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Symbiotic efficiency of indigenous common bean rhizobia isolated from Northwestern Croatia

Simbiozna učinkovitost autohtonih sojeva rizobija na grahu izoliranih iz sjeverozapadne Hrvatske

Ivana RAJNOVIĆ[1], Mihaela RAK[2], Sanja KAJIĆ[1] (✉), Sanja SIKORA

[1] University of Zagreb Faculty of Agriculture, Svetošimunska 25, 10000 Zagreb, Croatia

[2] EcoMission L.T.D., Ulica Hrvatske Republike 19H, 31000 Osijek, Croatia

✉ Corresponding author: skajic@agr.hr

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ABSTRACT

The strains of nodule bacteria differ greatly in their symbiotic properties and efficiency in nitrogen fixation and therefore, the selection of highly effective strains is of great importance when attempting to achieve a successful pre-sowing inoculation of common bean. The aim of this study was to determine the nodulation ability of indigenous rhizobial strains and to evaluate their compatibility and symbiotic efficacy with two common bean cultivars. A two-factorial pot experiment (12 indigenous rhizobial strains and two common bean cultivars) was set up in the greenhouse. In the full blossom phase, dry nodule biomass, shoot dry weight and N content in dry shoot was determined. The research revealed significant differences depending on the strain of nodule bacteria and common bean cultivars. The compatibility of indigenous and reference strains on the examined common bean cultivars was also observed. The cultivar Tresnjevac was significantly better than Slavonski zeleni in all measured parameters. The results revealed that the indigenous common bean rhizobial strains considerable differ in their symbiotic efficiency. The highest dry nodule biomass was determined when strain HO12 was applied, and the highest shoot dry weight with strains MB1 and DE7. The application of strain DE7 revealed the highest N content in dry shoot. The results indicate that these indigenous strains of common bean nodulating bacteria are characterized by the highest symbiotic efficiency and could be used for the pre-sowing inoculation of common bean after testing in field scale experiment.

Keywords: *Rhizobium* spp., symbiotic nitrogen fixation, Trešnjevac, Slavonski zeleni, greenhouse experiment

SAŽETAK

Sojevi kvržičnih bakterija značajno se razlikuju po svojim simbioznim svojstvima te učinkovitosti u fiksaciji dušika te je stoga selekcija visoko učinkovitih sojeva rizobija bitna je za uspjeh predsjetvene bakterizacije graha. Cilj ovog istraživanja bio je odrediti nodulacijsku sposobnost autohtonih sojeva kvržičnih bakterija koje noduliraju grah, procijeniti njihovu kompatibilnost i simbioznu učinkovitost sa dvije sorte graha. U svrhu istraživanja u plasteniku je postavljen dvofaktorijelni vegetacijski pokus (12 autohtonih sojeva *Rhizobium* spp. i dvije sorte graha). U punoj cvatnji određivana je masa suhe tvari kvržica, masa suhe tvari biljke te sadržaj ukupnog dušika nadzemnog dijela biljke. Istraživanjem su utvrđene signifikantne razlike ovisno o soju kvržičnih bakterija i sorti graha. Također je utvrđena kompatibilnosti autohtonih i referentnih sojeva sa ispitivanim sortama graha. Sorta Trešnjevac bila je signifikantno bolja po svim mjerenim parametrima u odnosu na Slavonski zeleni. Na temelju provedenog istraživanja utvrđeno je da se autohtoni sojevi razlikuju u svojoj simbioznoj učinkovitosti. Najveća masa suhe tvari kvržica utvrđena je uz primjenu soja HO12, dok je najveća masa suhe tvari biljke primjenom sojeva MB1 i DE7. Primjenom soja DE7 ostvaren je i najveći sadržaj ukupnog dušika u nadzemnom dijelu biljke. Rezultati ukazuju da se navedeni autohtoni sojevi odlikuju najvećom simbioznom učinkovitošću i te da predstavljaju potencijal za proizvodnju preparata za predsjetvenu bakterizaciju graha.

