PERIODICO di MINERALOGIA Volume 90, No. 1, 2021

The competitive ability of different *Rhizobium leguminosarum* bv. *trifolii* inoculant strains

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Abstract

Bacteria genus Rhizobium is soil microorganisms which induce nodule formatting on the roots of the leguminous plants. Within these nodules the bacteria fix atmospheric nitrogen and convert it to the ammonia, the form useful for the host plants. Rhizobia are usually used as microbiological fertilizers for leguminouses. Effective inoculant strain needs to be able to compete with the native rhizobia and to form high percentage of nodules. Many studies of rhizobial ecology require simultaneous detection of several strains in symbiosis with a plant. Differences between production of two types risobial exopolysaccharade: succinoglycan (EPS I) and galactoglucan (EPS II), intrinsic antibiotic resistance and heavy metal tolerance were used to investigate strain competition. 470C5 induced mutant was used as a strain marker and dual nodule occupancy were analyzed.

Keywords: Rhizobium sp., exopolysaccharide, succinoglycan, galactoglucan, calcofluor effect

Introduction

EPSs play the crucial role for nodule invasion and may play role in the host defenses and in providing protection from abiotic stress (draught, high acidity)[1]. Rhizobial EPSs can be either homopolymers or heteropolymers and may carry a variety of non-carbohydrate substituents. Rhizobia produce two types exopolysaccharade: succinoglycan (EPSI) and galactoglucan (EPSII).

Succinoglycan (EPSI) is composed of repeating octasaccharide subunits, each consisting of a backbone of three glucoses and one galactose, a side chain of four glucose and one pyruvyl, acetyl and succinyl modifications. EPSI are produced by strains of *Rhizobium, Sinorhizobium, Azospirollum, Agrobacterium, Alcaligens, Xantomonas* etc.

EPSII is a member of galactoglucans, and its backbones consist of alternating glucose and galactose moieties; they differ with respect to the non-carbohydrate modifications they carry. EPSII is produced by some of *Pseudomonas* species (*P. putida, P. fluorescens*), *Agrobacterium* (*A. radiobacter*), *Achromobacter* etc. Rhizobial EPSII consists of a disaccharide subunit of glucose and galactose carrying acetyl and pyruvyl modifications [2].

Bacterial competition studies require some specific methods for marking the competing strains. In this study we used differences between intrinsic phenotyping determinants of investigated strains: intrinsic antibiotic resistance (IAR), heavy metal tolerance (HMT) and differences in the Calcofluor fluorescence phenotype. The primary objective of this work was to examine the type of EPS production and to assess the possible advantage for competition. Bacteria producing succinoglycan exhibit a blue-green

fluorescence under ultraviolet light if grown on media containing the Calcofluor whitener. Calcofluor is a stilbene dye which binds cellulose and other β-linked polysaccharides. This work presented detection of the types of EPS producing by indigenous *Rhizobium* strains [3]. Selective staining of mutants by Sudan Black B solution enables the separation of EPSII and typization depending of EPSI/EPSII phenotype [4]. Typing of the rhizobial EPS production is the additional way for phenotyping characterization those strains, which make easier assessing during competitiveness investigations.

Materials and methods

We used culture media and growth conditions for *Rhizobium* strains as described earlier [5,6,7]. In this study we used differences between intrinsic phenotyping determinants of investigated strains: intrinsic antibiotic resistance (IAR), haevy metal tolerance (HMT) and differences in the Calcofluor fluorescence phenotype. Detection of mutants altered in EPSI or EPSII production was carried out by Sudan Black B staining [4]. Plants seeds were surface sterilized by ethanol 96% and 0,1%$HgCl_2$ and sown in plant growth tubes containing Jensen nutrient solution and inoculated with 10 exp8 of each bacteria in ratio 1:1. Un-inoculated controls were included. The plant-growth tubes were held at 24 C with twelve hour light period. The plants were harvested after six weeks [5]. Root nodules were sampled per plant, crushed and diluted in sterile distillated water, plated on YMA plate and replicated on YMA containing 0,02 % Calcofluor dye, appropriated antibiotic and heavy metals.

