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THE COMPETITIVENESS OF AZOTOBACTER, PSEUDOMONAS AND BACILLUSAPPLIED AS A MIXTURE INOCULUM IN RHIZOSPHERE OF FIVE MAIZE GENOTYPES ASSESSED BY GENOTYPING AND PHENOTYPING METHODS

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Abstract

The rhizosphere contain a different compounds produced by the plant roots. The plant growth–promoting rhizobacteria (PGPR) can colonize plant root and promote plant growth and some of them can reduce the incidence of soil–borne diseases. PGPRs are beneficial for agriculture and often used as biocontrol or biofertilizer inoculants. However, the variation in bacterial colonization and survival within the rhizosphere (rhizosphere competence) can cause inconsistency of the field results. In this study, the mixture of the selected bacteria (*Azotobacter, Pseudomonas* and *Bacillus*) has been used as inoculum to assess the competitiveness and effects on plant growth and yield of different maize genotypes. Rep–PCR using $(GTG)_5$ primer for BOX elements were applied. Phenotypic and PGP traits as well as persistance of inoculated strains in the total number of bacteria have been avaluated. The obtained results assessed by a combination of genotyping and phenotyping methods showed that mixture of strains (*Azotobacter, Pseudomonas* PS2 and *Bacillus* Q7) had better competitiveness to indigenous bacteria in the rhizosphere of all maize genotypes. Inoculation increased the total number of microorganisms by 61%, the number of N_2-fixing bacteria by 49% and the number of azotobacters by 5% compared to the non–inoculated control i.e. indigenous bacterial population. PGP traits of *Azotobacter, Pseudomonas* PS2 and *Bacillus* Q7 influenced a growth and quality of maize.

Key words: Plant growth–promoting rhizobacteria (PGPR); rhizosphere competence; *Azotobacter; Pseudomonas; Bacillus*.

Introduction

Plant growth promoting rhizobacteria (PGPR) are a heterogeneous group of bacteria (including species of *Pseudomonas, Bacillus, Azotobacter, Serratia, Azospirillum, Klebsiella, Arthrobacter* and *Burkholderia*), that can improve the plant growth both directly and indirectly. PGPR can be found in the rhizosphere, at root surfaces and in association with roots. Some of them can provide plant with PGP substances synthesized by the bacteria such as plant growth regulators – indoleacetic acid, gibberellic acid, cytokinins and ethylene (Joseph et al., 2007). PGPR improve plant growth by facilitating the uptake of certain plant nutrients from the environment during asymbiotic N_2 fixation, phosphate solubilization from insoluble mineral compounds and production of siderophores. In the indirect promotion of plant growth, PGPR can prevent deleterious effects of phytopathogenic microorganisms by production of antibiotics (Jamali et al., 2009; Jošic et al., 2012 *a*, *b*) and cyanide (Flaishman et al., 1996). To use in plant production, bacterial strains with several PGP traits need to be able to survive and colonize in the rhizospheric soil (Cattelan et al., 1999). Successful association

between introduced bacterial strains and plants cannot always be reproduced under field conditions, even good results were achieved in *in vitro* conditions (Zhender et al., 1999). The rhizospheric competence of introduced microorganisms depends on many factors, including the indigenous microbial flora in the soil (composition and activity) and environmental factors (climate, weather conditions, soil characteristics, etc).
In this study, the mixture of the selected bacteria (*Azotobacter, Pseudomonas* and *Bacillus*) was used as inoculum to assess the competitiveness and effects on plant growth and yield of different maize genotypes.

