Intercropped red beet and radish with green bean affected microbial communities and nodulation by indigenous rhizobia

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The impact of intercropping green bean (*Phaseolus vulgaris* L.) with red beet (*Beta vulgaris* L. *var. rubra*) and radish (*Raphanus sativus* L.), two non-legume plants, on the plants' yields, as well as the effect on occurrence and enumeration of microorganisms in the rhizosphere was studied. The intercrop efficacy evaluation, using Land equivalent ratio, revealed values above 1.0 for all intercropped treatments. Diversity of rhizobia from green bean nodules under different intercropping and fertilizing conditions was observed. On the basis of morphological and biochemical characteristics, 67 out of 158 isolates from green bean roots were selected as rhizobia (42.4%), confirmed by detection of 780 bp *nifH* gene fragments in *nifH*-PCR, and then clustered in 27 phenotype patterns. Production of exopolysaccharide succinoglycan was observed in 23 rhizobial isolates, while 6 were detected to solubilize tricalcium phosphate. Screening of genetic diversity using $(GTG)_5$-PCR fingerprinting showed presence of six different patterns on the 92% similarity level.

Key words: *Phaseolus vulgaris*, *Beta vulgaris*, *Raphanus sativus,* PCR, mixed cropping

Introduction

Intercropping is practiced in the production of food for humans and livestock throughout the world (Ghosh 2004). Due to the low level of mechanization of agricultural production and limited use of external inputs, intercropping is especially popular on the small farms in developing countries (Singh et al. 2010).

Intercropping is defined as the simultaneous growing of two or more species in the same area during the most of their life cycle (Vandermeer 1989). Comparing to sole crops, intercrops are able to provide many benefits. It was found that intercropping may increase yields (Zhang et al. 2011), income (Yildirim and Guvenc 2005, Singh et al. 2013), availability of plant-nutrients (Betencourt et al. 2012), reduce the occurrence of diseases (Gomez-Rodriguez et al. 2003), suppress weeds (Sharma and Banik 2013), and decrease the demands for fertilization and use of pesticides (Carruthers et al. 2000). It can be a helpful tool for soil remediation (Sun et al. 2011) and reduction of wind erosion (Chen et al. 2010). The effective examples of tree-based intercropping and agroforestry systems could be added to this list too (Rivest et al. 2009).

Microbial communities are a fundamental component of soil with impact on plant nutrient acquisition, growth and health. Microorganisms are fundamental in metabolism of organic matter, including biological nitrogen (N_2) fixation, which catalyzes the reduction of atmospheric N_2 gas to biologically available ammonium (Arp 2000, Madigan et al. 2000). The diazotroph populations in different environment capable to fix nitrogen are diverse and involve many bacterial genera. The *nifH* gene, encoding the dinitrogenase reductase enzyme, is one of the target genes most frequently used to amplify PCR products representing the diazotrophic community, which is spread through numerous phylogenetically distant groups (Zehr et al. 2003). One of the soil-bacteria groups harboring *nifH* gene is rhizobia. Able to establish nitrogen-fixing symbioses in root nodules of legume plants (*Fabaceae* family) and promoting plant growth and yield, rhizobia are often used as biofertilizers in the cultivation of most legume plants. Several studies have reported indigenous soil rhizobia to negatively affect successful symbiotic relationships with legume hosts through competition with inoculant strains (Lima et al. 2009). The reduced symbiotic efficiency between indigenous rhizobia, with N-fixed up to 72% comparing to the inoculant strain, have been reported (Ballard and Charman 2000). Also, the same authors reported better symbiotic efficiency with indigenous rhizobia, which form effective associations with their legume hosts, than introduced strains. Soil management practices also affect populations of rhizobia. Long-term monocultures reduce diversity of rhizobia compared to crop rotation with legume host plants (Depret et al. 2004, Grossman et al. 2011). The high diversity of bean rhizobia in no-till fields compared to conventional till fields are also reported (Kaschuk et al. 2006).

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Thanks to their ability to make symbiotic relationship with nitrogen-fixing bacteria, legume crops are good providers of nitrogen and the most frequent members of intercrops. Many trials were conducted in order to investigate influence of different species in intercrops, especially cereals and legumes, such as maize and beans (Fischler et al. 1999), maize and peanut (Xiong et al. 2013), wheat and chickpea (Banik et al. 2006, Betencourt et al. 2012), barley and pea (Launay et al. 2009).

Studies about intercrops consisted of other non-legume species are not so numerous (Yildirim and Guvenc 2005, Singh et al. 2010, Tosti and Thorup-Kirstensen 2010, Singh et al. 2013). Also, there is no available data about effect of intercropped green beans with radish or red beet on biodiversity of the soil bacteria and symbiotic microorganisms.

Recently, the interest for red beet and radish has arisen because of medical benefits of their principal components anthocyanins and glucosinolates (Krajka-Kuźniak et al. 2012, Gao et al. 2014). In terms of acreage, production or consumption, red beet and radish are not the world's major vegetables but they occupy unique niches in Europe, parts of Asia and North America (Goldman and Navazio 2008). Despite the good conditions for radish and red beet breeding in Serbia, production is apparently insufficient for local market. The data concerning production and yields in Serbia are not available (Statistical Office of the Republic of Serbia 2014), but there is data related to trading of root vegetables (beetroot, celery, radishes and similar edible roots). The annual import of root vegetables for 2012 was 535 tons and export was 902 tons (United Nations Commodity Trade Statistics Database 2014).