Results and discussion

Exopolysaccharide synthesis appears to be a common feature associated with numerous microorganisms. Results from a number of previous studies indicated that rhizobial exopolysaccharide production is necessary for successful nodulation of alfalfa, clovers and peas, as well as others leguminous plants [8]. Some of Rhizobia with mutation in *exo*, *muc* or *gum* genes, on chromosomes or plasmids, does not appear to be able to nodulate host-plants [9]. EPS increase the efficiency of nodulation and give advantages by improving nutrient acquisition or providing protection from environmental stress and host defenses [1]. We observed wild-type strains of *Rhizobium leguminosarum* bv. *trifolii* and their Calcofluor fluorescence effects. The Calcofluor phenotype of different *Rhizobium leguminosarum* bv. *trifolii* strains varied from no or dim fluorescence to very bright fluorescence. Some of the strains (404, 448 and 461) showed halo fluorescent effect and some slightly mucoid phenotype. We separated succinoglycan and galactoglucan producing strains. **Table 1.**

Rhizobium leguminosarum bv. *trifolii* strains produce both types of exopolysaccharides: 12 of 33 investigated strains produced succinoglycan and 18 strains produced galactoglucan. Four strains (407, 449, 452 and 457) produced neither EPSI nor EPSII. 470 strain showed very bright fluorescence, more then all investigated strains in this work.

The EPSII exopolysaccharide does not bind Calcofluor and detection of its mutants is very difficult. Mutants that do not produce succinoglycan can be isolated readily on the basis of lack of fluorescence on Calcofluor medium, even when there are no observable changes in the morphology of the colonies. Since 1998, the new Sudan Black B screening procedure has been used to isolate mutant deficient in either EPSI or EPSII production. Single colonies of all investigated strains were stained by Sudan Black B solution, and observed for the absence of Calcofluor phenotype that depends on mutation in the genes for production of

exopolisaccharides. Only one mutant altered in EPSI production by Sudan Black B staining are showed bluish black color and isolated (470C5).

Table 1. Exopolisaccharides typization of indigenous *Rhizobium leguminosarum* bv. *trifolii* strains

Rhizobium leguminosarum bv. *trifolii*					
	EPSI	EPSII		EPSI	EPSII
404	+++		436	+++	
407	-	-	**440**		++
408	++		441	+++	
409		+	442		+
410		+	443		+
412	+++		444		++
416		++	445	++	
418		+	**448**	+++	
420		+	449	-	-
421		+	452	-	-
422		+	453	+++	
424		+	457	-	-
427		+	458		++
428	++		460		+
429		++	**464**	++	
433		++	470	+++	
435		++	**470C5**	++++	

(-) no fluorescence;
+ dim fluorescence;
++ or +++ bright fluorescence;
++++ very bright fluorescence with slightly mucoid phenotype

Next, we observed differences between some phenotyping determinants of investigated strains - intrinsic antibiotic resistance (IAR) and heavy metal tolerance (HMT) for all of strains. **Table 2.** The most sensitive strains are 440 and 464. 470C5 mutant strain shows good resistance on 3 of 4 antibiotics and it is very tolerant on high concentration of heavy metals.

Table 2. Intrinsic antibiotic resistance (IAR) and heavy metal tolerance (HMT) of *Rhizobium leguminosarum* bv. *trifolii* strains used in competitiveness tests

	Intrinsic Antibiotic Resistance				Haevy Methal Tolerance					
Strains	Tet	Sm	Chl	Amp	Hg	Ni	Cd	Cd	Co	Mo
	μg/ml									
	5	1	50	20	2	20	20	40	40	3
427	S	R	R	S	S	S	S	S	S	S
433	R	R	S	S	T	T	T	S	T	T
440	S	S	S	S	S	S	S	S	T	S
448	S	S	S	R	T	T	T	S	T	T
464	S	R	S	S	S	S	S	S	S	S
470	R	R	R	S	T	T	T	S	S	T
470 C5	R	R	R	S	T	**T/80**	T	**T**	S	T

Abbreviation: S-susceptible; R- resistant; T- tolerant

In competitiveness investigation we used only high effective strains: 3 EPSI including 470C5 mutant and 3 EPSII producing strains. The results of competitiveness investigation are shown in **Table 3.**

