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SCREENING OF AZOTOBACTER ISOLATES FOR PGP PROPERTIES AND ANTIFUNGAL ACTIVITY

ABSTRACT: Among 50 bacterial isolates obtained from maize rhizosphere, 13 isolates belonged to the genus *Azotobacter*. Isolates were biochemically characterized and estimated for pH and halo tolerance ability and antibiotic resistance. According to characterization, the six representative isolates were selected and further screened *in vitro* for plant growth promoting properties: production of indole-3-acetic acid (IAA), siderophores, hydrogen cyanide (HCN), exopolysaccharides, phosphate solubilization and antifungal activity (vs. *Helminthosporium sp.*, *Macrophomina sp., Fusarium sp.*). Beside HCN production, PGP properties were detected for all isolates except Azt_7. All isolates produced IAA in the medium without L-tryptophan and the amount of produced IAA increased with concentration of precursor in medium. The highest amount of IAA was produced by isolates Azt_4 (37.69 and 45.86 μg ml^{-1}) and Azt_5 (29.44 and 50.38 μg ml^{-1}) in the medium with addition of L-tryptophan (2.5 and 5 mM). The isolates showed the highest antifungal activity against *Helminthosporium* sp. and the smallest antagonistic effect on *Macrophomina* sp. Radial Growth Inhibition (RGI) obtained by the confrontation of isolates with tested phytopathogenic fungi, ranged from 10 to 48%.

KEYWORDS: antifungal activity, *Azotobacter*, IAA, maize, PGP properties, rhizosphere

INTRODUCTION

Maize (*Zea mays* L.) is one of the three world's most widely grown crop with an annual global production of 1 billion t in 2013 [available at FAOSTAT]. In Serbia, maize is grown on about 1.2–1.4 million ha, with a total grain production between 4 and 7 million t per year [Jocković *et al*., 2010]. Besides genetic potential, achieving higher yields also demands appropriate fertilization.

Recently, plant growth promoting rhizobacteria (PGPR) have been used to enhance crop yield and improve agricultural sustainability. PGPR are directly involved in increased uptake of nitrogen through biological nitrogen fixation,

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synthesis of phytohormones, solubilization of minerals such as phosphorus and production of siderophores that chelate iron and make it available to the plant root [Ahemad and Kibret 2014].

The beneficial effect of *Azotobacter*, applied alone or in mixture with other PGPR strains, on vegetative growth and yield of maize was reported by numerous authors [Biari *et al.,* 2008; Gholami *et al.,* 2009; Jarak *et al.*, 2012]. Yield increase by *Azotobacter* inoculation is a result of nitrogen fixation, as well as the production of growth regulators, antibacterial and antifungal compounds [Mrkovački and Milić 2001; Wani *et al.*, 2013].

Efficiency of microbial preparations can be increased by using the best combination of beneficial microorganisms and it requires a clear definition of useful and necessary properties of a microorganism selected for specific environmental conditions and certain plants. Therefore, the aim of this study was to perform characterization of *Azotobacter* isolates from maize rhizosphere and selection of strains with potential environmental and plant growth properties, as well as antagonistic activities against phytopathogenic fungi, for the purpose of further field application.

MATERIAL AND METHODS

Isolation and characterization of Azotobacter strains

The rhizosphere soil samples were collected from the one-month-old maize plants (hybrid NS6010) grown on calcareous chernozem soil at Rimski Šančevi experimental field of the Institute of Field and Vegetable Crops, Novi Sad, Serbia. Chemical soil properties of the experimental field were: pH (in H_2O) – 8.42, nitrogen percentage – 0.152%, calcium carbonate percentage – 5.04%, humus content – 2.05%, available P and K contents – 12.8 and 17.3 mg 100 g^{-1} soil. The soil paste-plate method [Becking 1981] was used as isolation strategy for *Azotobacter*. The isolates were characterized by their morphological and biochemical characteristics using standard methods [Jarak and Đurić 2004].

The isolates were grown on N free medium adjusted with 1M HCl or 1M NaOH for pH tolerance (5.5 and 9.0) and supplemented with 3% and 7% NaCl for salt tolerance. Determination of the direct impact of antibiotics was performed by the diffusion method using different concentrations of antibiotics (μg ml^{-1}): ampicillin (10, 25), neomycin (10, 30), erithromicin (5, 15), streptomycin (10, 300), chloramphenicol (10), and kanamycin (30).