Ključne riječi: grah, *Rhizobium* spp., simbiozna fiksacija dušika, Trešnjevac, Slavonski zeleni

INTRODUCTION

Since nitrogen is the main limiting nutrient for most crops, symbiotic nitrogen fixation presents an important biological process for the sustainable agricultural production. The symbiosis between leguminous plants and nodule bacteria (rhizobia), which is the most important source of biologically fixed nitrogen (Laranjo et al., 2014), greatly contributes to the sustainability of agricultural systems. Genetic factors of both symbionts participate in this relationship which begins with mutual recognition of bacteria and plants. Infection of plants with bacteria is the next step resulting in the formation of nodules on the root system of plants, a new organ in which the process of symbiotic fixation of atmospheric nitrogen occurs. As a result, inaccessible nitrogen from the atmosphere becomes available for the plants, and after harvesting this nitrogen remains in the soil to sustain the needs of subsequent crops (Zahran, 1999). Studies on the origin and evolution of common bean microsymbionts have confirmed that common bean most often forms symbiotic relationships with the following species from the family Rhizobiaceae, genus *Rhizobium*: *Rhizobium leguminosarum*, *Rhizobium phaseoli*, *Rhizobium etli*, *Rhizobium gallicum*, *Rhizobium giardinii*, and *Rhizobium tropici* (Segovia et al., 1993; Amarger et al., 1997; Grange and Hungria, 2004).

The species *R. leguminosarum* was described by Frank (1889). This is a heterotrophic, gram-negative, aerobic bacterium that changes to a bacteroid form at the root of the host plant (Oke and Long, 1999). In a recent study (Rajnović et al., 2019) it was found that this species was a dominant common bean symbiont in soils of Northwestern Croatia. It was also found that the effective strains of this species significantly increase the shoot dry weight and the N content in dry shoot and with regard to other positive physiological characteristics have the potential to be applied as biofertilizer in common bean production (Rajnović, 2017). *R. tropici* differs from the species *R. leguminosarum* as determined by sequencing the 16S rDNA gene (Martinez-Romero et al., 1991). This species has the greatest resistance to the stress conditions caused by high temperatures and drought. It was first isolated in South America and Africa (Hungaria et al., 2000). *Rhizobium etli* is a species of nodule bacteria dominant in South America, but also found in European soils (Aguilar et al., 2006; Rodriguez-Navarro et al., 2000). It is genotypically divergent from *R. leguminosarum* using the 16S rDNA PCR - RFLP molecular method (Segovia et al., 1993). Species *R. phaseoli* has been isolated from some countries of common bean origin as well as from Ethiopia (Aserse et al., 2012), but it is one of the least common species in Europe.

Common bean (*Phaseolus vulgaris* L.) stands out as a very important and high-value vegetable crop from the legume family (*Fabaceae*), with the ability to establish symbiotic relationships with an extremely large number of nodule bacteria from the genus *Rhizobium*. Common bean is very valuable food, rich in proteins, potassium, phosphorus and vitamins B1, B2, B3, B5, B6 and C (Lešić et al., 2004).

In their research study on the origin and diversity of common bean in Croatia, Carovic-Stanko et al. (2017) found that 27.32% of accessions were of Mesoamerican origin, 68.31% of Andean, and 4.37% of accessions represent putative hybrids between gene pools. Common bean was introduced to Croatia (and western Balkan area) mainly from the Mediterranean Basin (Maras et al., 2015).

Although there are favorable agroecological conditions for growing common beans in the Republic of Croatia, this crop is neglected, with common bean production areas in constant decline, and with the market dominated by imported common beans. Therefore, it is important to encourage and improve the production of common bean in Croatia especially in a more environmental friendly approach including the pre-sowing inoculation, which is generally not carried out. Pre-sowing inoculation of common beans is an important agrotechnical measure that enables economically and ecologically acceptable production because the application of efficient strains ensures the supply of nitrogen to the plant and thus reduces the cost of mineral nitrogen fertilizers while increasing yields. Quantity of fixed nitrogen measured in biological fixation for the common bean growing systems was estimated to 55-85 kg/ha per year (Unkovich and Pate, 2000). In order to better understand and exploit the useful symbiosis in common bean cultivation, the characteristics of natural rhizobia populations should be investigated in order to select highly efficient strains which could serve as proper population for common bean in order to fix aerial nitrogen.

