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ORIGINAL PAPER

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POSSIBILITY OF ACHIEVING ORGANIC YIELDS FOR MEDICINAL AND AROMATIC PLANTS BY BIOFERTILIZATION WITH *Azotobacter chroococcum*

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ABSTRACT

The aim of this study was to examine the effects of management practices and biofertilization on microbial activity in rhizosphere and yield of medicinal and aromatic plants. Field experiment was performed using four plant species: peppermint (*Mentha × piperita* L.), pot marigold (*Calendula officinalis* L.), sweet basil (*Ocimum basilicum* L.), and dill (*Anethum graveolens* L.). Treatments were arranged in a split-plot layout in four replicates using basic plots under conventional and organic management, and subplots with and without biofertilizer (*Azotobacter chroococcum*). Organic management positively affected the microbial number and activity. Biofertilization increased the total microbial number (13–21%), number of ammonifiers (13–60%), nitrogen-fixing bacteria (7–36%), actinomycetes (36–50%), fungi (60–100%), cellulolytic microorganisms (57–217%), dehydrogenase (28–52%) and ß-glucosidase activity (15–39%). The effects of management practices and biofertilization were highly significant for the yield of examined plants. The yields were higher on inoculated treatments both in conventional (5–26%) and organic (7–15%) growing system.

Key words: *Azotobacter chroococcum*, dill, peppermint, pot marigold, sweet basil

INTRODUCTION

Medicinal and aromatic plants (MAPs) contain a wide range of essential oils and other biologically active compounds that are traditionally used in food, pharmaceutical industries and health care [Qasim et al. 2017]. Modern cultivation technologies such as using chemical fertilizers and pesticides may degrade the quality of medicinal plant products, lead to resource degradation and negatively affect soil ecological functions [Solaiman and Anawar 2015]. Additionally, chemical measures create imbalances in the microbial communities, which may be unfavorable to the activity of beneficial microorganisms [Lamsal et al. 2013].

Since MAPs are usually consumed without further processing after harvest, it is necessary to identify alternative growing systems, particularly those involving application of organic fertilizers and/or biofertilizers [Teixeira da Silva and Egamberdieva 2013]. Low-input systems such as organic management, substantially reduce the use of synthetic fertilizers, pesticides, energy and mechanic stress, and mitigate their negative impacts in order to improve the quality and fertility of agricultural soils [Gomiero et al. 2011]. Biofertilizers such as plant growth promoting rhizobacteria (PGPR), are soil bacteria able to colonize the surface of the root system and stimulate plant growth via associative nitrogen fixation, phosphate solubilization, production of phytohormones and siderophores, or enzymatic activities [Vacheron et al. 2013]. They can also protect the plant through inhibition of phytopathogens, based on antagonism or competition mech-

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anisms, and by elicitation of induced systemic resistance (ISR) [Bouizgarne 2013].

Azotobacter is one of the most widely reported PGPR and represents the main group of nitrogen-fixing bacteria present in rhizosphere of many plants [Wani et al. 2013]. In addition to nitrogen fixation ability (20−60 kg N ha^{-1} per year), it has been found that bacteria of the genus *Azotobacter* exhibit a series of PGP properties and lead to growth and yield improvement of several agricultural crops [Mrkovački and Milić 2001].

Replacement of mineral fertilizers with organic fertilizers and/or biofertilizers which contain *Azotobacter* is a well-justified practice from the perspective of energy, economy and ecology, and enables the production of high-quality organic food [Jnawalli et al. 2015]. Biofertilization causes proliferation of microorganisms in crop rhizosphere and leads to better plant growth and higher yield. Past medicinal plant research primarily focused on bioactive phytochemicals, yet their microbiome as well as rhizobacteria and plant interactions, remain poorly understood [Köberl et al. 2013]. Furthermore, the lack of data about biofertilization of MAPs is evident in literature, especially in the conditions of different management practices. Therefore, it is necessary to investigate the possibilities of using biofertilizers, namely *Azotobacter chroococcum,* in order to increase organic yields of medicinal and aromatic plants. The main objective of the present field experiment was to investigate the influence of biofertilization with *Azotobacter* on microbial activity in rhizosphere and yield of four MAPs grown under conventional and organic management.

