DIVERSITY *Rhizobium leguminosarum* bv. *trifolii* FIELD POPULATION FROM CHERNOZEM, PSEUDOGLEY AND HYDROMORPHIC BLACK SOIL

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ABSTRACT: The presence of indigenous nitrogen fixing microsymbionts of leguminous plants in different type of soil are of great agricultural and environmental significance. To achieve beneficial effect of plant inoculation we need consider the characheristics of rhizobial field population. To investigate the diversity level of rhizobial population, we isolated 80 indigenius Rhizobium leguminosarum bv. trifolii from soil type chernozem, 27 from hydromorphic black soil and 92 from pseudogley. High level of genetic diversity was found among indigenous Rhizobium leguminosarum bv. trifolii population. Diversity of indigenous plant isolates from two types of soil in Serbia will be monitoring in the different enviromental conditions.

Key words: *Rhizobium leguminosarum* bv. *trifolii*, RAPD analysis, genetic diversity, Calcofluor effect, IAR, HMT

INTRODUCTION

The phenomennon of biological nitrogen fixation can be used to increase plant biomass production. The most effective in nitrogen fixation are Gram-negative bacteria of the genus *Rhizobium*. Rhizobia can live in soil or can invade specific host plants. *Rhizobium leguminosarum* bv. *trifolii* is microsymbiont *Trifolium pratense* and *Trifolium repens,* very important legumes in Serbia.The presence of indigenous nitrogen fixing microsymbionts of leguminosis in different type of soil are of great agricultural and environmental significance. To achieve beneficial effect of plant inoculation we need consider the characheristics of rhizobial field population.

MATERIAL AND METHODS

We isolated 199 indigenius *Rhizobium leguminosarum* bv. *trifolii*: 80 from soil type chernozem (ch), 27 from hydromorphic black soil (hbs) and 92 from pseudogley (psy) to investigate the diversity level of rhizobial population. Rhizobia were rescused from nodules of *Trifolium repens* and *Trifolium pratense* from 22 different locations.Culture

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media and growth conditions for *Rhizobium* isolates described by Vincent (1970). Phenotypic analysis performed by growth on YEM medium containing following concentrations of: Tetracycline (Tet) –3; Streptomycin (Str) –4 and 5; Chloramphenicol (Chl) –40 and 50; Ampiciline (Amp) –30 and 40; Gentamycin 3 and 5 mg/ml for intrinsic antibiotic resistance (IAR) and Mercury (Hg) –2 and 3; Nickel (Ni) –15 and 25; Cadmium (Cd) –20; Cobalt (Co) –35 and 40; Zinc – 100 and 120 mg/ml for heavy methal tolerance.

To obtained the differences in Calcofluor fluorescence phenotype we used culture media and growth conditions for *Rhizobium* strains described by Long *et al*. (1988) and Gonzales *et al*. (1996). Detection of mutants altered in EPS I or EPS II production was carried out by Sudan lack B staining – Liu *et al*. (1998). Total genomic DNAs were isolated as described by Chen and Kuo (1993) and used as a DNA template in PCR reactions as described by Laguerre et al. (1996).

RESULTS AND DISCUSSION

Using the IAR (Intrinsic Antibiotic Resistance), HMT (Heavy Methal Tolerance) and Cf (Calcofluor) effect as phenotyping markers, we obtained 28 different isolates from chernozem, 10 from hydromorphic black soil, and 31 different isolates from pseudogley. Isolates from hydromorphic bleck soil was more sensitive on Hg , Chl and Sm then others isolates, but less sensitive and very tolerante on Co. Isolates from pseudogley was very tolerante on Ni 25 mg/ml, more than isolates from other soil types, but very sensitive on Gen. All isolates exhibited uniformly low level of tolerance to nickel (25mg/ml). Our results are different from data reported by Tong and Sadowsky (1994) for *Rhizobium leguminosarum* bv. *trifolii* tolerance on Ni (80 mg/ml).

On the basis of IAR-HMT sensitive paterns, we chose one representative isolate per cluster group for each type of soil: 4 for chernozem, 4 for pseudogley and 3 for hydromorphic black soil and we compared it. (Figure 1.)

