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THE EFFECT OF INOCULATION WITH *Azotobacter chroococcum* ON MICROORGANISMS IN RHIZOSPHERE AND SUGAR BEET YIELD IN ORGANIC FARMING

ABSTRACT: The effect on sugar beet yield parameters and microbiological soil status was studied using two techniques of sugar beet inoculation with strains of *Azotobacter chroococcum*. Cultivar "Drena" was used in the study, and field trial was set under the conditions of organic farming system in Bački Petrovac. A mixture of three strains of *Azotobacter chroococcum* was used as microbial fertilizer. Inoculation was performed by: (A) incorporation of strains into soil before sowing; and (B) repeated incorporation of strains into soil two weeks after sowing. PGP characterization of the strains confirmed the ability of producing indole-3-acetic acid (IAA) from 12.63 μg ml^{-1} to 14.95 μg ml^{-1}, nitrogen fixation, and P-solubilization. Positive effects on the number of azotobacter and free nitrogen fixers in rhizosphere were obtained by inoculation, as well as positive effects on the tested sugar beet yield parameters. The largest increase in root yield, yield of crystal sugar, and yield of polarised sugar compared with the control was obtained by repeated soil inoculation, ranging from 22 to 23%.

KEYWORDS: abundance of microorganisms, *Azotobacter chroococcum*, organic production, root yield, sugar beet, sugar yield

INTRODUCTION

Production of mineral fertilizers requires a significant amount of non-renewable energy sources and great financial expenses, with negative effects of their application on the environment. Rationalization of mineral fertilizer application can be achieved using N-fixing and P-solubilizing bacteria as microbial fertilizers, which transform the macroelements essential for plant nutrition (nitrogen and phosphorus) into plant-accessible forms [Milić *et al.*, 2004; Mi-

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lošević *et al*., 2006]. Interaction between plants and microorganisms are becoming increasingly significant in the systems of sustainable organic agriculture, above all for the purpose of transformation and mobilisation of nutrients from limited soil nutrient supply, so that plants can adopt these nutrients in order to achieve their full genetic potential. Therefore, more work has recently been made in the use of microbial preparations as addition or replacement for mineral fertilizers and pesticides, and increased efficiency of these preparations using the best combinations of useful bacteria.

Among the plant growth-promoting rhizobacteria – PGPR, bacteria of the genus *Azotobacter* is well known for promotion of growth of non-leguminous plants, resulting in increased plant growth, increased dry weight, higher total nitrogen content, and often in significant yield increase [Govedarica *et al*., 1993; Jarak *et al*., 2012; Milošević *et al*., 2012]. The results of our previous research revealed a significant effect of *Azotobacter chroococcum* on productive and technological traits of sugar beet [Čačić *et al*., 2003; Mrkovački *et al*., 2008], and a significant increase in biogenicity of sugar beet rhizosphere [Mrkovački and Mezei 2003; Kuzevski *et al*., 2011]. Besides the ability to bind atmospheric nitrogen, bacteria of the genus *Azotobacter* have a positive influence on growth and yield of plants due to their P-solubilization ability as well as the ability to produce phytohormones, exopolysaccharides, siderophores, and antibiotics [Bjelić *et al*., 2015].

With the application of PGPR a limited yield increase can be achieved, due to variability of the factors which contribute to the survival of PGPR strains in soil. In addition to the selection of optimal bacterial strains and defining their useful traits, it is necessary to additionally examine different techniques of inoculant application. Therefore, the aim of our research was to examine the effect of inoculation and repeated inoculation with *Azotobacter chroococcum* strains on microbial abundance in rhizosphere and yield of sugar beet grown in the system of organic farming.

MATERIALS AND METHODS

Bacterial strains. Strains of *Azotobacter chroococcum* (strains 5, 8, 14) used in this study were taken from the collection maintained at the Department of Microbiological Preparations, Institute of Field and Vegetable Crops, Novi Sad (WDCM754). *A. chroococcum* was cultured for 72 hrs in Burk's N-free broth, at optimal temperature of 28 °C, at a shaking rate of 150 rpm.

PGPR properties of Azotobacter strains. Quantitative analysis of IAA production was performed as described by Glickman and Dessaux [1995]. The potential of strains to grow on Döbereiner nitrogen-free culture medium [Döbereiner 1988] indicated their N_2-fixation ability. Phosphate solubilization capacity was determined by spot inoculations on Pikovskaya medium – PVK [Pikovskaya 1948] and National Botanical Research Institute's phosphate medium – NBRIP [Nautiyal 1999] with 0.5% TCP [$Ca_3(PO_4)_2$].

