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Characterization of *Rhizobium* Sp. Isolated from Bean

Çiğdem KÜÇÜK[1], Merih KIVANÇ[2], Engin KINACI[3]

[1]Osmangazi University, Institute of Natural and Applied Sciences, Eskişehir - TURKEY

[2]Department of Biology, Faculty of Science, Anadolu University 26470 Eskişehir - TURKEY

[3]Osmangazi University, Faculty of Agriculture, Eskişehir - TURKEY

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Abstract: Thirty nodule isolates from bean (*Phaseolus vulgaris* L.) grown in Eskişehir were studied for their physiological and biochemical characteristics. Most isolates produced abundant extracellular polysaccharides, tolerated high salt concentration (5% NaCl), grew at a temperature of 42 °C, and synthesized melanin. They were able to grow at pHs ranging from 3.5 to 9.0. The majority of the isolates showed an intrinsic resistance to the antibiotics chloramphenicol (20 and 50 µg ml^{-1}), erythromycin (30 µg ml^{-1}), kanamycin (10 µg ml^{-1}), and streptomycin (40, 80, and 100 µg ml^{-1}).

Plasmid DNA profiles of the isolates were identified and it was determined that 2 isolates contained plasmid DNA from 1 to 3.

Key Words: *Rhizobium* sp., bean (*Phaseolus vulgaris* L.), isolation, isolate

Fasülyeden İzole Edilen *Rhizobium* sp.'nin Karakterizasyonu

Özet: Eskişehir'de yetiştirilen fasulyelerden elde edilen otuz nodül izolatının fizyolojik ve biyokimyasal özellikleri çalışılmıştır. İzolatların çoğu ekstrasellüler polisakkarit üretmiş, yüksek tuz konsantrasyonuna (% 5 NaCl) toleranslı bulunmuş, 42 °C'lik sıcaklıkta gelişmiş ve melanin sentezlemiştir. İzolatlar 3,5'den 9'a kadar değişen pH'da gelişebilmiştir. İzolatların önemli bir kısmı kloramfenikol (20 ve 50 µg ml^{-1}), eritromisin (30 µg ml^{-1}), kanamisin (10 µg ml^{-1}) ve streptomisin (40, 80 ve 100 µg ml^{-1}) antibiyotiklerine dirençlilik göstermiştir.

İzolatların plasmid DNA profileri incelenmiş ve iki izolatın 1 ile 3 arasında plasmid DNA içerdiği belirlenmiştir.

Anahtar Sözcükler: *Rhizobium* sp., Fasulye (*Phaseolus vulgaris* L.), İzolasyon, İzolat

Introduction

Dry bean (*Phaseolus vulgaris* L.), herein referred to simply as bean, is grown throughout Anatolia, where it is an important source of protein for human consumption (1). Bean is considered a lower N_2 fixer pulse in comparison to other grain legumes (2-5). Sparse nodulation or a lack of response to inoculation in field experiments has been frequently reported worldwide, raising doubts about the benefits of inoculation (7). This fact could be related to the promiscuity observed in bean (8), or to other limiting nodulation factors, like the high rate of nitrogen fertilizer used in intensive agriculture, which is particularly detrimental to bean (9).

In Eskişehir, the crop occupies 1.312 ha and contributes about 25% of the population's protein consumption; however, the yield is low, averaging 1379 kg ha^{-1}, primarily due to poor cropping practices, such as inefficient application of nitrogen fertilizers (6). Since most Eskişehir agricultural soils are nitrogen deficient, N_2 fixing with *Rhizobium* bacteria could be a low-cost method to increase yield and protect water resources from nitrate pollution (6).

The bacteria able to nodulate and establish an effective symbiosis with bean are fast-growing rhizobia first isolated from Brazil (4), initially classified as *Rhizobium phaseoli* (2). Today, they are classified as *Rhizobium trifolii*, *R. etli*, and *R. phaseoli* (4).

The presence of *Rhizobium* in bean nodules in Eskişehir has not been previously reported. In this work, we investigated and characterized the diversity of 30 *Rhizobium* isolates from root nodules of bean grown in Eskişehir.

Materials and Methods

Isolation of *Rhizobium* sp.

The *Rhizobium* isolates were obtained from root nodules of bean. Nodules located on the roots were spherical (2-4 mm in diameter) and pink. Root nodules were sterilized in 95% (v/v) ethanol for 10 s and then washed 7 times with sterile distilled water. Individual nodules were crushed with sterile glass rods and streaked onto yeast extract mannitol (YEM) agar containing 0.0025% (w/v) Congo red. After incubation for 2-3 days at 30 °C, single colonies were selected and restreaked on YEM agar for purity (2).