This study was carried out with a purpose to investigate the impact of intercropping green bean (*Phaseolus vulgaris* L. var. *nanus*) and two non-legume plants: red beet (*Beta vulgaris* L. var. *rubra*) and radish (*Raphanus sativus* var. *major* L.) (i) on the plants' yields, (ii) on occurrence and enumeration of microorganisms in the rhizosphere of green bean, radish and red beet and (iii) on indigenous rhizobia from green bean nodules. Diversity of rhizobia from green bean nodules under different cropping and fertilizing condition was observed using phenotypic and genotyping methods.

Material and methods

Experimental design and field management

The field experiment was carried out in the growing season 2010 at the Institute for vegetable crops, located in Smederevska Palanka in central Serbia (44° 22' N, 20° 57' E, elevation 101 m). The experiment was conducted on the vertisol soil type with following properties: pH 6.7; percentage of organic matter 3.13, nitrogen 0.16, calcium carbonate 0; available phosphorus 374.2 ppm and available potasium 335.6 ppm (Pivic et al. 2011). Phosphorus and potassium were extracted with ammonium lactate (AL) solution (Egner et al. 1960, Pivic et al. 2011). The preceding crop was wheat.

The experiment had a completely randomized block design with ten treatments and three replicates making a total of 30 plots. Green bean (*Phaseolus vulgaris* L. var. *nanus* cv. 'Palanacka rana'), red beet (*Beta vulgaris* L. var. *rubra* cv. 'Palanacka crvena') and radish (*Raphanus sativus* L. cv. 'Zimska bela') were grown alone without (K, A and D, respectively) or with NPK fertilizer (N, B and E, respectively). Bean was intercropped with red beet and radish without (C and R, respectively) or with NPK fertilizer (P and F, respectively). The amounts of 75 kg ha^{-1} of nitrogen, phosphorus and potassium were applied by commercial mineral fertilizer (N-P-K 15-15-15).

The experimental plot consisted of 12 rows. The distance between rows was 0.4 m, both in the sole and intercrop treatments. The sowing densities were 250×10^3, 125×10^3 and 125×10^3 plants per hectare for sole green beans, radish and red beet, respectively. The intercrops were created according to the method of replacement series (two rows of green beans and two rows of radish or red beet). The size of the experimental plots was 12.5 m^2 (2.5 × 5.0 m). The experimental plots were separated from one another by 0.5 m spacing. After seeding of green bean (2 August 2010), red beet and radish were sown as intercrops and sole crops (3 and 4 August, respectively). Weeds were controlled manually. Plots were watered several times during the growing season. All applied measures, except fertilizing (control), were according to standards concerning organic production. Average monthly temperatures and precipitations during the trial are shown in Table 1.

Table 1. Average temperatures (AT), precipitation (P), cumulative daily mean temperatures (CDMT) and total precipitations during the growing season.

	July	August	September	October	CDMT* / Total**
AT (°C)	23.0	23.7	17.0	10.1	1969.5*
P (mm)	59.3	17.2	57.8	60.9	195.2**

At the maturity stage for each crop (8 October 2010 green bean, 15 October 2010 radish and red beet), yields of inner rows were measured. The yields of samples which were taken previously were added and Land Equivalent Ratio (LER) index was calculated for intercrops (Willey 1979). LER is usually used for an intercrop efficacy evaluation. When the competition for light, water and soil resources among intercropped species is not significant, values of LER are above 1.0, which means the intercrop is favored and if the value is below 1.0, the monocultures are favored. LER is the sum of the relative yields (RY) of intercrops: LER= $RY_{green\ bean}$ + $RY_{red\ beet}$ or LER = $RY_{green\ bean}$ + RY_{radish} (Vandermeer 1989). The relative yields of green bean, red beet and radish were calculated using the following equation: RY = P / M (P is yield of some crop per hectare in intercrop and M is its yield per hectare in monoculture).

Microbial populations in rhizosphere

The rhizosphere soil for microbiological analysis was sampled at the flowering stage of green bean. Rhizosphere samples were collected from the depth of 15–30 cm adhering very closely to plants' roots. Samples from all 10 variants were ten-fold serially diluted in sterile saline (0.9% sodium chloride) and appropriate dilutions were spread in triplicates on the appropriate nutritive medium for each microbial group. Total number of microorganisms was counted on soil agar, the fungi on Czapek agar, actinomyces on the synthetic agar with sacharose according to Krasilynikov, ammonifiers in the liquid medium with asparagines as the source of nitrogen, azotobacters and oligonitrophiles on Fiodorov medium and nitrificators on soluble medium (Jarak and Đurić 2004, Rasulić et al. 2012). Colony forming units (CFU) were counted after 2 or 5 days of incubation at 26 °C and calculated per gram of dry soil.

Fenotyping of bacteria from green bean nodules

At the flowering stage nodules from green bean roots from 6 variants: K, N, C, P, R and F were taken for sampling. Five plants per plot from each variant were randomly chosen and three nodules per plant (45 nodules per variant) were sterilized with $HClO_4$ and washed in ethanol and sterile water. Yeast mannitol agar (YMA) medium was used for isolation of bacterial colonies from nodules, morphology testing and propagation of isolates. The 25 randomly chosen colonies per variant were used for further investigation. For additional testing, YMA medium was supplemented with 3% $CaCO_3$ for acidification test, 0.1% Congo red for dye absorption test, 4% NaCl for salt tolerance and Hg (3 and 5µg ml^{-1}) for heavy metal (Hg) tolerance test. For pH tolerance test on various pH (from 5.5 to 8), YMA was adjusted with 1M HCl or 1M NaOH. Intrinsic Antibiotic Resistance (IAR) pattern was observed using: tetracycline (5 and 25µg ml^{-1}), kanamycin (1 and 3µg ml^{-1}) and chloramphenicol (1 and 3µg ml^{-1}). King's B agar medium was used to separate rhizobia from other endophytic bacteria. Pikovskaya medium was used for tricalcium phosphate (TCP) solubilization and clear halo appearance around colonies after 5 days of incubation was scored as a positive result (Djuric et al. 2011). Calcofluor fluorescence (CF) was used for assessment of succinoglycan production as one of phenotypic character of isolates as recommended by Leigh and Coplin (1992).