Experimental design. Research of the effects of different inoculation techniques using *Azotobacter chroococcum* strains on the parameters of yield and microorganisms of sugar beet rhizosphere was carried out at the locality of Bački Petrovac. The experiment was set in the system of organic production as a randomized block design with four replications, using basic plots 10 m long and 2 m wide. Sowing was done mechanically, using inter-row spacing of 50 cm x 10 cm, with the correction of planting density after sprouting to inter-row spacing of 20 cm. Seed of sugar beet cultivar "Drena" developed at the Institute of Field and Vegetable Crops in Novi Sad was used for sowing. Two techniques for inoculation with *Azotobacter chroococcum* strains were used in the trial: (A) incorporation of strains into soil before sowing, (B) repeated incorporation of strains into soil two weeks after sowing. A mixture of liquid cultures of *Azotobacter chroococcum* strains was used for soil treatment (strains 5, 8 and 14). The capacity of inoculum (density of 10^9 cells per ml) was calculated per trial surface (1 l inoculum + 300 l water ha^{-1}). Untreated soil was used as the control.

Microbiological analysis. Rhizosphere soil samples were taken for microbiological analyses at two dates (June and September) during 2015. Samples were analysed by the serial–dilution method followed by plating on different selective media. A total number of microorganisms (TNM) was determined on an agarized soil extract (dilution 10^7). Nitrogen–free medium was used for determination of free N-fixing bacteria (N-fix) (dilution 10^6) and *Azotobacter* sp. (AZT) (dilution 10^2). Ammonifiers (AMN) were determined on a mesopeptone agar (dilution 10^6). All microbiological analyses were performed in three replications and the average number of microorganisms was calculated at 1.0 g of absolutely dry soil [Jarak and Đurić 2006].

Soil chemical analysis. Soil samples were taken for determination of soil chemical characteristics at the end of the experiment, in late October. Samples were collected from the depth of 0–30 cm, air-dried and ground to a particle size <2 mm, after which the basic chemical characteristics were determined in the laboratory of the Institute of Field and Vegetable Crops.

Yield analysis. Plants were dug up at the end of October, after which root weight and number of plants were determined. The samples containing twenty sugar beet plants from each replication were examined for their sugar content and non-sugar content (K, Na, and amino N), which was determined in the laboratory of the Institute of Field and Vegetable Crops in Novi Sad for the purpose of sugar beet root analysis. The obtained data were used for calculation of root yield per surface unit, yield of polarized sugar, and yield of crystal sugar.

Statistical analysis. The variables were analysed in accordance with the analysis of variance (ANOVA) using software *STATISTICA* (StatSoft Inc. 2012). Means between the levels of the factors were separated by Duncan's multiple range test (DMRT) and letter groupings was generated using 0.05 level of significance.

RESULTS AND DISCUSSION

Azotobacter is one of the most widely reported among the different bacterial genera that have been established as PGPR [Mrkovački and Milić 2001].

Azotobacter represents the main group of heterotrophic free living nitrogen-fixing bacteria present in rhizosphere of many plants (free nitrogen fixation), and occasionally at the root surface (associative nitrogen fixation) [Wani *et al*., 2013]. The isolated culture of *Azotobacter* fixes about 10 mg nitrogen g^{-1} of carbon source under *in vitro* conditions [Jnawali *et al*., 2015]. The amount of nitrogen taken by *Azotobacter* under field conditions is about 20–60 kg ha^{-1} per year [Hajnal *et al*., 2012], depending on soil conditions. PGP characteristics of strains used in this research are shown in Table 1. Strains produced IAA on the agar with added L-triptophan. N-fixing ability was determined for all strains, while P-solubilizing ability was recorded in AC5 and AC8 strains. Similarly, the variability within the PGPR properties in different isolates was recorded by Cakmakci *et al*. [2009], while plant-growth response was variable and dependent on the inoculant strain, plant species, and evaluated growth parameters.