Growth on Yeast Extract Broth

Growth in YEM broth incubated at 250 rev min^{-1} was determined by measuring the optical density at 600 nm every 2 h. The generation time was calculated from the logarithmic phase of growth.

Phenotypic Characterization

All tests were carried out in triplicate. Before inoculation, isolates were grown on YEM liquid medium to log phase (10^8 cell min^{-1}). When test plates were used, inoculation was performed with 30 µl of these cultures. The results were scored after 5 days of incubation at 30 °C.

Colony Morphology

The colony morphology of isolates was examined on both YEM agar plates. After an incubation of 2-3 days at 30 °C, individual colonies were characterized based on their color, shape, Gram stain reaction, and capacity to produce exopolysaccharide gum (2).

Sodium Chloride and pH Tolerance

The ability of the *Rhizobium* isolates to grow in basic or acidic media was tested by streaking them on YEM agar plates with pH adjusted to 3.5, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0 with HCl or NaOH (2,10).

The ability of the isolates to grow in different concentrations of salt was tested by streaking isolates on YEM medium containing 0%, 0.5%, 1%, 2%, 3%, 3.5%, 4%, and 5% (w/v) NaCl (11,12).

Temperature Tolerance

Tolerance to high temperatures was tested by typing on YEM broth and incubating at 37, 40, 42, and 45 °C (10,13).

Detection of Melanin

Melanin production by the isolates was determined as described by Cubo et al. (14).

Growth with Different C and N Sources

For C and N assimilation measurement, sugars and organic acids were prepared as 10% (w/v) solutions, which were sterilized by autoclaving or by 0.45 µm filtration and then added to the basal salt liquid medium to a final concentration of 1% (w/v). After 5 days of incubation, growth scores were made visually with reference to the control plates with carbohydrate omitted. A 4-category system was used to denote no response through abundant growth, designated as follows: -, +, ++ and +++, respectively (15-17).

For the evaluation of C sources, casein, dulcitol, citrate, D(-) fructose, D(+) galactose, D(+) glucose, D(+) mannitol, sucrose, starch, succinate, αL-rhamnose, and malate were added to the medium.

For N sources, the medium was supplemented with mannitol, and L-asparagine, L-glutamine, L-tryptophan, thymine, and glycine compounds were added.

Intrinsic Resistance to Antibiotics and Heavy Metals

All isolates were tested for sensitivity to heavy metals and intrinsic resistance to antibiotics, essentially as described by Hungria et al. (17,18). The following antibiotics and heavy metals were tested (µg ml^{-1} at the concentrations in parentheses): streptomycin (40, 80, and 100), kanamycin (10 and 100), erythromycin (30

and 60), chloramphenicol (20, 50, 100, and 200), $CuCl_2.2H_2O$ (50 and 100), $HgCl_2$ (2.5, 5, and 10), and $CdCl_2.2H_2O$ (5, 10, and 20).

Plasmid identification

Plasmid profiles of the isolates were determined using a modified Eckhardt procedure (19). Each isolate was grown in 10 ml of YEM broth for 24 h at 30 °C with shaking. The cells were centrifuged at 10,000 µg for 10 min at 4 °C. The resulting pellet was resuspended in TE buffer (50 mM Tris, 20 mM EDTA, pH 8.0) and again centrifuged for 10 min at 10,000 µg at 4 °C. The resulting pellet was resuspended in 100 µl of Tris borate buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.2) containing 20% Ficoll 400 and placed on ice. A 50 µl sample of the cell suspension was transferred to a 1.5 µl microcentrifuge tube on ice and mixed with lysozyme. A 25 µl sample of this mixture was loaded into the sample well of a vertical gel (1% agarose) (20). After electrophoresis, the gel was stained with ethidium bromide.

Results and Discussion

Differences between isolates were verified using some morphological parameters, but a high production of mucus was verified in 80% of the isolates; 23% were white and 70% opaque colonies. All retrieved isolates were Gram-negative and moderately motile, and most contained granules of poly-ß-hydroxybutyrate (data not shown).

A study conducted in Spain reported that 50 isolates from root nodules of bean plants grown in agricultural areas were also characterized as producing copious amounts of exopolysaccharide slime containing granules of poly-ß-hydroxybutyrate (9).