Phytopathogenicity assay

In order to differentiate beneficial from deleterious isolates, phytopathogenicity tests were performed by *ex vivo* methods (Moragrega et al. 2003) using bacterial inoculation of disinfected bean pods. Sterile distilled water was used as negative controls. Phytopathogenic *Bacillus* sp. Q13 (strain ISS 608 from ISS WDCM 375) strain was used as positive control. After incubation at 25 °C for 3–5 days in controlled environment chamber, the pathogenicity of isolates was assessed.

Detection of *nifH* gene

Nitrogenase reductase genes (*nifH*) were chosen as a nitrogen fixation marker in different rhizobia. The primers are designed for amplification of about 780 bp of the 890 bp *nifH* gene on the basis of known *nifH* sequences for *R. leguminosarum* bv. *trifolii*, *R. etli* bv. *phaseoli*, *S. meliloti*, *Sinorhizobium* sp. NGR234, *B. japonicum*, *Azorhizobium caulinodans*, *Azospirillum brasilense*, *Azotobacter chroococcum*, *Rhodobacter sphaeroides*, *Rhodobacter capsulatus* and *Rhodospirillum rubrum* (Laguerre et al. 2001). On the basis of morphological and growing properties, 67 isolates were assumed to be rhizobia. Confirmation of isolates harboring *nifH* genes was performed using *nifH*-PCR (Laguerre et al. 2001). Total DNA was isolated from cultures (OD_{600} =0.625) of the purified isolates grown at 26°C in YEM medium, following the protocol described by Laguerre et al. (1994). Amplification of *nifH* gene was performed in 25 µl reaction volume using Dream Taq Grean Master Mix (Thermo Scientific, Lithuania) and 0.1 µl M primers *nifHF*/*nifHI* (Metabion International AG, Martinsried, Germany). All 158 isolates were subjected to amplification in two replications.

Genotyping of rhizobia from green bean nodules

Isolates selected as rhizobia were used for further analysis. *Rhizobium leguminosarum* bv. *phaseoli* strain ISS 122 (ISS WDCM 375) was used for comparison. Total genomic DNA was extracted using a boiling method. Screening of genetic diversity was performed using BOX analysis by $(GTG)_5$ primer (Versalovic et al. 1994). All PCR reactions were performed using DreamTaq Green Master Mix (Thermo Scientific, Lithuania) in 25µl reaction mixture and undertaken in an Eppendorf Master Cycler Personal (Germany). Products of amplifications were separated on 1.5% agarose gels, stained with 5% ethidium bromide (EtBr) and visualized on UV transilluminator (MacroVue UVis-20, Hoefer).

Statistical analyses

Phenotypic and PCR fingerprint results were converted to binary form and cluster analysis of isolates were done using STATISTICA 7 program.

Results

Plants' yields and LER index

Values of LER index were above 1.0 for all intercropped treatments, as shown in Table 2.

Table 2. Yields of sole crops (SC), relative yields (RY) and land equivalent ratio (LER) of intercropping (IC) green bean with red beet and green bean with radish.

IC	Treatment	SC yield 10^3 kg ha^{-1}	SE	RY	SE	LER	SE
Green bean	Control	13.75	0,2367	0.52	0.0133	1.06	0.0115
Red beet		13.87	0,1313	0.54	0.0067		
Green bean	NPK fertilizer	16.25	0,3014	0.51	0.0153	1.08	0.0219
Red beet		17.50	0,4117	0.57	0.0088		
Green bean	Control	13.75	0,2367	0.43	0.02	1.19	0.0120
Radish		40.78	1,1936	0.76	0.0145		
Green bean	NPK fertilizer	16.25	0,3014	0.45	0.0176	1.23	0.0120
Radish		48.69	2,2275	0.79	0.0145		

SE - standard error; NPK- mineral fertilizer (N-P-K 15-15-15)

Microbial populations in rhizosphere. The total number of bacteria and fungi, and specific groups: actinomyces, ammonifiers, nitrificators and azotobacters, were investigated in all treatments of both monocultured and intercropping systems (Table 3).

Table 3. Microbial populations in rhizosphere of green bean, red beet and radish

Sample	Bacteria[a] CFU[c] × 10^6 g^{-1}	Actinomyces[a] CFU × 10^4 g^{-1}	Ammonifiers[b] CFU × 10^6 g^{-1}	Azotobacters[b] CFU × 10^4 g^{-1}	Nitrificators[b] CFU × 10^4 g^{-1}	Fungi[a] CFU × 10^4 g^{-1}
green been (K)	75.00	36.00	45	110	140	17.67
green been + NPK (N)	77.67^{ns}	13.00**	15**	25**	140^{ns}	17.67^{ns}
red beet (A)	23.33**	40.00**	45^{ns}	25**	110**	11.67**
red beet +NPK (B)	33.00**	26.33**	15**	45**	110**	12.33**
green bean + red beet (C)	49.00**	38.67*	45^{ns}	15**	25**	9.67**
green bean + red beet + NPK (P)	50.33**	24.33**	7.5**	9.5**	15**	13.00**
radish (D)	46.00**	28.33**	15**	110^{ns}	140^{ns}	9.67**
radish + NPK (E)	57.33**	25.33**	20**	25**	140^{ns}	16.67^{ns}
green bean + radish (R)	48.33**	41.67**	25**	110^{ns}	110**	12.33**
green bean + radish + NPK (F)	67.33**	18.33**	25**	45**	110**	13.67**
$LSD_{0.05}$	3.925	2.37	5.189	4.785	8.033	1.068
$LSD_{0.01}$	5.583	3.372	7.38	6.805	11.426	1.519