MATERIAL AND METHODS

The experiment set up

The vegetation experiment was set up in the greenhouse (Department of Vegetable Growing, Faculty of Agriculture, University of Zagreb) in order to determine the symbiotic efficiency of indigenous strains of common bean rhizobia. A two-factorial experiment (2 × 14) was set up in three replications according to the completely randomized block design. The experimental factors were common bean cultivars (Slavonski zeleni and Trešnjevac) and strains of *Rhizobium* spp. (12 indigenous strains isolated from the area of Northwestern Croatia, one reference strain and non-inoculated control). Indigenous rhizobial strains were isolated from the common bean producing areas of Northwestern Croatia. They are part of collection belonging to Department of Microbiology, Faculty of Agriculture, University of Zagreb and were previously identified on the basis of 16S rRNA sequencing as *Rhizobium* spp. In the experiment they were labeled MB1, LS2, CA3, FE4, KP6, DE7, PE8, ZA9, VI10, VI11, HO12 and GU13. The reference strain 3622 (*R. leguminosarum* bv. *phaseoli*) was obtained from the Institute of Grassland Environmental Research UK, and it was labeled with R.

The common bean cultivars

The common bean cultivar Slavonski zeleni is widespread in Slavonia. The height of the stem ranges from 29.86 to 35.32 cm. The color of the flower is white, and flowering begins 33 to 37 days after emergence and lasts 18 to 28 days. The vegetation period of low grain common beans of the Slavonski zeleni cultivar lasts 89 days. The pods are slightly bent, green, have a thin thread, and contain 3.52 to 4.13 grains of medium size, elliptical shape. The grain is greenish with yellowish-brown tinges. According to Vidović and Todorović (1988), this cultivar of common beans contains 25.52% protein.

Trešnjevac cultivar is widespread in continental Croatia. The average height of the stem is about 31.09 cm, and the number of fertile nodules is about 3.5. The flowers are light pink, and their flowering begins between 31 and 34 days after germination and lasts from 16 to 28 days. The vegetation period lasts 89 days. The pods are straight to slightly bent with a thin thread, and later, pale pink stripes appear on them.

Figure 1. Seeds of „Trešnjevac“(left) and „Slavonski zeleni“ (right) cultivars

The grains are elliptical shape, pale pink and dotted with purple-red streaks. There are two brown rings around the hilum. According to Vidović and Todorović (1988), this common bean cultivar contains 28.8% protein.

Seed inoculation

Surface sterilization of the common bean seeds was performed according to Somasegaran and Hoben (1994), so that the seeds were immersed for a one minute in 96% ethanol then 3 minutes in 5% sodium hypochlorite (NaClO) and then washed several times in sterile distilled water and placed in sterile Petri dishes. To prepare the inoculum, pure cultures of each strain were grown in liquid nutrient medium containing 0.1% K_2HPO_4, 0.5% KH_2PO_4, 0.25 % K_2SO_4, 0.1% $CaCl_2$, 0.1% NaCl, 1% D – mannitol, 5% yeast extract and $FeSO_4$, $MnSO_4$, $Na_2B_4O_7$, Na_2MoO_4 in traces. Selected strains were grown for 5 days at 28 °C to achieve approximately 10^6 CFU/ml. Surface sterilized seeds were inoculated with pure cultures of 12 different indigenous strains and one reference strain. A non-inoculated control was included in the experiment, the plants were watered with a nitrogen-free nutrient solution (Macary, 1989). Common beans were grown in a greenhouse in the plastic pots filled with quartz sand until the full flowering phase (Figure 2). Light and temperature regime were as follows: 16 h light at 24 °C and 8 h darkness at 18 °C.

In order to assess the symbiotic efficiency of each strain, dry nodule biomass, shoot dry weight and the N content in dry shoot were measured. Analysis of variance was performed with the statistical package SAS 9.4 for Windows (SAS Institute Inc.). Differences between mean factors were tested by Tukey's multiple comparison test.

RESULTS AND DISCUSSION

The results of the analysis of variance for the investigated traits: dry nodule biomass, shoot dry weight and N content in dry shoot are shown in Table 1. Statistical analysis showed significant differences for all the investigated traits depending on the applied strain of rhizobia and common bean cultivars. The interaction (strain x cultivar) was not significant for the dry nodule biomass and the shoot dry weight, while only for the N content in dry shoot, a significant interaction was found.

