *Rhizobium* isolates capable of growing at NaCl concentration of up to 0.500 M have been isolated from a *Melilotus* plants (Lloret *et al.,* 1995). In this study all the isolates of *Rhizobium* showed confluent growth at a salt concentration of 0.1%. Salt concentrations of 2-2.5% were tolerated by only a three isolates of *Rhizobium*, while others were sensitive (Table 2). The results of Ali *et al.* (2009) agree with the above finding. They reported that root nodule isolates of *Leucaena leucocephala* could tolerate NaCl concentration upto 4.5 %. In our study, 3 % of salt concentration was found to be inhibitory to all of the isolates.

The symbiotic performance of isolate Mo4 was determined with *Melilotus officinalis*. Results in Table 3 summarize the nodulation parameters of isolate with *Melilotus officinalis*. Shoot dry weights were greater with isolate Mo4 than in the non-inoculated and non-N fertilized control. However, the non-inoculated N-fertilized control resulted in greater shoot weight then all other treatments.

Table 3. Nodulation and shoot weight of selected *Rhizobium* isolate with *Melilotus officinalis*

Treatments	No. of nodules per plant	FW of shoot (g plant^{-1})
Control	0	3.2
Inoculated with isolate	5	4.6
Isolate and N	2	5.2
N fertilized	0	4.3

IV – Conclusions

The phenotypic studies were necessary for the characterization and selection of isolates provided information about their genetic diversity and adapted to climatic conditions. Rhizobium isolates with tolerance stress factors for increased tolerance to temperature on salinity could enhance production of forage in legume in semi-arid regions. Further studies are in progress to characterize plasmids. Understanding to contribution of plasmids to the overall symbiotic potential of isolates will allow the development of improved isolates better adapted to the plant, soil and climate conditions in Şanlıurfa, Turkey.

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