MATERIAL AND METHODS

Experimental design. Field experiment was carried out at the locality of Bački Petrovac (45°21'N, 19°35'E, 86 m), Vojvodina, northern Serbia. The experiment was set on the Haplic Chernozem (loamic) soil [IUSS Working Group WRB 2014]. Four MAPs: peppermint (*Mentha* × *piperita* L.), pot marigold (*Calendula officinalis* L.), sweet basil (*Ocimum basilicum* L.), and dill (*Anethum graveolens* L.), were grown under conventional and organic management. Experimental treatments were arranged in a split-plot layout with four repetitions, using basic plots 10 m long and 2.8 m wide. The conventional plot treatments were fertilized with 400 kg NPK ha^{-1} (N, P_2O_5 and K_2O – 15 : 15 : 15), while the organic plots were established with 15 t manure ha^{-1} in 2011, and the crop rotation (soybean – wheat) afterwards. Half plots of each treatment were inoculated with *Azotobacter*, while the other halves were not inoculated.

Biofertilization. This research was performed with *Azotobacter chroococcum* strains from the collection of Institute of Field and Vegetable Crops Novi Sad, Serbia. Strains were cultured for 72 h in Burk's N-free broth, at optimal temperature of 28°C and shaking rate of 150 rpm (Edmund Bühler Shaker SM-30 B). A mixture of liquid cultures of these strains was used for soil treatment. Inoculation was performed by incorporation of strains into soil immediately after sowing. The capacity of inoculum (density of 10^9 cells per mL) was calculated per trial surface (1 L inoculum + 300 L water per ha). Untreated soil was used as control.

Microbial analysis. Rhizosphere samples for microbiological analyses were collected at two dates during 2016 (July 7 and August 10), in four replications for each treatment. In the rhizosphere samples, the number of microorganisms and activity of enzymes were determined.

Microbial number was analyzed by the indirect dilution method followed by plating of soil suspension on selective nutritive media: soil agar for the total number of microorganisms, meat peptone agar for the number of ammonifiers, N-free medium for the number of nitrogen-fixing bacteria, synthetic medium for the number of actinomycetes, Czapek Dox agar for the number of fungi, and Waksman-Carey medium for the number of cellulolytic microorganisms. Incubation temperature was 28°C, while incubation time depended on the tested group of microorganisms. All microbiological analyses were performed in three replications and the average number of microorganisms was calculated at 1.0 g absolutely dry soil (CFU g^{-1} soil) [Briones and Reichardt 1999].

Microbial activity was analyzed spectrophotometrically (Agilent Cary 60, Agilent Tehnologies) by determining dehydrogenase (DHA) and ß-glucosidase (BGL) activity. Activity of DHA (EC 1.1.1.) was determined by measuring the extinction of colored triphenyl-formazan (TPF) formed by reducing a col-

orless triphenyl-tetrazolium chloride (TTC) [Casida et al. 1964]. Five grams of fresh soil was weighed into glass vials, then 2 mL of 3% TTC in 2 mL of Tris-buffer (pH 7.8) were added. After 24 h incubation of soil suspensions in the dark at 37°C, 20 mL of methanol was added to each vial. The vials were shaken in the dark and then filtered in 50 mL volumetric flasks. The remaining soil in the vials as well as the filter papers were washed twice with methanol. To extract all the TPF produced, filtrates were poured up to mL with methanol. TPF concentration was measured at 485 nm and the average DHA for all samplings were calculated per 1.0 g of soil (μg TPF g^{-1} soil).

Activity of BGL (EC 3.2.1.21) was determined by measuring the extinction of colored p-nitrophenol formed by reducing a colorless p-nitrophenyl β-d-glucoside (PNG) [Hayano 1973]. A 0.5 g amount of moist soil was placed in test tube and 0.1 mL toluene was added. After 10 min 0.9 mL of distilled water, 1.5 mL of McIlvaine buffer (pH 4.8), and 0.6 mL of PNG were added, vortexed briefly and then incubated at 30°C for 1 h. After filtration and addition of 2 mL of 2 M Tris solution, the intensity of the yellow colored p-nitrophenol was measured at 400 nm and the average BGL for all samplings were calculated. One unit of enzyme was defined as 1 μmol of p-nitrophenol released per min at 30°C.