The pattern of antibiotic resistance of *Rhizobium* isolates was a stable trait by which rhizobia could be recognised. IAR markers have been successfully used for strain differentiation as already reported (Shishido and Pepper, 1990; Moawad et al., 1998).

The RAPD fingerprint patterns were scored for *R. leguminosarum* bv. *trifolii* isolates for each of 3 type soil by recording the presence or absence of bands.PCR of representative isolates yelded multiple DNA products of molecular sizes ranging from 160 to 1200 bp with AP 10 primer and 440 to 1500 with SPH 1 primer. As a molecular weight marker was used 1kb DNA ladder (Amersham biosciences). Groupings of *R. leguminosarum* bv. *trifolii* strains obtained by the PCR based methods investigated in this study confirmed diversity data obtained by conventional techniques. (Fig. 2)

Bacteria producing succinoglycan exhibit a blue-green fluorescence under ultraviolet light if grown on media containing the Calcofluor whitener. Calcofluor dye can bind cellulose and other b-linked polysaccharides. We separated succinoglycan and galactoglucan producing isolates; we didn't observe mutants in EPS production wich are able to incorporate the Sudan Black B dye and show black colour. (Tab.1.)

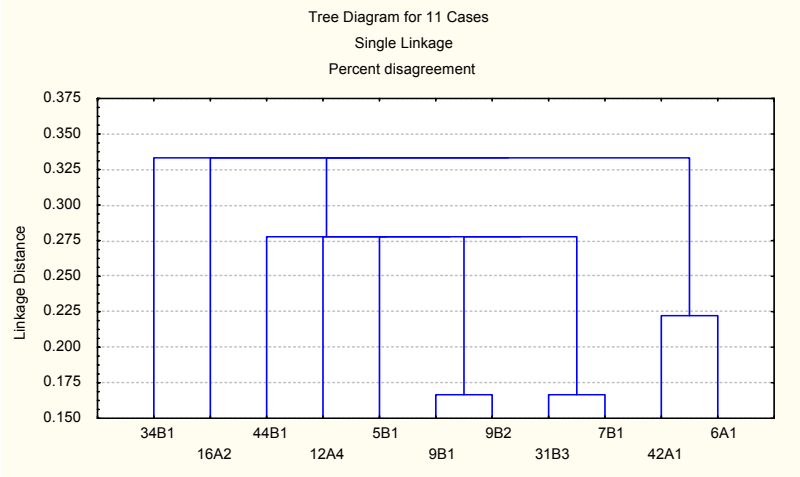

Figure 1. Similarity of intrinsic antibiotic resistance (IAR) and haevy methal tolerance (HMT) of representative isolates Rhizobium leguminosarum bv. trifolii