Table 1. Plant growth promoting properties of *Azotobacter* strains

Strain	IAA (μg ml^{-1})		N_2-fix	P – sol	
	0 μg ml^{-1}	250 μg ml^{-1}		PVK	NBRIP
AC5	0.37 ± 0.07	14.95 ±0.13	+	+	+
AC8	0.07 ± 0.12	12.99 ± 0.22	+	+	+
AC14	0.32 ± 0.09	12.63 ± 0.35	+	–	–

IAA: values are average of three replicates (mean ± SD); N_2-fixation: (-) negative reaction; (+) positive reaction; P-solubilization: (-) without clear zone (+) 1–4 mm diameter of clear zone formed around the bacterial colony as a result of solubilization of tri-calcium phosphate

The presence of *Azotobacter* sp. in soils has beneficial effects on plants, but the abundance of these bacteria is related to many factors, soil physico-chemical (e.g. organic matter, pH, temperature, soil moisture) and microbiological properties. *Azotobacter* presence in our climatic region goes from several hundred to several thousand cells, primarily inhabiting neutral or alkaline soils. The population of *Azotobacter* is generally low in the rhizosphere of crop plants, and in uncultivated soils. However, a higher presence of *Azotobacter* sp. was recorded in rhizosphere comparing with the surrounding soil. Previous results have confirmed that the application of bacteria in plant production increases the number and enzymatic activity of microorganisms, which results in higher production ability of soil [Đurić *et al*., 2004; Jarak *et al*., 2012].

Our research revealed that the incorporation of strains in soil before and after sowing lead to increase in the number of *Azotobacter* sp. and free nitrogen fixers in sugar beet rhizosphere. The number of *Azotobacter* increased compared with the control in both sampling periods, and the largest number was obtained in the variant of repeated incorporation of strains into soil in the second period, which was higher than control by 23.2%. In the first period, the number of ammonifiers was higher compared with the control in both variants of inoculation. However, unlike the number of azotobacters and similar to the

total number of microorganisms, the number of ammonifiers decreased in the second sampling period. The highest total number of microorganisms and of ammonifiers was obtained in the control variant in the second sampling period. The number of free nitrogen fixers increased in the first sampling period compared with the control, and decreased in the second sampling period. These microorganisms were the most abundant (higher than control by 33.9%) in the variant with repeated inoculation, similarly to the number of azotobacters in the second period (Table 2). Increase in the abundance of microorganisms in sugar beet rhizosphere as a result of inoculation with *Azotobacter chroococcum* was also obtained in our previous studies [Mrkovački *et al.*, 2012].

Table 2. Effect of inoculation with *Azotobacter* on microbial number in sugar beet rhizosphere

Treatment	Sampling	Microbial group (CFU ml^{-1} g^{-1} absolutely dry soil)			
		TMN x 10^7	AZT x 10^2	AMN x 10^6	N-fix x 10^6
Ø	I	68 ± 44 abc	63 ± 46 b	111 ± 20 a	247 ± 89 a
	II	119 ± 29 a	102 ± 33 ab	221 ± 10 a	99 ± 50 b
Inoculation	I	97 ± 18 ab	87 ± 19 ab	129 ± 14 a	251 ± 73 a
	II	39 ± 19 c	109 ± 21ab	148 ± 40 a	65 ± 74 b
Repeated Inoculation	I	79 ± 19 abc	70 ± 15 b	116 ± 2 a	330 ± 25 a
	II	53 ± 30 bc	126 ± 8 a	117 ± 29 a	41 ± 31 b

Values are average of four replicates (mean ± SD); means followed by the same letter are not statistically different at 0.05 level according to Duncan's multiple range test (DMRT)

Chemical analyses of soil have shown that humus content and total nitrogen content were higher in inoculated variants compared with the control. Therefore, the repeated incorporation of strains into soil had the best effect on these traits (Table 3), suggesting that microorganisms play a very important role in supplying nutrients to crop plants by improving soil fertility through a number of processes. Microorganisms enable processes of humification and dehumification, nitrogen fixation, and release of certain nutrients present in organic matter (N, P, C, S). They also affect plant nutrition by the products of their life activity, thereby participating in the creation and maintaining of soil fertility, growth, yield and health of plants [Milošević *et al.*, 2006].

Table 3. Soil chemical properties

Treatment	pH		$CaCO_3$ %	Humus %	Total N %	AL-P_2O_5 mg/100g	AL-K_2O mg/100g
	in KCl	in H_2O					
Ø	7.55	8.29	2.82	2.61	0.194	29.8	26.8
Inoculation	7.53	8.19	2.11	2.79	0.207	27.6	26.4
Repeated Inoculation	7.50	8.24	1.61	3.18	0.218	24.8	26.8

Incorporation of *Azotobacter chrooccum* strains in soil before and after sowing significantly affected the studied parameters of sugar beet yield (Table 4). Root yield was increased by inoculation compared with the control (7.52–8.47 t ha^{-1}). Inoculation affected the increase in root yield by 20%, and repeated inoculation increased it by 23%. Higher yields of polarized and crystal sugar obtained after inoculation ranged between 20–21%, while repeated inoculation caused the increase of the studied parameters by 22–23%.