Yield analysis. Harvest was performed in technological maturity of plants, at the optimal stage for each species. The yield of fresh mass was measured and calculated in kilograms per hectare (kg ha^{-1}).

Statistical analysis. The variables were analyzed using two-way analysis of variance (ANOVA), followed by mean separation according to Tukey's test at the $p < 0.05$ level of probability. Correlation analysis was used to determine whether there was a significant relationship between microbial and yield properties. All analyses were performed with STATISTICA 12.0 package computer program (StatSoft).

RESULTS

Management practices significantly affected the total microbial number, number of nitrogen-fixing bacteria, and cellulolytic microorganisms in the rhizosphere of pot marigold and basil, as well as the number of fungi in the rhizosphere of peppermint, basil and dill (Tab. 1). Dehydrogenase and ß-glucosidase activity in the rhizosphere of all investigated plant species also varied significantly depending on the growing system (Tab. 1).

On average, the number of actinomycetes (7–36%), fungi (20–167%), cellulolytic microorganisms (18–100%), as well as activity of dehydrogenase (43–129%) and ß-glucosidase (38–64%) in rhizosphere of examined MAPs were higher in organic growing system compared with the conventional. Organic management also positively affected the number of ammonifiers (23%) and nitrogen-fixing bacteria (3%) in peppermint rhizosphere (Tab. 1).

Biofertilization significantly increased the number of nitrogen-fixing bacteria, fungi, cellulolytic microorganisms and enzyme activities in peppermint rhizosphere (Tab. 2).

Inoculation also led to a significant increase in the number of cellulolytic microorganisms and activity of enzymes in rhizosphere of marigold, as well as number of fungi, cellulolytic microorganisms and ß-glucosidase activity in basil rhizosphere. On average, inoculation increased the total microbial number (13–21%), number of ammonifiers (13–60%), nitrogen-fixing bacteria (7–36%), actinomycetes (36–50%), fungi (60–100%), cellulolytic microorganisms (57–217%), dehydrogenase (28–52%), and ß-glucosidase activity (15–39%) (Tab. 2). Observed by plant species, the effect of biofertilization on microbial number and activity in rhizosphere was the highest for peppermint.

The results showed statistically significant influence of biofertilization and management practices on the yield of MAPs (Tab. 3). The application of *Azotobacter* had a positive impact on the yield of all tested MAPs, both in the conventional (5–26%) and organic (7–15%) growing system. Higher yields were achieved by biofertilization of peppermint and dill under conventional management (14% and 26%), while yields of pot marigold and basil were higher in organic growing system (10% and 15%) (Tab. 3).

On average, organic management led to a significant increase in the yield of peppermint and dill, while the yields of pot marigold and basil were higher in conventional growing system (Tab. 3). Similarly, inoculation increased the yield of examined MAPS (8–15%), while the effect of biofertilization was better in peppermint and dill (Tab. 3).

Table 1. Effect of management practices on microbial number and activity in MAPs rhizosphere

Plant	Peppermint		Pot marigold		Sweet basil		Dill	
System	CON	ORG	CON	ORG	CON	ORG	CON	ORG
Total number (CFU × 10^6 g^{-1})	223 a	200 a	309 a	190 b	282 a	183 b	258 a	254 a
Increase (%)	–10		–39		–35		–2	
Ammonifiers (CFU × 10^6 g^{-1})	75 a	92 a	103 a	75 a	92 a	83 a	84 a	63 a
Increase (%)	23		–27		–10		–25	
N_2-fixers (CFU × 10^5 g^{-1})	120 a	124 a	201 a	127 b	267 a	133 b	172 a	164 a
Increase (%)	3		–37		–50		–5	
Actinomycetes (CFU × 10^3 g^{-1})	19 a	23 a	14 a	15 a	23 a	24 a	14 a	19 a
Increase (%)	21		7		4		36	
Fungi (CFU × 10^3 g^{-1})	5 a	7 a	10 a	12 a	6 b	11 a	3 b	8 a
Increase (%)	40		20		83		167	
Cellulolytic MO (CFU × 10^5 g^{-1})	11 a	13 a	12 b	24 a	15 b	23 a	10 a	12 a
Increase (%)	18		100		53		20	
Dehydrogenase (μg TPF g^{-1})	384 b	550 a	179 b	410 a	220 b	435 a	167 b	375 a
Increase (%)	43		129		98		125	
ß–glucosidase (mU g^{-1})	16 b	22 a	17 b	26 a	16 b	26 a	14 b	23 a
Increase (%)	38		53		63		64	