[a]Number of microorganisms per g of absolute dry soil; [b]MPN – Most probable number of microorganisms per g of absolute dry soil; [c]CFU-colony forming units; NPK- mineral fertilizer (N-P-K 15-15-15); LSD- Least Significant Difference *p<0.05 ** p < 0.01

Phenotyping of bacteria from green bean nodules

Total of 150 colonies were included in analyses: 25 colonies from each variant were randomly chosen and the same number of pure isolates were obtained for variants C, P, R and F. Additional 8 isolates were obtained during separation of isolates from the same colony from variant K (additional 5 isolates) and variant N (additional 3 isolates). All bacterial isolates from green bean nodules were tested for growth on YMA and KB mediums. All isolates showed ability to grow on YMA medium with a pH range from 6.0 to 8.0. Abundant growth on KB agar medium showed 57.6% of isolates (91). Out of 158 isolates, 67 isolates which grew only on YMA were selected for comparison as potential rhizobia (Table 4). The highest number of rhizobia- 16 out of 30 (53.3%) was encountered in variant K – green been grown as a sole crop without fertilizer. Minimal number of rhizobia - 6 out of 28 (21.4%), was obtained in control fertilized variant N. Out of 25 isolates per variant for intercropping with red beet and radish, the numbers of rhizobia were 13 (52%) for both C and P, followed by 12 (48%) and 7 (28%) for F and R variants, respectively.

Selected rhizobia were negative for growth on YMA supplemented with 4% NaCl, tetracycline 5 and 25µg ml^{-1}, kanamycin 3µg ml^{-1}, chloramphenicol 3µg ml^{-1} and have no ability to solubilize $CaCO_3$. Isolated rhizobia showed differences in tolerance to Hg (3 and 5µg ml^{-1}), growth in different pH, absorption of Congo red dye, TCP solubilization and fluorescence in the presence of Calcofluor dye in medium.

Detection of *nifH* gene and phytopathogenicity assay

Morphological and biochemical characteristic of 67 rhizobial isolates from green bean nodules were confirmed by detection of 780 bp *nifH* gene fragments. Two isolates grown on KB, belonging to *Bacillus* genera, yielded several products of unexpected sizes. In order to test isolates for their potential phytopathogenic effect on plants and avoid selection of isolates with deleterious effects, phytopathogenicity tests were performed by *ex vivo* method on young bean pods. None of the selected 67 rhizobial isolates caused necrosis on the bean pods, while the bean pods inoculated with *Bacillus* sp. Q13 strain (positive control) become necrotic after 24h and disease progression is clearly observed during 5 days.

Table 4. Several phenotypic traits of isolates harboring *nifH* gene and negative for growth on KB medium and phytopathogenicity

Isolates	Phenotype (No of isolates)	Growth on YMA[a]								
		pH 5.5	pH 8.5	Hg[b] 3 µg ml^{-1}	Hg 5 µg ml^{-1}	Kan[c] 1 µg ml^{-1}	Chl[d] 1 µg ml^{-1}	Congo red apsorption	Calcofluor fluorescence	TCP[e] solubilization
K38, K297, C69, C264, C266	a (5)	–	–	–	–	–	–	–	–	–
K121, K126, K186, K286, K288, K401; P272, P210, P273	b (9)	–	–	–	–	–	–	–	+	–
K122, K289	c (2)	+	–	+	–	–	–	+	–	–
K200, C174, C212, C218; N118, F443	d (6)	–	–	+	–	–	–	–	–	–
K214, C88	e (2)	+	–	+	–	–	+	+	–	–
K215	f (1)	+	–	–	–	–	+	–	–	–
K224, R199	g (2)	–	–	+	+	–	–	–	–	–
K225	h (1)	–	–	+	+	–	+	–	–	–
K285	i (1)	–	–	–	–	–	–	+	–	–
N220, N221	j (2)	+	–	+	+	–	–	+	+	–
N248, P216, F128, F149, F204, F235, F435, F437; R101, R104, R107	k (11)	–	–	–	–	–	+	–	–	–
N251, P217, P303	l (3)	+	–	–	–	–	–	–	+	–
N305, P211, P269, P293, P304	m (5)	+	–	+	–	–	–	–	+	–
C89	n (1)	–	–	+	–	–	–	–	–	+
C112	o (1)	–	–	–	–	–	–	+	+	–
C213	p (1)	–	–	+	–	–	–	+	–	+
C227, C300	q (2)	+	–	+	–	–	–	–	–	–
C263	r (1)	+	+	+	–	–	–	–	–	–
P209	s (1)	+	–	+	+	–	–	+	–	+
P271	t (1)	+	–	+	–	–	–	–	+	+
P309	u (1)	+	–	+	+	–	+	+	–	–
R197	v (1)	–	–	–	–	+	–	–	–	–
R232	w (1)	+	–	+	+	–	+	–	–	–
R238	x (1)	+	–	+	+	+	–	–	–	–
F187, F243a	y (2)	–	–	+	+	–	–	–	–	–
F229, F437a	z (2)	–	–	+	+	+	–	–	+	+
F268	š (1)	–	–	+	+	–	–	+	–	–
Number of phenotype patterns	27									