In our study, 10 isolates (R11, R12, R13, R92, R100, R102, R103, R104, R107, and R108) were fast growers (60 min), while other isolates were slow growers (12 h). There are no previous reports of *Rhizobium* isolates being isolated from bean nodules in Eskişehir. In our study, fast growing *Rhizobium* isolates were detected. They showed convex elevation in YEM medium, as described by Jordan (2) for *Rhizobium phaseoli*; however, Hungria et al. (18) also reported copious extracellular polysaccharide slime production.

Some physiological and biochemical properties of the isolates are presented in Table 1. In general, the Eskişehir isolates were able to use several compounds as sole sources of C, as reported for other bean *Rhizobium* isolates (2). All isolates were able to grow well in the presence of D(-) fructose, D(+) galactose, D(+) glucose, D(+) mannitol, sucrose, starch, succinate, αL-rhamnose, and malate. The isolates we tested were not able to use citrate and dulcitol, which is similar to other *Rhizobium* bacteria (2). Bean rhizobial isolates utilized a wide range of carbohydrates and salts of organic acids as carbon sources (18).

All isolates were grown in YEM medium with pH values of 5 and 8, but differences were detected at pH 4 (Table 1). Of all the isolates, 19 (R4, R9, R11, R12, R13, R20, R28, R48, R51, R61, R80, R92, R100, R102, R103, R104, R107, R108 and R118) even grew at a basic condition as high as pH 9.

It is known that salt stress significantly reduces nitrogen fixation and nodulation in legumes. Hashem et al. (12) have proposed that salt stress may decrease the efficiency of the *Rhizobium*-legume symbiosis by reducing plant growth and photosynthesis, and hence nitrogen demand, by decreasing survival and proliferation of rhizobia in the soil and rhizosphere, or by inhibiting very early symbiotic events, such as chemotaxis and root hair colonization, thus directly interfering with root nodule function. To date, some rhizobial isolates have been shown to grow under high salt conditions. R. phaseoli is one of the most halotolerant rhizobia and several isolates have been reported to grow at high salt concentrations (4%-5%) (18). Most of the other halotolerant *Rhizobium* isolates that have been isolated originate from leguminous trees (12,15).

In our study, the 30 rhizobia isolated from bean grew in 3%-3.5% (w/v) NaCl, 20 isolates grew in 4% (w/v) NaCl, and 10 isolates even grew in 5% NaCl (Table 1). As suggested by previous research (11,15,17,18), we found that fast growing isolates were generally more tolerant to high NaCl concentrations than slow growing isolates. Furthermore, the majority of our isolates had similar demands for carbohydrates as several fast growing rhizobia (10,13). Among the isolates we studied, 18 (R4, R11, R12, R13, R15, R20, R28, R32, R45, R51, R57, R100, R102, R103, R107, R108, R110, and R118) were able to at grow 37 and 40 °C, whereas 5 isolates (R45, R61, R102, R104, and R107) showed only minimal growth at 42 and 45 °C (Table 1).

Table 1. Tolerance of the isolates to acidity and alkalinity, high temperature and NaCl in vitro.