Values with different letters within the same row differ significantly (Tukey test, $p < 0.05$). CON − conventional management, ORG − organic management

Positive correlation was obtained between the number of N_2-fixers and the yields of all examined MAPs, while significant correlation was observed for pot marigold and sweet basil (Tab. 4). The number of nitrogen-fixing bacteria was also positively correlated to total microbial number, number of ammonifiers, actinomycetes and cellulolytic microorganisms in the rhizosphere of tested MAPs (Tab. 4). All microbial parameters were positively correlated with N_2-fixers in the rhizosphere of peppermint, while negative correlation was observed between the number of nitrogen-fixing bacteria and number of fungi, activity of dehydrogenase and ß-glucosidase in the rhizosphere of pot marigold, sweet basil and dill.

DISCUSSION

Microbial indicators are more susceptible than physical and chemical properties to changes imposed to the environment like soil use and management, and for this reason they can be used to predict the effects of ecosystem perturbations by organic and conventional management practices [Cardoso et al. 2013]. In this study, management practices significantly affected the microbial number and activity in rhizosphere of MAPs, while higher values of examined parameters were recorded in organic growing system compared with the conventional. General increase of microbial biomass and higher enzyme activities have been

Table 2. Effect of biofertilization on microbial number and activity in MAPs rhizosphere

Plant	Peppermint		Pot marigold		Sweet basil		Dill	
Biofertilizer	– AC	+ AC	– AC	+ AC	– AC	+ AC	– AC	+ AC
Total number (CFU × 10^6 g^{-1})	191 a	231 a	231 a	269 a	218 a	248 a	240 a	272 a
Increase (%)	21		16		14		13	
Ammonifiers (CFU × 10^6 g^{-1})	72 a	94 a	82 b	96 a	67 a	107 a	69 a	78 a
Increase (%)	31		17		60		13	
N_2-fixers (CFU × 10^5 g^{-1})	103 b	140 a	159 a	170 a	181 a	219 a	162 a	173 a
Increase (%)	36		7		21		7	
Actinomycetes (CFU × 10^3 g^{-1})	17 a	25 a	12 a	18 a	25 a	23 a	14 a	19 a
Increase (%)	47		50		–8		36	
Fungi (CFU × 10^3 g^{-1})	5 b	8 a	8 a	13 a	6 b	12 a	6 a	5 a
Increase (%)	60		63		100		–17	
Cellulolytic MO (CFU × 10^5 g^{-1})	6 b	19 a	14 b	22 a	14 b	23 a	8 a	14 a
Increase (%)	217		57		64		75	
Dehydrogenase (μg TPF g^{-1})	381 b	552 a	235 b	354 a	260 a	395 a	238 a	304 a
Increase (%)	45		51		52		28	
ß-glucosidase (mU g^{-1})	17 a	22 a	18 b	25 a	20 b	23 a	17 a	21 a
Increase (%)	29		39		15		24	

Values with different letters within the same row differ significantly (Tukey test, $p < 0.05$). – AC, without *Azotobacter chroococcum*; + AC, with *Azotobacter chroococcum*

reported in other studies when organic or minimum conventional practices were compared to conventional management [Kaschuk et al. 2010]. Conventional management implies the continuous use of synthetic fertilizers and pesticides that causes changes in physical, chemical, and biological properties of the soil. Reduced conventional practices as well as organic systems can improve soil properties, such as soil structure, nutrient availability, and the number, diversity and activity of microbial populations, while reducing soil disturbance [Heidari et al. 2016].