On the roots of the Trešnjevac cultivar, significantly higher dry nodule biomass was found compared to the Slavonski zeleni cultivar. Figure 3 shows the average weight values for the dry nodule biomass. The highest dry nodule biomass (0.170 g) was determined when the indigenous strain HO12 was applied. The lowest dry nodule biomass per plant was determined applying the indigenous strain GU13. No nodules were observed on the non-inoculated control variants.

Figure 1. Vegetation experiment in full blossom phase

Table 1. Results of analysis of variance - significance of the tested factors and interactions of the tested properties

Source of variability	Dry nodule biomass	Shoot dry weight	N content in dry shoot
Rhizobial strain	***	***	***
Common bean cultivar	*	**	***
Strain x common bean cultivar	ns	ns	**

1* significance level *P*<0.05; *** significance level *P*<0.001; ns – not significant

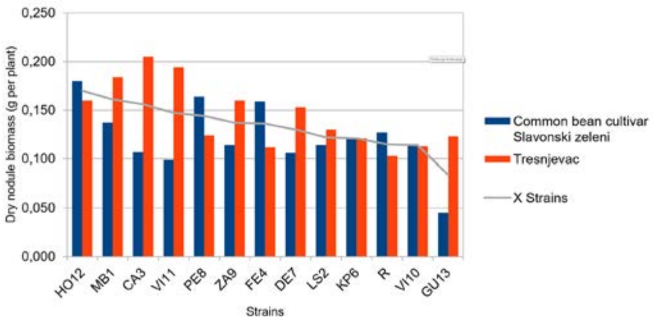

Figure 3. Dry nodule biomass (g per plant)

The differences between the reference strain and the indigenous strains were not statistically significant, for this tested trait, although the application of the reference strain resulted in lower values compared to most indigenous strains.

As no statistically significant differences were found between applied indigenous strains, it can be concluded that all of them possess great ability to nodulate both common bean cultivars. However, the ability to nodulate the legume is not positively correlated with symbiotic efficiency (Pohajde et al., 2016), and therefore it is necessary to access other traits to estimate strain's efficiency.

Significantly higher shoot dry weight was found for the cultivar Trešnjevac then for Slavonski zeleni. Figure 4 shows the average values for the shoot dry weight (g per plant). The highest shoot dry weight was determined when strains MB1 and DE7 were applied (8.883 g and 6.673 g respectively). No statistically significant differences between these two strains were found. The strain DE7 did not differ significantly from other applied strains, while only strain MB1 differed significantly from all other strains. Although the average lower shoot dry weight was obtained in non-inoculated variants, these values were not significantly different from those in which inoculation was applied. The obtained results for shoot dry weight indicate that indigenous strains MB1 and DE7 are the most efficient while indigenous strains VI10 and FE4 show weaker symbiotic efficiency.

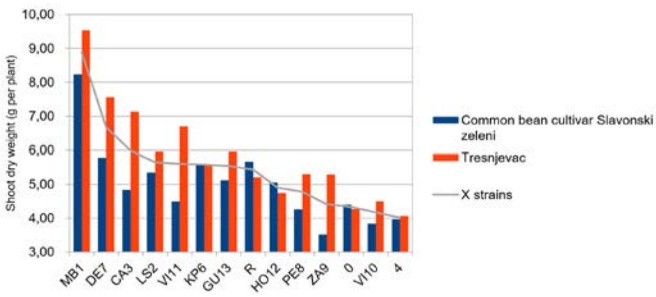

Figure 4. Shoot dry weight (g per plant)