Material and Methods

Bacterial isolation. *Pseudomonas* isolates were tested for fluorescence on King B medium (KB). *Bacillus* isolates were collected on Nutrient agar (NA) plates after incubation of soil suspension at 80^0C for 15 min. *Azotobacter* isolates were screened on solid Fjodorov medium (FA) (Anderson, 1965). Isolates from each maize genotype (20 colonies per bacterial group) were tested for PGP traits and enzymatic activity. Isolates with the same or similar phenotypic properties (more than 80%) were subjected to genotyping using $(GTG)_5$ primer.
Enzymatic activities. Celulase, pectinase and protease activity were estimated as described by Milagres et al. (1999). Gelatinase activity was detected by liquefied solid gelatin, urease activity observed by color change using urea agar base supplemented with urea and amylase by zones on starch agar plates (Jha et al., 2008).
PGP traits. PSB trait was tested on Pikovskaya agar with 0.5% tricalcium phosphate $[Ca_3(PO_4)_2]$ by plating method. After 7 days of incubation, phosphate solubilization was verified by clear halo appearance around colonies (Pikovskaya et al., 1949). Siderophore production was estimated using the chrome azurol S (CAS) assay, described by Schwyn & Neilands (1987) and modified by Milagres et al. (1999). Bacteria were grown on one half of the plate containing KB, NA or FA medium and simultaneously the siderophore production was detected on the other half containing CAS–blue agar.
PCR assay. Total DNAs were isolated from bacterial isolates and strains using heat–lysed method. Bacterial colonies, grown on KB (*Pseudomonas*), NA (*Bacillus*) and ON (*Azotobacter*) for 48 h at 25^0C, were suspended in 300 µl of distilled sterile water, boiled for 8 min. and incubated on ice for 5 min. The lysed suspensions were centrifuged for 3 min at 13000 rpm and supernatants were used in PCR reactions. PCR reactions were carried out in a 25 µl volumes using Green Taq Dream master mix (Thermo Scientific, Lithuania) with 1µl of template DNA and 0.1 µmol of $(GTG)_5$ primer. Amplifications were performed in Eppendorf MasterCycler personal (Germany) with the temperature profile: an initial denaturation at 95^0C for 7 min followed by 35 cycles of a three–step PCR program (94^0C for 1 min, 52^0C for 1 min and 65^0C for 8 min) and a final extension at 65^0C for 16 min (de Bruijn, 1992). Amplified products were separated by electrophoresis for 2h at 5V/cm in 1,2% (w/v) agarose gel with GeneRuler DNA Ladder mix SM0331 (Thermo Scientific, Lithuania).

Results and Discussion

Plant growth promoting rhizobacteria (PGPR) can influence plant growth by various mechanisms. Effects of PGPR strains in different crops were already demonstrated (Wu et al., 2005). Bacterial inoculants are able to increase plant growth, protect plants from disease, improve seedling emergence and responses to external stress factors (Lugtenberg et al., 2002).
The present study was designed to assess the mixture of selected rhizospheric bacterial strains *Bacillus* Q7, *Pseudomonas* PS2 and three *Azotobacter* strains (NM5, NM8 and NM 14) for their competitiveness and effects on plant growth and yield of different maize genotypes. The

enzymatic activities and PGP traits were used for obtaining the specific phenotype patterns (tab. 1). Isolates from different maize genotypes showing similar phenotypic patterns (more than 7 traits) were included in genotypic analysis. Rep–PCR (BOX type) using $(GTG)_5$ primer was appropriate for amplification of DNA fragments which form different patterns within the groups of applied bacteria in the mixture. Specific $(GTG)_5$ patterns were used to evaluate the persistence of inoculated strains in the same bacterial genera and to estimate colonization within the rhizosphere (tab. 2).

Table 1. Plant growth promoting traits, enzymatic activities and BOX type of introduced *Bacillus, Pseudomonas* and *Azotobacter* strains

Bacterial strain	Enzymatic activities [a]						PGP trait		Phenotype pattern	BOX type $(GTG)_5$
	Celulase	Pectinase	Protease	Gelatinase	Urease	Amylase	Phosphate solubilizaton ability[b]	Siderophore production[c]		
Bacillus Q7	-	-	-	+	-	-	-	-	BI	B–A1
Pseudomonas PS2	-	-	+	-	+	-	+++	++	PII	P–A2
Azotobacter NM5	-	-	-	±	-	-	-	-	AIII	A–A1
Azotobacter NM8	-	-	-	±	-	-	++	-	AIII	A–A2
Azotobacter NM14	+	-	-	±	-	+	+	-	AIV	A–A3

[a]Protease, gellatinase, cellulase, pectinase, urease and amylase activities were determined by plate assay (+) hydrolysis; (-) no hydrolysis.
[b]Efficacy of phosphate solubilization evaluated according to halo diameter and colony diameter: (+) 1-4 mm/day; (++) 4-7 mm/day; (+++) ≥ 7 mm/day
[c]Siderophore activity on CAS medium: (+)1-5 mm wide of orange zone; (++) 5-20 mm wide of orange zone; (+++) ≥ 20 mm wide of orange zone.