In vitro screening for plant growth promoting properties

Phosphate solubilization. The ability of isolates to dissolve sparingly soluble inorganic phosphate was determined by spot inoculations on PVK (Pikovskaya medium) [Pikovskaya 1948] and NBRIP (National Botanical Research Institute's phosphate growth medium) [Nautiyal 1999] with 0.5% TCP ($Ca_3(PO_4)_2$).

Siderophores production. Bacterial ability to produce siderophores was assayed on a chrom-azurol S (CAS) medium by protocol of Milagres *et al.* [1999].

Exopolysaccharides (EPS) production. In order to test the production of EPS, isolates were grown on the appropriate media supplemented with 0.02% Calcofluor color (Calcofluor White M2R, Sigma) [Reed *et al.*, 1991].

*Hydrocyanic acid (HCN) production***.** HCN production was tested on HCN induction medium supplemented with glycine (4.4 g l^{-1}) [Ayyadurai *et al.*, 2007].

Indole acetic acid (IAA) production. For quantitative analysis of IAA production, a 100 µl 24h-old bacterial suspension was inoculated in liquid N-free medium, supplemented without and with 2.5 and 5 mM of L-tryptophan (as precursor of IAA) and incubated for 48h at standard temperature. Salkowski reagent was mixed with the supernatant (2:1 v/v) and intesity of the developed color was measured at 530 nm [Glickman and Dessaux 1995].

*Antifungal activity assay***.** The ability of the isolates to inhibit the growth of the phytopathogenic fungi (*Helminthosporium sp.*, *Macrophomina sp.* and *Fusarium sp.*) was determined by the method of dual culture [Rodriguez *et al.*, 2000]. Radial Growth Inhibition (RGI) was caluculated according to formula: RGI (%) = $[(r_1-r_2) / r_1] \times 100$; where: r_1 = radius/growth of mycelium in the control and r_2 = radius/growth of mycelium confronted with a bacterial isolate.

*Statistical Analysis***.** The statistical variation in IAA production by *Azotobacter* isolates and antifungal activity were analyzed using the analysis of variance (ANOVA), followed by mean separation according to Duncan's Multiple Range test ($p<0.05$).

RESULTS AND DISCUSSION

Among 50 bacterial isolates obtained from maize rhizosphere, according to morphological and biochemical characterization, 13 representative isolates were grouped into genus *Azotobacter* as described in Bergey's Manual of Determinative Bacteriology [Holt *et al.*, 1994]. General properties of test isolates were presented in Table 1. All the isolates were gram-negative and able to use the examined carbohydrate and citrate as carbon sources.

Table 1. *Screening of isolates for morphological and biochemical characteristics*

Morphology	C utilization	Isolates %	Test reaction	Isolates %
Colony Slimy, glistening, brown to yellow-green on aging, medium to large-size *Cell* Gr−ve rod-shaped to coccoid cells	Glucose	69	Citratase	84
	Galactose	100	Gelatinase	92
	Sucrose	69	Amilase	0
	Fructose	100	Catalase	100
	Lactose	92	Urease	69
	Mannitol	69	Nitrate reduction	61

Soil properties have a strong impact on a range of processes that influence crop yield, including microbial activity. Bacteria investigated in our study expressed good potential for adaptation. Four isolates had optimal growth on the medium with pH 5.5. All isolates were tolerant to concentration of 3% NaCl, while on the medium with the addition of 7% NaCl growth was not recorded. Resistance to antibiotics depended on the tested isolates, types and concentrations of antibiotics. Neomycin and streptomycin had the largest inhibitory effect, while the most tolerant isolates were *Azt*$_1$, *Azt*$_5$ and *Azt*$_7$ (Table 2).