Table 3. Percentage nodule occupancy of investigated *Rhizobium leguminosarum* bv. *trifolii* strains

Strains A + B	Basis of separation of strains	No of nodules / plant (10)	Nodule occupancy A (%)	Nodule occupancy B (%)	Dual nodule occupancy	
					AB (%)	A/B (%)
470C5 + 427	Cf + / Cf -	7,8	94,23	2,84	2,93	89,91 / 10,09
470C5 + 440	Cf + / Cf -	6,1	61,12	34,03	4,85	77,03 / 22,97
470C5 + 433	Cf + / Cf -	7,3	73,44	19,32	7,24	78,92 / 21,08
464 + 427	Cf + / Cf -	7,7	79,82	15,68	4,50	86,31 / 13,69
464 + 440	Cf + / Cf -	5,3	43,15	51,78	5,07	22,04 / 77,96
464 + 433	Cf + / Cf -	5,7	65,55	25,03	9,42	60,26 / 39,74
448 + 427	Cf + / Cf -	8,2	84,29	10,34	5,37	87,98 / 12,02
448 + 440	Cf + / Cf -	6,4	69,93	24,72	4,35	52,30 / 47,70
448 + 433	Cf + / Cf -	6,8	73,58	18,41	8,01	71,83 / 28,17
470C5 + 448	Cd 40 T / S Tet 5 R / S	6,1	71,17	22,28	6,55	68,22 / 31,78
470C5 + 464	Cd 40 T / S Tet 5 R / S	8,6	81,58	12,96	5,46	84,17 / 15,83

The percentage of nodule occupancy the mutant 470C5 strain and strains which produce both of types EPSs are observed. Basis of separation was Calcofluor fluorescent effect in EPSI/EPSII strain producing combination. Possibility of detection the spontaneous mutations in EPSI production was eliminated by Sudan Black B staining. Mutants able to incorporate the dye appeared bluish black color.

In combination of 2 EPSI strains, we used differences in HTM and additionally IAR, supposing that the spontaneous mutation witch led the change in both of characteristics is very small. 470C5 mutant and 448 wild type strain were better competitor than 464 EPSI strain and other EPSII strains. Only 440- EPSII producing strain was better then 464 EPSI strain. Strain 427 is a poor competitor, especially against strain 470C5. This mutant showed better competition ability then 448 and 464 in competitions of two strains of EPSI type.

Use of marker genes such as **GFP** (green fluorescent protein), ***gas*A** (β glucuronidase) and ***cel*B** (thermostable β glucosidase) has several advantages in studying rhizobial competition compared to classical approaches. The greatest advantage is that this reporter genes can be detected and localized simultaneously on a plants roots (using up or blue light-GFP or double staining –gasA and celB). However, many countries have strict regulation concerning the release of GMO. Calcofluor fluorescent methodology may be used as easy and fast methods for investigating some rhizobial competition.

Conclusions

Rhizobium leguminosarum bv. *trifolii* strains produce both types of exopolysaccharides: 12 of 33 investigated strains produced succinoglycan and 18 strains produced galactoglucan. Four of the strains (407, 449, 452 and 457) produced neither EPS I nor EPS II. 470 strain shows bright fluorescence, especially its mutant 470C5, more then all investigated strains in this work. Some of strains (404, 448 and 461) show halo fluorescent effect.

470C5 mutant and 448 wild type strains were better competitor than 464 wt strain (EPSI) and other EPSII strains. Only 440- EPSII producing strain was better then 464 EPSI strain. Strain 427 is a poor competitor, especially against strain 470C5. In competitions two EPSI type of strains, 470C5 mutant shown better competitive ability then 448 and 464. The induced mutant 470C5 showing good chloramphenicol 50μg/ml, tetracycline 5μg/ml resistance, very good nickel 80μg/ml and cadmium 40μg/ml tolerance and over production of succinoglycan with very bright Calcofluor fluorescent phenotype, are useful for further competitiveness investigations.

This methodology does not require expensive equipment and may be used as easy and fast method for investigating some rhizobal competition.

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