[a]all positive for growth on Yeast Mannitol Agar (YMA) pH 6.0–8.0; all negative for growth on YMA supplemented with 4% NaCl, tetracycline 5 and 25µg ml^{-1}, kanamycin 3µg ml^{-1} and chloramphenicol 3µg ml^{-1}; all negative for solubilization of $CaCO_3$. [b]Hg- mercury (added as $HgCl_2$); [c]Kan- kanamycin; [d]Chl- chloramphenicol; [e]TCP- tricalcium phosphate

On the basis of morphological, phenotypic and *nifH*-PCR results, 67 isolates from green bean roots were selected as rhizobia, grouped into two groups: without and with fertilizing, and compared on the basis of phenotypic properties. Rhizobia from 3 variants without fertilizing – K, C and R, involved 36 isolates showing 17 different phenotypic patterns, divided in two major clusters with 60% similarity, as shown in Figure 2. Both clusters include isolates from all variants.

Out of 31 rhizobial isolates from nodules of green bean grown on fertilized soil (N, P and F variants), 13 were phenotypically different (Fig. 3). Three major clusters with 60% similarity are formed. One of them contains only few isolates from F variants (4), while the second contains 5 isolates from all variants. The major cluster is divided into 2 subclusters: one includes 7 isolates from P and 2 from N variants, while the second includes the majority of isolates from all variants, very similar mutually.

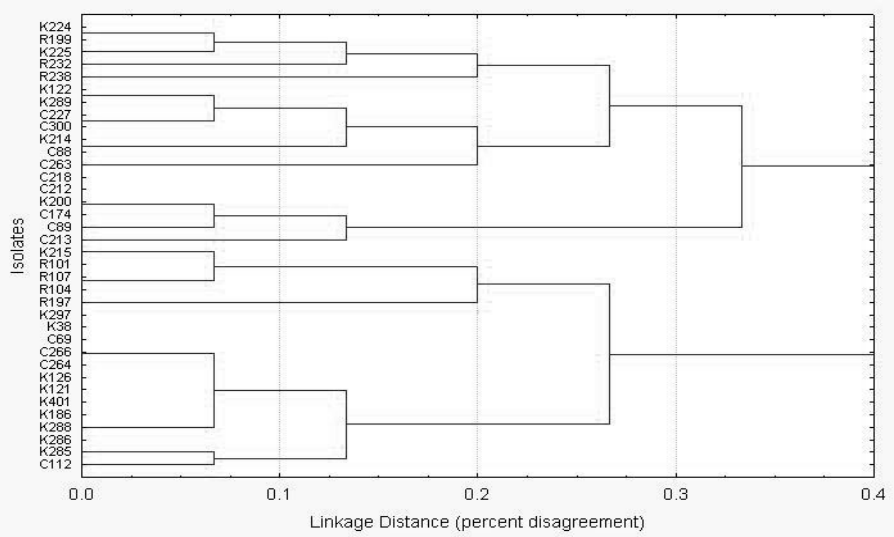

Fig. 1. Phenogram of isolates from nodules of green bean grown in the soil without a fertilizer: as a sole crop (K), intercropped with red beet (C) and radish (R)

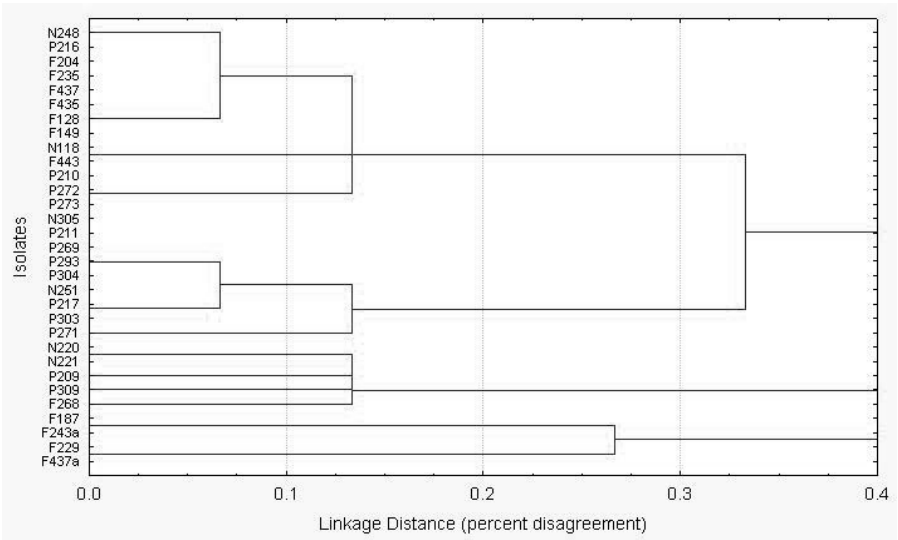

Fig. 2. Phenogram of isolates from nodules of green bean grown in the soil with a fertilizer: as a sole crop (N), intercropped with red beet (P) and radish (F).