Table 2. *Screening of isolates for pH and halo tolerance and antibiotic resistance*

Isolates	pH and halo tolerance				Antibiotics (μg ml^{-1})									
	pH		NaCl (%)		Amp		Ery		Neo		Str		Kan	Chl
	5.5	9.0	3	7	10	25	5	15	10	30	10	300	30	30
Azt$_1$	-	-	+	-	r	r	r	s	s	s	s	s	r	r
Azt$_2$	+	-	+	-	s	s	s	s	r	s	s	s	s	s
Azt$_3$	-	-	+	-	s	s	s	s	s	s	s	s	s	s
Azt$_4$	+	-	+	-	s	s	s	s	s	s	s	s	s	s
Azt$_5$	+	-	+	-	r	r	s	s	s	s	s	s	s	r
Azt$_6$	-	-	+	-	s	s	s	s	s	s	s	s	s	s
Azt$_7$	-	-	+	-	r	s	r	s	s	s	s	s	s	r
Azt$_8$	-	-	+	-	s	s	s	s	s	s	s	s	s	s
Azt$_9$	-	-	+	-	s	s	s	s	s	s	s	s	s	s
Azt$_{10}$	+	-	+	-	s	s	s	s	s	s	s	s	s	s
Azt$_{11}$	-	-	+	-	s	s	s	s	s	s	s	s	s	s
Azt$_{12}$	-	-	+	-	s	s	s	s	s	s	s	s	s	s
Azt$_{13}$	-	-	+	-	s	s	s	s	s	s	s	s	s	s

pH and halo tolerance: (+) growth; (-) growth absent; Antibiotic resistance: (s) sensitive; (r) resistant

According to environmental properties, the six representative isolates were selected for further screening on the basis of PGP properties and antifungal activity (Table 3). Poor solubility of TCP on PVK and NBRIP has been determined, with the width of the solubilization zone between 1 and 4 mm, whereas a larger solubilising zone, from 4 to 7 mm, was measured only for isolate *Azt*$_7$ on the NBRIP medium. The ability of bacteria to produce siderophores was detected in all isolates except *Azt*$_7$. Larger orange zone, from 5 to 15 mm, was measured for isolates *Azt*$_5$ and *Azt*$_{10}$, while the zone of color change for other isolates was between 1 and 5 mm. All isolates except *Azt*$_7$ produced exopolysaccharides, while production of HCN was detected in isolates *Azt*$_5$ and *Azt*$_{10}$.

Diverse PGPR produce IAA and other metabolically active substances, which lead to an increase in root length, height of above ground plant parts and yield. Bacteria use this phytohormone to interact with plants as part of their colonization strategy, including phytostimulation and circumvention of basal plant defense mechanisms [Spaepen *et al.*, 2007]. In this study, the quantity of produced IAA depended on the applied concentration of L-tryptophan and tested isolates. All isolates produced IAA in the medium without L-tryptophan and the amount of produced IAA increased with concentration of precursor in medium. Isolate Azt_{10} was the best IAA producer in the medium without precursor (26.16 μg ml^{-1}), while isolates Azt_4 (37.69 and 45.86 μg ml^{-1}) and Azt_5 (29.44 and 50.38 μg ml^{-1}) produced the largest amounts in medium supplemented with 2.5 and 5 mM L-tryptophan. This is in accordance with the investigation of Govedarica *et al.* [1993]. In their study, *Azotobacter* strains isolated from chernozem soil had the ability to produce auxins, gibberelins and phenols and thus increase planth length, mass and nitrogen content of tomato plants. Similarly, variability within the same PGPR properties in different isolates was recorded by Mahalakshmi and Reetha [2009], while Suresh *et al.* [2010] concluded that the most isolates from maize rhizosphere possessed PGPR characteristics, and therefore should be used as potential biofertilizers.