Table 4. Effect of inoculation with *Azotobacter* on the yield of sugar beet roots (t ha^{-1})

Treatment	Root yield (t ha^{-1})	Polarized sugar yield (t ha^{-1})	Crystal sugar yield (t ha^{-1})
Ø	37.43 ± 6.10 b	5.19 ± 0.64 b	4.34 ± 0.76 b
	100%	100%	100%
Inoculation	44.95 ± 1.08 a	6.23 ± 0.14 a	5.27 ± 0.26 a
	+ 20%	+ 20 %	+ 21%
Repeated Inoculation	45.90 ± 1.79 a	6.37 ± 0.16 a	5.28 ± 0.23 a
	+ 23%	+ 23%	+ 22%

Values are average of four replicates (mean ± sd); means followed by the same letter are not statistically different at 0.05 level according to Duncan's multiple range test (DMRT)

Previous results [Mrkovački and Mezei 2003] obtained after two years of testing the effects of inoculation with *Azotobacter* in several sugar beet cultivars showed the increase in root yield by about 5.9%, and increased yield of crystal sugar by 7.9–8.2% (536–660 kg ha^{-1}). The results of Čačić *et al.* [2003] showed a statistically significant increase in crystal sugar yield of three sugar beet cultivars at two localities after inoculation with *A. chroococcum*. Yield increase due to *Azotobacter* inoculation ranged from 2–45% in vegetables, 9–24% in sugar cane, and 0–31% in maize, sorghum etc. [Pandey and Kumar, 1989]. Results of Amirhandeh *et al.* [2012] suggested that *Azotobacter chroococcum* is a suitable inoculant, due to its positive response in crop production and it could be part of a strategy in achieving sustainable agriculture. It is necessary that some future researches further explore the potentiality of *Azotobacter* in crop production, especially in organic growing systems. The success of PGPR inoculants will depend on our ability to manage the rhizosphere in order to enhance survival and competitiveness of these beneficial microorganisms.

CONCLUSION

The research confirmed that incorporation of *Azotobacter chroococcum* strains into the soil affected the increase in sugar beet production and soil biogenicity. Incorporation of strains into the soil before and after sowing lead to the increase in abundance of *Azotobacter* sp. and free nitrogen fixers in sugar beet rhizosphere. Root yield increased after inoculation compared with

the control (7.52–8.47 t ha^{-1}). Inoculation increased root yield by 20%, and repeated inoculation caused a 23% increase. Increase in the yield of crystal sugar obtained after inoculation was 21%, while repeated inoculation caused the increase of 22–23%.

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УТИЦАЈ ИНОКУЛАЦИЈЕ СА *Azotobacter chroococcum* НА МИКРООРГАНИЗМЕ У РИЗОСФЕРИ И ПРИНОС ШЕЋЕРНЕ РЕПЕ У ОРГАНСКОЈ ПРОИЗВОДЊИ

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РЕЗИМЕ: Испитан је ефекат два начина инокулације шећерне репе са сојевима *Azotobacter chroococcum* на параметре приноса шећерне репе и микробиолошки статус земљишта. У испитивањима је коришћена сорта Дрена, а експеримент је постављен у систему органске производње у Бачком Петровцу. Као микробиолошко ђубриво коришћена је смеша три соја *Azotobacter chroococcum*. Инокулација је извршена на два начина: (А) инкорпорација сојева у земљиште пре сетве, (Б) поновљена инкорпорација сојева у земљиште две недеље након сетве. PGP карактеризацијом коришћених сојева утврђена је способност продукције индол-3-сирћетне киселине (IAA) од 12.63 μg ml^{-1}до 14.95 μg ml^{-1}, азотофиксације и фосфосолубилизације. Инокулацијом је добијен позитиван ефекат на број азотобактера и слободних азотофиксатора у ризосфери, као и на испитиване параметре приноса шећерне репе. Највеће повећање приноса корена, приноса кристалног и поларизационог шећера добијено је на варијанти поновљене инокулације земљишта и кретало се од 22 до 23% у односу на контролну варијанту.

КЉУЧНЕ РЕЧИ: *Azotobacter chroococcum*, бројност микроорганизама, органска производња, принос корена, принос шећера, шећерна репа