Enhancement of microbial processes can be achieved through biofertilization with PGPR which cause proliferation of microorganisms in crop rhizosphere [Sharma et al. 2012]. In this study, biofertilization led to a significant increase in the number of microorganisms and activity of enzymes in rhizosphere of MAPs. Similar findings which confirm a possible application of *Azotobacter chroococcum* and other PGPR as biofertilizers in the MAPs production have been reported in other studies. Ordookhani et al. [2011] found an increase in shoot and root dry weight, nitrogen (N), phosphorus (P) and potassium (K), and essential oils content in *Ocimum basilicum* inoculated with *Pseudomonas putida* and *Azotobacter chroococcum*. Similar observations were reported by Hosseinzadah et al. [2011] who showed that *Azospirillum lipoferum*, *Azotobacter chroococcum*, and *Pseudomo-*

Table 3. Effect of management practice and biofertilization on MAPs fresh mass yield (kg ha^{-1})

Management/Biofertilization		Peppermint	Pot marigold	Basil	Dill
CON	– AC	16290 c	7337 a	22395 a	10575 b
	+ AC	18496 b	7776 a	23439 a	13332 a
	Increase (%)	14	6	5	26
ORG	– AC	19710 b	5800 c	18342 b	13786 a
	+ AC	21850 a	6380 b	21060 ab	14802 a
	Increase (%)	11	10	15	7
Average	CON	17393 B	7557 A	22917 A	11954 B
	ORG	20780 A	6090 B	19701 B	14294 A
	Increase (%)	19	–19	–14	20
Average	– AC	18000 B	6569 B	20369 B	12180 B
	+ AC	20173 A	7078 A	22250 A	14067 A
	Increase (%)	12	8	9	15

Values with different letters within the same column differ significantly (Tukey test, $p < 0.05$). CON − conventional management, ORG − organic management; − AC, without *Azotobacter chroococcum*; + AC, with *Azotobacter chroococcum*

Table 4. Correlation coefficients (r) for relationships between number of nitrogen-fixing bacteria and microbial number and activity in rhizosphere and yield of MAPs

Rhizosphere	Peppermint	Pot marigold	Sweet basil	Dill
Variable		N_2-fixers		
Total microbial number	0.406	0.707*	0.873*	0.558*
Ammonifiers	0.438	0.698*	0.212	0.093
Actinomycetes	0.392	0.058	0.122	0.441
Fungi	0.500*	–0.242	–0.268	–0.476
Cellulolytic microorganisms	0.691*	0.698*	0.713*	0.101
Dehydrogenase	0.583*	–0.691*	–0.530*	–0.167
ß-glucosidase	0.508*	–0.442	–0.770*	–0.267
Fresh mass yield	0.460	0.896*	0.647*	0.111

*$p < 0.05$

nas fluorescens increased the dry weights of shoot and root, as well as the NPK content of leaves and roots of *Calendula officinalis*. El-Hadi et al. [2009] established that microbial parameters (total microbial number, number of fungi and nitrogen-fixing bacteria), as well as vegetative growth and essential oil content of *Mentha* plants were increased by inoculation with *Azospirillum lipoferum* and *Azotobacter chroococcum*. Darzi [2012] demonstrated that the mixture of *Azotobacter chroococcum* and *Azospirillum lipoferum* had a significant effect on dry weight and seed weight of *Anethum graveolens*.

The expression of plant-beneficial properties depends upon ability of PGPR to survive and multiply in soils and colonize plant roots [Berg and Smalla 2009]. Positive effects of biofertilization on microbial number and activity in this study also indicated high competitiveness of the introduced *Azotobacter chroococcum* strains against indigenous soil microbiota.

Each plant species harbors a specific rhizosphere colonized in high abundances by microorganisms that are of great importance for plant nutrition, health, and quality [Mendes et al. 2013]. Medicinal plants provide an enormous bioresource of structurally divergent bioactive secondary metabolites that are most likely responsible for the high specificity of the associated microorganisms and establishment of a characteristic microbiome in their rhizosphere [Qi et al. 2012]. In this study, biofertilization led to the highest increase of the total microbial number, number of N_2-fixers and cellulolytic microorganisms in the rhizosphere of peppermint. Increase in the number of ammonifiers, fungi, and dehydrogenase activity was the highest in rhizosphere of basil. The number and activity of microorganisms may has been intensified as a result of the close relationship between peppermint and basil who both belong to the *Lamiaceae* family and produce similar bioactive metabolites. Similar colonization patterns of the total bacterial and fungal communities between the medicinal plants *Matricaria chamomilla* and *Calendula officinalis*, both from the *Asteraceae* family, were observed by Köberl et al. [2013].