Table 2. Appearance (%) of introduced bacterial strains in rhizosphere of different maize genotypes

Bacterial strain	Introduced strain patterns	Introduced strain pattern (%) in 5 maize genotypes				
		3014	4015	5043	6010	6030
Bacillus Q7	B–I	85	75	**95**	80	95
	B–A1	80	65	**95**	80	90
Pseudomonas PS2	P–II	65	70	**85**	65	65
	P–A2	55	45	**70**	50	55
Azotobacter NM5	A–III	**25**	80	85	70	75
	A–A1	**21**	5	5	5	10
Azotobacter NM8	A–III	25	80	**85**	70	75
	A–A2	2	75	**80**	65	65
Azotobacter NM14	A–IV	**75**	20	15	30	25
	A–A3	**75**	20	10	25	20

To develop the efficient mixture of strains for promoting maize growth and yield in field conditions we tested different maize genotypes and their impact on rhizosphere competence of applied strains. The higher percent of applied strain appearance was observed in *Bacillus* Q7 strain in combination with maize genotype 5043, than genotype 6030. Also, *Pseudomonas*

PS2 and *Azotobacter* NM8 were the most frequent in maize genotype 5043, while the maximal number of *Azotobacter* NM14 and *Azotobacter* NM5 was found in the rhizosphere of maize genotype 3014.
To achieve good growth promoting interaction between introduced PGP strains and maize genotype in the presence of other microorganisms, we estimated plant parametres: plant weight, 1000 seed weight, N, P and K content and yield (unpublished data).
The phosphate–solubilizing activity of microorganisms is enabled by production of organic and inorganic acids, converting insoluble mineral phosphates into soluble forms (Kravchenko et al., 2004). Solubilization of different form of phosphates often leads to increasing of mass and productivity of agriculture plants (Lugtenberg & Kamilova, 2009). *Pseudomonas* PS2 and *Azotobacter* NM8 showed substantial acid production and tricalcium phosphate solubilization. *Azotobacter* NM14 was able to solubilize this mineral phosphate very slowly (1.5 mm/day). Siderophore production was detected only by *Pseudomonas* PS2 strain and in several indigenous isolates. Isolate PS2 cause hyphal deformation of several phytopatogenic fungi (*Alternaria tenuissima, Curvularia lunata, Fusarium semitectum, F. equiseti* from *Salvia officinalis L., F. equiseti* from *Matricaria chamomilla L., Myrothecium verrucaria, Verticillium* sp., *Diaporte eres complex* and *Sclerotinia sclerotiorum*) and effectively inhibit mycelial growth, due to production of chitinases, siderpohores, lytic enzymes and several antibiotics (Đuric et al., 2011; Jošic et al., 2012 *a*, *b*). All *Azotobacter* strains (*Mrkovački & Milic*, 2001) and *Bacillus* Q7 strain (unpublished data) were able to stimulate growth of several agriculture plants. Pseudomonades PS2, being suitable for application in maize cultivation as biocontrol agent, and *Azotobacter* and *Bacillus* Q7, as good PGP strains, were involved in this competitiveness field trial investigation. All applied bacterial strains, except *Azotobacter* NM5, showed high percent of appearance in rhizosphere of all maize genotype and well adaptation to particular soil environment and extreme weather conditions (drought).

Conclusion

The obtained results assessed by a combination of phenotyping and genotyping methods showed that a mixture of *Azotobacter* NM8 and NM14, *Pseudomonas* PS2 and *Bacillus* Q7 had better competitiveness to indigenous bacteria in the rhizosphere of all maize genotypes. The mixture can be further tested for application as maize growth and yield promoting inoculum on different soil types.

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