Table 3. *Screening of isolates for PGP properties and antifungal activity*

Isolates	P-sol		Siderophore	HCN	EPS	IAA production (μg ml^{-1})			Radial Growth Inhibition (%)		
						mM L-trypthophan					
	PVK	NBRIP				0	2.5	5	Macrophomina	Helminthosporium	Fusarium
Azt1	+	+	+	-	+	4.57^{d}	28.71^{c}	36.20^{c}	19.61^{a}	46.76^{ab}	38.43^{ab}
Azt2	+	+	+	-	+	2.96^{e}	23.73^{f}	24.38^{f}	18.03^{a}	42.28^{ab}	39.21^{a}
Azt4	+	+	+	-	+	7.26^{b}	37.69^{a}	45.86^{b}	10.59^{a}	29.36^{b}	35.29^{bc}
Azt5	+	+	++	+	+	5.26^{c}	29.44^{b}	50.38^{a}	10.98^{a}	40.79^{b}	27.06^{bc}
Azt7	+	++	-	-	-	4.13^{d}	24.66^{e}	28.79^{e}	21.96^{a}	48.25^{a}	34.50^{bc}
Azt10	+	+	++	+	+	26.16^{a}	27.09^{d}	30.83^{d}	18.82^{a}	42.79^{ab}	30.58^{c}

P-sol: (+) 1-4 mm of halo diameter; (++) 4-7 mm of halo diameter; siderophore: (-) no color change (+) 1-5 mm wide of orange zone; (++) 5-15 mm wide of orange zone; HCN, EPS: (+) positive reaction; (-) negative reaction; IAA: The different letter above the number inicates a significant difference at $P < 0.05$

Azotobacter isolates showed the highest antifungal activity against *Helminthosporium* sp. and the smallest antagonistic effect on *Macrophomina* sp. RGI, obtained by confrontation of isolates with tested pathogens, ranged from 10 to 48%. The largest decrease in growth of *Macrophomina* sp. and *Helminthosporium* sp. was obtained by the confrontation with isolates Azt_1 (19.61%

and 46.76%) and Azt_7 (21.96% and 48.25%). The highest antifungal activity against *Fusarium* sp. was registered through confrontation with the isolates Azt_1 and Azt_2 (38.43% and 39.21%). Other isolates had equally good antagonistic effect against the tested pathogens. Similar findings about fungal growth inhibition and posibble application of *Azotobacter* isolates as biocontrol agents were obtained in numerous studies. Subba Rao [2001] proved that isolates of *Azotobacter chroococcum* produced an antibiotic which inhibited the growth of several pathogenic fungi. Investigating the effect of *Azotobacter* isolates against *Apergillus flavus*, *Cercospora* sp., and *Fusarium oxysporum*, Ponmurugan *et al*. [2012] determined the larger inhibition zone at a higher suspension of culture.

CONCLUSION

This study confirmed the occurence of *Azotobacter* sp. in maize rhizosphere. Results are of practical importance because we demonstrated that the tested isolates produce a considerable amount of IAA, and have a good antifungal activity, partly due to production of siderophores and HCN. Further studies on the performance of these isolates in soil-plant system are needed to establish which traits of selected isolates are useful and necessary for certain environmental conditions and different hybrids.

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ИСПИТИВАЊЕ PGP СВОЈСТАВА И АНТИФУНГАЛНЕ АКТИВНОСТИ ИЗОЛАТА АЗОТОБАКТЕРА

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РЕЗИМЕ: Међу 50 изолата бактерија из ризосфере кукуруза, 13 изолата припадали су роду *Azotobacter*. Изолати су биохемијски карактерисани и испитана је толерантност према реакцији средине, концентрацији соли и резистентност на антибиотике. Након карактеризације, одабрано је шест репрезентативних изолата за даља испитивања PGP својстава: продукције индол-3-сирћетне киселине (IAA), сидерофора, цијановодоничне киселине, егзополисахарида, фосфосолубилизације и антифунгалне активности (према *Helminthosporium* sp., *Macrophomina* sp., *Fusarium* sp.). Осим продукције HCN, PGP својства утврђена су за све изолате осим Azt_7. Највећу количину IAA продуковали су изолати Azt_4 (37,69 и 45,86 μg ml^{-1}) и Azt_5 (29,44 и 50,38 μg ml^{-1}) у подлози са додатком L-tryptophan-a (2,5 и 5 mM). Изолати су испољили највећу антифунгалну активност према *Helminthosporium* sp., а најмањи антагоностички ефекат према *Macrophomina* sp. Проценат инхибиције раста (RGI) добијен суочавањем изолата са испитиваним фитопатогеним гљивама кретао се од 10 до 48%.

КЉУЧНЕ РЕЧИ: антифунгална активност, *Azotobacter*, IAA, кукуруз, PGP својства, ризосфера