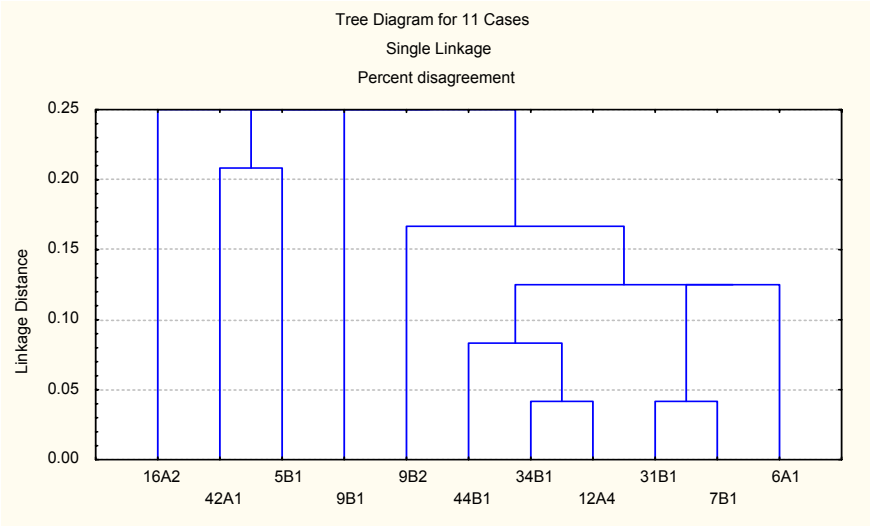

Figure 2. RAPD of representative R. leguminosarum bv. trifolii isolates

R.leguminosarum bv. *trifolii* isolates produce both types of exopolysaccharides: 55 isolates produced succinoglycan (24/28 from ch; 8/10 from hbs; 23/31 from psy); 14 isolates produced galactoglucan (4/28 from ch; 2/10 from hbs; 8/31 from psy). Calcofluor phenotype the most of *R. leg.* bv. *trifolii* isolates (55 of 69 investigated isolates EPS I type) varied from no or dim to very bright fluorescence. Some of the isolates showed slightly mucoid phenotype.

Table 1. Exopolisaccharides typization of indigenous R. leguminosarum bv. trifolii isolates

Isolate	EPS prod.	Cf efect	SBB dye	EPS type	Isolate	EPS prod	Cf. efect	SBB dye	EPS type
chernozem					pseudogley				
1A1	+	*	–	I	21A1	++	*	–	I
1A2	+	*	–	I	21A2	++++	–	–	II
1B1	+	***	–	I	21A3	++	–	–	II
1B2	++	**	–	I	21B1	+++	**	–	I
2A1	++++	–	–	II	22A1	+++	–	–	II
2A2	+	*	–	I	22A2	++	**	–	I
2B1	++++	**	–	I	22A5	+++	**	–	I
2B2	+++	**	–	I	22B1	+++	–	–	II
4A1	++	**	–	I	31A1	++	–	–	II
4A2	+++	****	–	I	31B1	++	*	–	I
4A3	++	**	–	I	31B3	++++	*	–	I
5A1	++++	–	–	II	32A2	+++	**	–	I
5B1	+++	–	–	II	32A3	++++	–	–	II
5B2	++	**	–	I	33A2	++	**	–	I
6A1	++	*	–	I	33B1	++	***	–	I
6A2	+++	**	–	I	33B3	++	**	–	I
7A1	+++	**	–	I	34A1	+	*	–	I
7A2	+++	*	–	I	34A3	++	****	–	I
7B1	++	**	–	I	34B1	+++	–	–	II
7B2	+++	***	–	I	36A1	+++	**	–	I
8A1	++	**	–	I	36A3	++	***	–	I
8A2	+++	****	–	I	36B1	+++	–	–	I
8B1	++	***	–	I	36B3	+	*	–	I
10A1	+++	****	–	I	41A1	+++	*	–	I
16A1	+++	*	–	I	41B1	++++	***	–	I
16A2	+++	***	–	I	42A1	++	***	–	I
16B1	++	–	–	II	42A2	+++	–	–	I
16B3	+++	**	–	I	42B1	+	***	–	I
hydromorphic black soil					44A1	++	***	–	I
					44A3	+	***	–	I
9A1	+	***	–	I	44B1	++	–	–	II
9A2	+++	*	–	I	hydromorphic black soil				
9B1	+++	***	–	I	11A3	++	*	–	I
9B2	+	–	–	II	12A1	+	***	–	I
11A1	+++	***	–	I	12A4	+++	–	–	II
11A2	+	***	–	I	12B4	+++	***	–	I

Production of EPSs: (–) no; + low ; ++ intermediate; +++ abundant; ++++ very abundant production with mucoid phenotype
Fluorescence: (–) no; * dim; **or *** bright; **** very bright fluorescence

CONCLUSIONS

- The results of the present study show the wide diversity of *R. leguminosarum* bv. *trifolii* field populations from 3 soil type with different IAR and HMT patterns;
- The most of *R leguminosarum* bv. *trifolii* isolates (55 of 69 investigated isolates) produced succinoglycan and their Cf efect varied from no or dim fluorescence to very bright fluorescence, since 14 isolates produced galactoglucan without Cf efect.
- RAPD fingerprint patterns of *R leguminosarum* bv. *trifolii* showed significante differences between isolates with similar IAR-HMT pattern. RAPD methods are efficient means for rapidly typing a large number of strains and estimation of their diversity.

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