Majority of the total microbial population in the soil and rhizosphere consists of bacteria important for nutrient cycling, while the dominance of certain microbial groups directs the processes to either synthesis or decomposition of organic and inorganic matter which gets into soil [Liang and Balser 2011]. Investigated microbial parameters in this study are suitable indicators of nitrogen (N) and carbon (C) cycling in soil. Ammonifiers decompose organic nitrogen compounds, while nitrogen-fixing bacteria reduce atmospheric nitrogen, transforming them into plant-available forms. Actinomycetes and fungi are active decomposers of organic matter, while cellulolytic microorganisms are important in decomposition of plant biomass. Besides microbial number and composition, biochemical indicators such as soil enzymes are involved in several metabolic processes and can be useful indicators of soil health and changes due to its use and management [Cardoso et al. 2013]. Dehydrogenases play a significant role in biological oxidation of soil organic matter by transferring hydrogen from organic substrates to inorganic acceptors [Wolinska and Stepniewska 2012]. ß-glucosidases help in the hydrolysis of various beta-glucosides which are frequently supplied to soil from plant residues [Bégiun and Aubert 1994].

Additionally, establishment of a sufficient number of effective PGPR in rhizosphere leads to better root growth and even an increase in plant biomass and yield [Vacheron et al. 2013]. The results showed statistically significant influence of biofertilization and management practices on the yield of MAPs. Organic yields are usually lower than conventional yields (from 5% to 34%), but the yield differences are very dependent on system and site characteristics [Seufert et al. 2012]. Same authors established that under certain conditions, such as good management practices, particular crops and growing conditions, organic systems can nearly match conventional yields. In this study, higher yields were achieved by biofertilization of peppermint and dill under conventional management, while yields of pot marigold and basil were higher in organic growing system. These findings confirmed that *Azotobacter* could be used as biofertilizer for improving MAPs productivity.

Correlation analysis was performed in order to determine whether the increased number of nitrogen-fixing bacteria due to *Azotobacter* application influenced the other tested parameters. Positive correlation was obtained between the number of N_2-fixers and the yields of all examined MAPs. The number of nitrogen-fixing bacteria was also positively correlated to total microbial number, number of ammonifiers, actinomycetes and cellulolytic microorganisms in the rhizosphere of tested MAPs. Precisely these microbial parameters generally had the highest percentage of increase depending on the management practices and biofertilization, indicating that *Azotobacter* could potentially affect soil N and C cycling in conventional and organic growing systems. All microbial parameters were positively correlated with N_2-fixers in the rhizosphere of peppermint, while negative correlation was observed between the number of nitrogen-fixing bacteria and number of fungi, activity of dehydrogenase and ß-glucosidase in the rhizosphere of pot marigold, sweet basil and dill. Overall, effect of biofertilization on microbial and yield parameters was the highest in peppermint. This effect may has been due to that pot marigold, basil and dill are annual herbal medicinal and aromatic plants, while peppermint is a perennial plant whose rhizosphere is inhabited by a more stable associated microbiome.

CONCLUSIONS

Organic management featuring biofertilization resulted in increased number and activity of microorganisms in rhizosphere and caused yield improvement of examined plant species, thereby confirming that *Azotobacter* could be a suitable tool in achieving organic yields for medicinal and aromatic plants. Biofertilization had a positive impact on the yield of examined plant species both in the conventional and organic growing system, and to our knowledge, this is the first experimental confirmation on using *Azotobacter* in cultivation of medicinal and aromatic plants in Serbia. The obtained study results may be helpful in recommending *Azotobacter* as prospective biofertilizer in the production of peppermint, pot marigold, sweet basil and dill.

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