Figure 5 shows the average values for the N content in dry shoot (%). Higher values for N content in dry shoot were found for the cultivar Trešnjevac in relation to the cultivar Slavonski zeleni. Differences in the total nitrogen content in dry shoot could also be observed by leaf color between non-inoculated (light green) and inoculated variants (darker green) (Figure 2). Significant differences were found for the N content in dry shoot between individual strains. Significantly highest value for this characteristic was determined applying strain DE7 (3.073%). Second group in analysis of shoot was comprised of strains KP6 and PE8 with no significant differences from strains MB1, LS2 and VI11. The lowest content of total nitrogen was determined on the non-inoculated control, as well as with the variant with applied strain FE4. Therefore, this result confirmed that the strain FE4 was the least symbiotically effective and can be grouped with non-inoculated control. The indigenous strains MB1, LS2, CA3, ZA9, VI11 and HO12 showed similar symbiotic efficiency as the reference strain, as no statistically justified difference was found between those strains. Kawaka et al. (2014) proved the same by testing the symbiotic efficiency of indigenous strains of common bean nodulating bacteria in western Kenya. In their research on the effectiveness of commercial inoculum, Mungai and Karubiu (2012) also found indigenous strains that had similar symbiotic efficiency compared to commercial inocula.

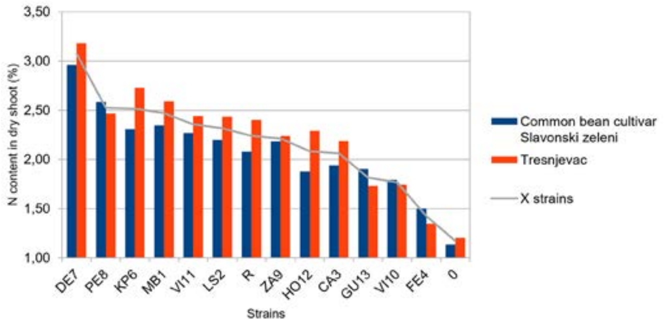

Figure 5. N content in dry shoot (%)

When observing the interaction of cultivars x strains, the highest values for the N content in dry shoot on cultivars Trešnjevac and Slavonski zeleni were determined applying strain DE7. The results of these studies confirmed the differences in symbiotic efficiency between the applied strains of nodule bacteria, so it is very important to choose the most effective strain to be applied. The obtained results indicate that indigenous strains MB1 and DE7 with the highest symbiotic efficiency, and KP6 and PE8 with slightly weaker symbiotic efficiency could be used in further field research with the potential to be applied as inoculum for pre-sowing inoculation of common bean in Northwestern Croatia. Strain HO12 showed the best performance when testing the ability to nodulate common bean (ie. highest dry nodule biomass was obtained). On the contrary, by application of this strain, lower shoot dry weight, and lower N content in dry shoot were obtained. It was found in previous research that those features do not have to correlate (Rajnović, 2017). The part of nitrogen that rhizobia convert in a symbiotic relationship, plants utilize for the development of their cellular structures which increases their biomass. Therefore, the efficiency in symbiotic nitrogen fixation can also be assessed by comparing the shoot dry weight of inoculated plants and the shoot dry weight produced by an uninoculated plant. Also, comparing indigenous strains with the reference strain, it was found that most indigenous strains have a great ability to nodulate common bean and differ in their symbiotic efficiency. In order to maximize the pre-sowing inoculation and more successful cultivation of common bean, it is necessary to conduct additional research in field experiment.

CONCLUSIONS

The current research aimed at estimating the symbiotic efficacy of indigenous common bean rhizobia isolated from Northwestern Croatia. Based on the obtained results of this experiment it can be concluded that the tested strains differ in their symbiotic efficiency. Most of the applied indigenous strains, in comparison with the control variants, have a positive effect on the tested properties, especially on the N content in dry shoot. By comparing indigenous strains with the reference strain, most indigenous strains of nodule bacteria have a high nodulation ability and show good symbiotic efficacy with both common bean cultivars. It was found that inoculation of common beans with indigenous strain HO12 produced the highest value for the dry nodule biomass. Since no statistically significant differences were found between strain HO12 and other indigenous strains, it was proved that all these strains possess a high ability to nodulate both cultivars of common beans. The highest shoot dry weight was achieved with the use of indigenous strains MB1 and DE7, while the highest total nitrogen content in dry shoot was achieved with the use of strain DE7. The interaction of indigenous and reference strains with the Trešnjevac cultivar was significantly better than with Slavonski zeleni in all measured parameters. The indigenous high-quality strains MB1 and DE7 should further be evaluated in the field trial as potential application as pre-sowing common bean inoculation.

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