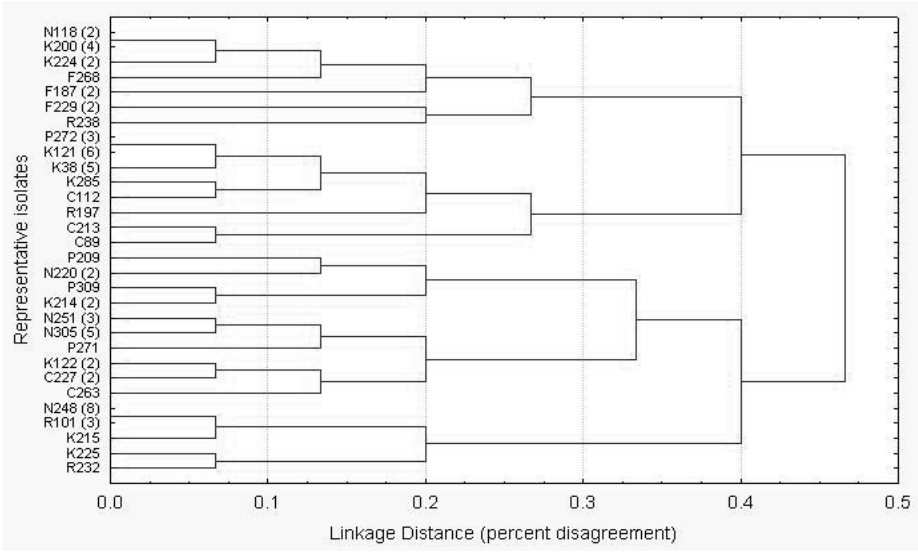

Fig. 3. Phenogram of representative isolates from nodules of green bean grown as: a sole crop without (K) and with fertilizer (N); intercropped with red beet without (C) and with fertilizer (P); intercropped with radish without (R) and with fertilizer (F). The frequencies of isolates are marked in parentheses.

All rhizobial isolates from nodules of green bean that were grown in all six variants, showed 27 patterns, as shown on the phenogram of representative isolates (Fig. 4).

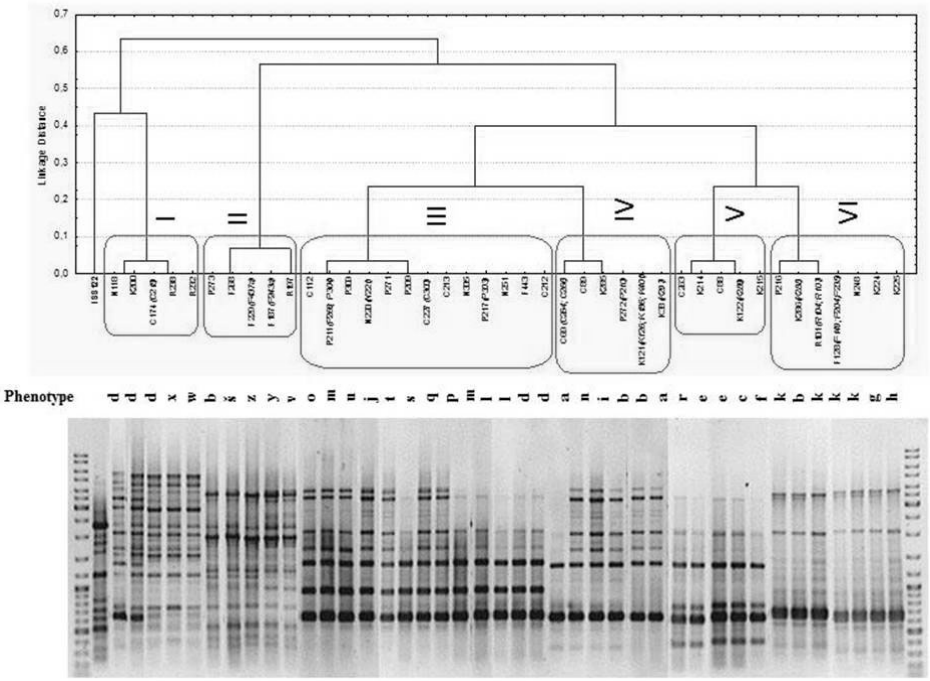

Fig. 4. Dendrogram of representative isolates from nodules of green bean based on $(GTG)_5$-PCR fingerprinting and phenotype groups of isolates (isolates with the same genotype and phenotype patterns originated from the same variants are marked in parentheses). The scale bar represents percent disagreement.

Genotyping of rhizobia from green bean nodules

Screening of genetic diversity using BOX analysis by $(GTG)_5$ primer showed presence of six different patterns (Fig. 5). Unsuccessful $(GTG)_5$-PCR fingerprinting was obtained for *nifH*-PCR positive isolates from nodules of green bean grown intercropped with radish (F435, F437 and R199) and one isolate from nodules of green bean grown intercropped with fertilized red beet (P293). *Rhizobium leguminosarum* bv. *phaseoli* strain ISS 122 was used for comparison.

Discussion

Periodic selection of rhizobia in nodules could increase the number of the better-adapted bacteria for a region and cultivar and may constitute the basis for co-evolution (Martinez-Romero 2003). Selection of well-adapted isolates may determine the success of these bacteria in beans and improve bean nodulation capacity or competitiveness. In our study, the abundance of several microbial population, and particularly the presence and diversity of indigenous rhizobia affected by different variants of cultivation (monoculture, intercrop and fertilizing) were observed.

LER index values obtained in our trial, used for an intercrop efficacy evaluation, were above 1.0 for all intercropped treatments (1.06−1.23). The lower relative yield of green bean was recorded but it was neutralized by higher radish relative yields and values of LER index were above 1.0 on fertilized and unfertilized treatment. The highest value of LER was recorded when green bean was fertilized and intercropped with radish, whereas the lowest values occurred in unfertilized green bean/red beet intercropping. LER index values were 1.06 and 1.08 for unfertilized and fertilized red beet/green bean intercropping, respectively. With additional nutrients, relative yield of red beet was increased and relative yield of green bean has gently decreased, probably due to the higher competitiveness of the red beet. Similarly, trials in rhizotrone tubes confirmed that the red beet root system is more competitive comparing to legumes (Tosti and Thorup-Kristensen 2010). Red beet is rarely examined as a member of intercrops (Filho et al. 2003, Tosti and Thorup-Kristensen 2010). Subjected to the time of establishment, values of LER index for intercropped red beet and roquette (rucola) were 1.01 to 1.27 (Filho et al. 2003).