Isolate	No Plasmid DNAs	NaCl (%)			Temperature (°C)				pH						
		0.5-3.5	4.5	5	37	40	42	45	3.5	4	5	6	7	8	9
R4	2	++	+	-	++	++	++	++	-	+	+	++	++	++	++
R9	1	++	++	++	++	++	-	-	-	+	+	++	++	++	++
R11	2	++	++	++	++	++	++	++	-	-	+	++	++	++	++
R12	2	++	++	++	++	++	++	++	-	++	++	++	++	++	++
R13	2	++	++	++	++	++	++	++	-	++	++	++	++	++	++
R15	3	++	-	-	++	++	++	++	-	-	++	++	++	++	++
R20	3	++	+	+	++	++	++	++	-	++	++	++	++	++	++
R28	1	++	++	++	++	++	++	++	-	++	++	++	++	++	++
R32	1	++	-	-	++	++	-	-	-	-	++	++	++	++	++
R34	1	++	-	-	++	++	-	-	-	-	++	++	++	++	++
R42	1	++	-	-	++	++	-	-	-	-	++	++	++	++	++
R45	1	++	-	-	++	++	+	+	-	-	++	++	++	++	++
R48	1	++	+	-	++	++	-	-	-	++	++	++	++	++	++
R51	1	++	+	-	++	++	++	++	-	-	++	++	++	++	++
R54	1	++	-	-	++	++	-	-	-	-	++	++	++	++	++
R57	2	++	-	-	++	++	-	-	-	-	++	++	++	++	++
R61	1	++	+	-	++	++	+	+	-	+	++	++	++	++	++
R76	1	++	-	-	++	++	-	-	-	-	++	++	++	++	++
R80	1	++	+	-	++	++	-	-	-	-	++	++	++	++	++
R91	1	++	-	-	++	++	-	-	-	-	++	++	++	++	++
R92	1	++	++	++	++	++	-	-	-	-	++	++	++	++	++
R95	1	++	-	-	++	++	-	-	-	-	++	++	++	++	++
R100	1	++	+	-	++	++	++	++	-	++	++	++	++	++	++
R102	1	++	+	-	++	++	+	+	-	+	++	++	++	++	++
R103	1	++	++	++	++	++	++	++	-	++	++	++	++	++	++
R104	2	++	+	-	++	++	-	-	-	+	++	++	++	++	++
R107	1	++	++	++	++	++	+	+	-	+	++	++	++	++	++
R108	2	++	+	-	++	++	+	+	-	++	++	++	++	++	++
R110	1	++	-	-	++	++	++	++	-	-	++	++	++	++	++
R118	1	++	+	-	++	++	++	++	-	++	++	++	++	++	++

Absence of growth (-) and indicates(+) weak growth

All isolates were characterized as melanin-producing in vitro. Most isolates produced melanin, but this is a widespread characteristic of unknown origin among rhizobial isolates (14) and thus is not frequently used to characterize rhizobia groups (3).

Differences between isolates were verified. Most isolates were resistant to low levels of erythromycin (30 μg ml^{-1}), kanamycin (10 μg ml^{-1}), chloramphenicol (20 and 50 μg ml^{-1}), and streptomycin (40 μg ml^{-1}) (Table 2). In general, resistance to the streptomycin levels tested

Table 2. Minimum inhibitory concentration of four antibiotics with 30 isolates of bean *Rhizobium* sp. (2 dry isolates, 28 wet isolates)

Antibiotics	Concentration (µg ml-1)	No isolates with a '—' score	Colony type	
			Dry	Wet
Streptomycin	40	30	2	28
	80	29	1	2
	100	28	0	28
Kanamycin	10	30	2	28
	100	19	0	19
Erythromycin	30	14	2	12
	60	8	1	7
Chloramphenicol	20	30	2	28
	50	28	1	27
	100	10	0	10
	200	4	0	4

was high. There are 3 known determinants of bacterial permeability to an antibiotic: hydrophobicity, electrical charge, and amount of the antibiotic (10,18). Exopolysaccharides and lipopolysaccharides also influence the transport of antibiotics into bacteria (2,10). The basis for the correlation found here between antibiotic resistance and rhizobial colony morphology remains to be determined, but it is probable that the *Rhizobium* that showed a high level of resistance did not take up the antibiotics. Streptomycin, erythromycin, chloramphenicol, and kanamycin were generally more effective against the wet *Rhizobium* isolates than they were against the dry ones; however, most of the wet and dry isolates were resistant to relatively high levels of streptomycin and chloramphenicol. All isolates were sensitive to the heavy metals.

Plasmid DNA profiles of isolates and molecular weights of plasmid DNA are given in Table 1. Additionally, the number of plasmids in *Rhizobium* species field isolates correlated positively with the growth rates in laboratory cultures (20). *Rhizobium* isolates R4, R11, R12, R13, R57, R104, and R108 had 2 plasmids, while R15 and R20 had 3 plasmids. The common phenomenon of *Rhizobium* sp. losing some of their ability to nodulate or fix nitrogen is more likely due to genomic instability than actual plasmid loss.

Additional research needs to be undertaken to determine the functions of these plasmids and if their expression is affected following culturing. No relationship could be established between the plasmid DNA of the isolates and some phenotypic features. The phenotypic measurements were necessary for the characterization and selection of isolates adapted to climatic conditions and provided information about their genetic diversity. Further work is needed with *Rhizobium* isolates from bean at the genetic level to confirm whether they represent different biovars or, perhaps, different species.

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Corresponding author:
Merih KIVANÇ
Anadolu University, Faculty of Science,
Department of Biology,
26470 Eskişehir - TURKEY
E-mail address: mkivanc@anadolu.edu.tr

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