In our study, the highest value of LER index was recorded when green bean was fertilized and intercropped with radish. Yildirim and Guvenc (2005) studied intercrops with several *Brassicaceae* species. They reported value of LER index higher than 1.0 for intercropped cauliflower and green bean. Our results are in accordance with the results of Kocacaliskan (2001) that radish express a decreased growth and yield of other crops due to the allelopathic effects.

We tested microbial population in rhizosphere of different intercropping variants, knowing that microorganisms in soil depend on many abiotic factors (pH, temperature, soil moisture, presence of different harmful substances), including the type of fertilizers, but also on soil types and growing plants. Compared with monoculture, intercropping significantly decreased the quantity of soil microorganisms in rhizosphere of green bean and increased this quantity in beetroot areas in this study. Actinomycetes were more abundant in plants' rhizospheres without fertilization and their numbers ranged from 36–40 × 10^4 g^{-1}. The higher number of fungi was observed in green bean rhizosphere independently of fertilization. Plants can induce a proliferation or inhibition of microorganisms in rhizosphere by producing easily degradable substrates in root exudates (Bais et al. 2006). In this study, intercropping significantly decreased the number of fungi comparing to rhizosphere of green bean as a sole crop, especially in samples without fertilization. The most abundant group of tested microorganisms was ammonifiers with the highest number of CFU (45 × 10^6 g^{-1}) in samples from non-fertilized soils taken from green bean rhizosphere as sole and intercropped plant. The numbers of soil nitrificators and azotobacters showed significant differences in the root areas of intercropped and monocultured plants; it was, however, lower in intercropped than in monocultured root area. Compared to the non-fertilized intercropping, fertilization slightly increased the fungi number, but significantly reduced the numbers of ammonifiers, nitrificators, actinomyces and azotobacters.

The effects of nitrogen supply levels on the rhizosphere microorganism of crops were studied in wheat and broad bean intercropping. The amount of rhizosphere microorganisms increased with the increase of nitrogen supply, but decreased in rhizosphere of both crops with the supply of high nitrogen. Intercropping with the nitrogen treatment significantly increased the amount of rhizosphere microorganisms of wheat, but had the inhibitory influence on the broad bean (Wei et al. 2008). Our results showed lower number of soil bacteria in green bean rhizosphere and higher number in beet rhizosphere in intercropping variants and those numbers were independent from fertilizing.

The phenotyping of bacteria from green bean nodules showed the highest number of rhizobia in green been grown as a sole crop without fertilizer (53.3%), than variants of intercropping with red beet (52%) in both fertilizing variants. Only 21.4% of bacteria belonging to rhizobia was obtained in control fertilized variant N.

Growth on YMA pH 5.5 showed 25 rhizobial isolates from different variants, while only C263 grew at pH 8.5; more than 50% and 20% of isolates were tolerant to 3 and 5 µg ml^{-1} Hg, respectively. Intrinsic antibiotic resistance to kanamycin (1µg ml^{-1}) showed only 4 isolates, all from green bean nodules intercropped with radish. Isolates resistant to 1µgml^{-1} of chloramphenicol (17) belong to all variants.

Rhizobia produce two types of exopolysaccharide: succinoglycan (EPSI) and galactoglucan (EPSII), which both play a crucial role for nodule invasion, in the host defenses and in providing protection from abiotic stress (draught, high acidity) (Leigh and Walker 1994). Calcofluor fluorescence (CF) is characteristic for succinoglycan production (Leigh and Coplin 1992). In this study, CF fluorescence was observed in 23 rhizobial isolates belonging to 7 phenotypic patterns, suggesting EPSI production. Congo red absorption (observed in 12 isolates) and CF fluorescence are important phenotypic traits in rhizobia. Only 3 isolates showed both phenotypes.

Isolates were tested for their ability to solubilize tricalcium phosphate (TCP). Out of 67 rhizobia, only 6 formed zone of TCP solubilization (~ 9%). Four of them were originated from intercrop with red beet - two from unfertilized and two from fertilized variants. Sridevi and Mallaiah (2007) reported TCP solubilization zones in 26 out of 46 rhizobial isolates (56.5%) isolated from root and stem nodules of 20 different legume hosts. Studies on phosphate solubilizing ability of rhizobia reported that several species are involved in phosphate solubilization (Daimon et al. 2006, Rivas et al. 2006, Sridevi and Mallaiah 2007). Availability of phosphate in soil is greatly enhanced through microbial production of metabolites and release of phosphate from organic and inorganic complexes. Phosphorus deficiency in soil can limit plant growth productivity. In leguminous plants, lack of phosphorus may affect both symbionts: the plants and rhizobia, and this may have a deleterious effect on formation, development and function of nodules. In addition to nitrogen fixation, phosphate solubilization ability of rhizobia may lead to great beneficial nutritional effect for legume (Peix et al. 2001).

The 67 rhizobial isolates from green bean nodules were confirmed by detection of 780 bp *nifH* gene fragments. Similarly, in addition to high specificity between hairy vetch and *R. leguminosarum* biovar *viciae*, *nifH*-PCR is used

for rhizobia confirmation during investigation of large number of isolates (Mothapo et al. 2013). However, two *Bacillus* isolates from radish rhizosphere yielded several products of unexpected sizes. Since *nifH* encodes the highly conserved Fe protein of nitrogenase and has been used as a marker gene for nitrogenase, we assumed that amplicons of different size may not represent fragments of *nifH* gene.

Rhizobia grouped into two groups: without (36 isolates) and with fertilizing (31 isolates) were compared on the basis of phenotypic traits. The first group showed 17 phenotypic patterns divided in two major clusters with 60% similarity and both contained isolates from all tested variants. The second group with 13 different patterns clustered in 3 major clusters with 60% similarity. One of them contains only 4 isolates from F variants. The major cluster is divided into 2 subclusters: one includes 7 isolates from P and 2 from N variants, while the second includes the majority of isolates from all variants, very similar mutually. All isolates from all six variants showed 27 patterns, which are divided into two clusters (34 and 33 isolates) with 53% similarity. The frequencies of isolates were different and ranged from 1 to 9 (1.5 to 13.4%). Three groups of isolates from fertilized soils showed identical phenotypic pattern as isolates from variants without fertilizing.

Genotyping was done using $(GTG)_5$-PCR. Among the six fingerprint patterns (I–VI) obtained on the 92% similarity level, only one (I) clustered together with *Rhizobium leguminosarum* bv. *phaseoli* strain ISS 122, which was used as the marker strain in this study. Six isolates (five from all unfertilized variants and one from variant N) formed cluster I with 43% dissimilarity to marker strain ISS122 and 63% with other isolates. Pattern II, detected in six isolates from green been intercropped with radish and one isolate from green been intercropped with fertilized red beet, showed 44% similarity with patterns III–VI. The majority of isolates from nodules of green bean grown intercropped with red beet clustered together and formed fingerprint patterns III(12) and IV(6). Only two out of 13 isolates forming pattern IV originated from one fertilized variant (P), while 12 out of 17 isolates forming pattern III belong to all fertilizing variants: N(4), P(7) and F(1). Isolates from variant K amplified patterns I(1), IV(7), V(4) and VI(4). Pattern VI, clustered with V (showing 24% differences) and contained seven isolates from nodules of green bean grown intercropped with radish. Isolates from phenotypic group d were found among fingerprinting patterns I and III, while isolates from phenotypic group b were dispersed in genotype patterns II, IV and VI. All other isolates of the same phenotype belong to the same genotype pattern.

Diversity of rhizobia isolated from *Phaseolus vulgaris* has been examined almost worldwide. High diversity of isolates was observed in Argentina using REP-PCR. BOX A1R-PCR clustered bean nodule isolates from Ecuador and Peru are distinctive from the Mexican isolates (Bernal and Graham 2001). In Europe, narrow genetic diversity of *R. leguminosarum* bv. *phaseoli* strains was correlative to beans being an introduced crop (Laguerre et al. 1993). Some bean nodule isolates from Spain have been found to be very similar to *R. leguminosarum* bv. *viciae* and bv. *trifolii* (Velázquez et al. 2001). The limited genetic diversity of bean isolates *R. etli* and *R. tropici* in Africa has been related to the fact that beans are an introduced crop (Diouf et al. 2000).

Populations of rhizobia in nodules are determined by the environmental conditions and the agricultural practices (Palmer and Young 2000). Our results are in agreement with this finding showing genotypic patterns dependable on plants used for intercropping. Only 42.4% of all of the isolates in this study harbored *nifH* gene and, on the basis of their biochemical and morphological characteristics, selected as rhizobia. This finding may be interpreted as evidence that there is a strong selective pressure imposed by plants intercropped with the green bean as plant host, which favors endophytic bacteria from other genera (57.6%). The low numbers of rhizobial isolates from nodules of green bean grown intercropped with radish (16 out of 50) may depend on the sensitivity to exudates of radish. Intercropping with radish caused lower relative yield of green bean and may be connected with the higher number of endophytic bacteria and lower number of rhizobia in nodules belonging mostly to $(GTG)_5$-PCR fingerprint patterns II and VI, comparing to genetically diverse isolates found in monoculture (patterns I, III, IV, V and VI). Higher yields of radish and LER values for both unfertilized (1.19) and fertilized (1.23) variants probably may be explained by the other microorganisms in rhizosphere. Improvement of the red beet yield and LER index of 1.06 (unfertilized – C variant) and 1.08 (fertilized – P variant) are lower than those in green bean/radish intercrop. The 38% of isolates originated from nodules of green bean grown intercropped with red beet and 75% of them formed $(GTG)_5$-PCR fingerprint patterns III and IV. After future effectiveness testing, it may be possible to explain effect of rhizobia from III and IV genotypic groups and minor decreasing of green bean relative yield. Tosti and Thorup-Kristensen (2010) reported red beet root system more competitive than legumes such as crimson clover (*Trifolium incarnatum* L.) and faba bean (*Vicia faba* L. *minor* Beck). In this study, red beet root system increased the number of rhizobial isolates in green bean nodules and favored genotypic pattern III for unfertilized (R) and pattern IV for fertilized variant (P).

Caballero-Mellado and Martínez-Romero (1999) reported the diminished genetic diversity encountered in bean nodules under chemical fertilization in doses recommended for usage in agricultural fields. In this study, fertilizing itself reduced the number of rhizobial isolates in nodules of green bean in both control and variant intercropped with radish. Intercropping with red beet stimulated nodulation with rhizobia even in fertilized variants.

Taken together, these results indicate that the number and diversity of indigenous rhizobia in green bean nodules depend on fertilizing and plants used for intercropping. The further research is needed to examine the effectiveness of indigenous rhizobial isolates in order to select the most promising representative isolates from each genotypic group. Intercropping with green bean and benefits of nitrogen fixation using indigenous selected rhizobia is the environmentally friendly and low cost way for the improvement of vegetable yields and quality.

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