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INDIGENOUS PSEUDOMONADS FROM RHIZOSPHERE OF MAIZE GROWN ON PSEUDOGLEY SOIL IN SERBIA

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Abstract

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Pseudomonads are distributed widely in temperate soils and often predominate among bacteria from plant rhizosphere. The present study revealed the genodiversity among 67 pseudomonads isolates from maize rhizosphere (39) and non-rhizospheric (28) nutrient poor pseudogley soil. Results from the screening for plant growth promoting (PGP) traits (phosphate solubilization, siderophore and indoleacetic acid production, enzymatic activity) and rep-PCR fingerprinting of isolates using ERIC and $(GTG)_5$ primers indicated low diversity existing in this selective environment. We selected five different isolates – two of them were non-phytopathogenic isolates with protease, lipase and phospholipase activity, ability to solubilize tricalcium phosphate and produce indoleacetic acids. All isolates inhibited growth of some of seven investigated phytopathogenic fungi. Isolate Q4m showed high enzymatic activity, good phosphate solubilization and production of siderophores and high amount of indoleacetic acids (from 8.6 μg/ml without tryptophan in medium to 26 μg/ml with 5 mM tryptophan added in medium). This selected strain has the potential to enhance plant growth and participate in phosphorus and iron availability in rhizosphere.

Key words: pseudomonads, PGPR, rhizosphere, siderophores, PCB, rep- PCR, genodiversity
Abbreviations: PSB - Phosphate-Solubilizing Bacteria; IAA – Indole Acetic Acid; PGPR - Plant Growth Promoting Rhizobacteria; CAS - Chrome Azurol S ; IAR - Intrinsic Antibiotic Resistance; rep-PCR - repetitive sequence-based polymerase chain; reaction; ERIC- enterobacterial repetitive intergeneric consensus

Introduction

Plant growth promoting rhizobacteria (PGPR) are beneficial microorganisms able to enhance plant growth and increase yield, improve plant health and crop protection. Plant hormone production, nutrient mobilization and plant protection are the generally accepted mechanisms for plant growth promotion (Cattelan et al., 1999; Ryu et al., 2006; Ahn et al., 2007).

Phosphorus and iron are some of the major essential macronutrients for biological growth and

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development. Most agricultural soils contain large reserves of total P, a part of the accumulated P depends on regular application of chemical fertilizers or sludge from wastewater treatment. Both P fixation and precipitation occur in soil, because of the large reactivity of phosphate ions with numerous soil constituents. The concentration of soluble P in soil is usually very low and pool of immediately available P is extremely small and must be replenished regularly to meet plant requirements. Interest has been focused on the inoculation of phosphate-solubilizing bacteria (PSB) into the soil to increase the availability of native, fixed P and to reduce the use of fertilizers. Phosphate solubilization occurs by carboxylic acids synthesized and released by microorganisms. These carboxylic acids also may decrease pH (Puente et al., 2004; Rodriguez et al., 2006).

Bioavailability of iron in soil is limited by low solubility of Fe3+, the predominant state of iron in every environment. Accumulation in mineral phases such as iron hydroxides cannot be utilized by plants. Some bacteria and fungi, when grow in iron deficient environment, synthesize siderophores, iron chelating agents which bind with Fe3+ ions and form soluble complexes that can be transported into the cells. Siderpophores act as biocontrol agents by limiting iron availability to the pathogens and lead to induction of plants host resistance against pathogens (Meziane et al., 2005).

PGPR also produce many secondary metabolites and hydrolytic enzymes, which act as antifungal factors and can degrade the structural matrix of fungal cell walls (Cattelan et al., 1999; Wolf et al., 2002; Hu, 2005; Zarrin et al., 2009). Rhizobacteria can produce or change the concentration of plant growth stimulating hormones such as indoleacetic acid (IAA), gibberellic acid, cytokinins and ethylene and exert beneficial effect on plants. IAA produced by bacteria can induce increasing the number of root hairs and lateral roots (Okon and Kapulnik, 1986).

We investigated maize rhizospheric and non-rhizospheric soil from the same location, looking for indigenous isolates, which grew on unpolluted rural soil. The objective of the present study was to assess the plant growth promoting traits of indigenous pseudomonads isolates, estimate their genodiversity and select the most promising isolates for application in plant production on nutrient poor soils.

Materials and Methods

Isolation of pseudomonads from rhizosphere and IAR pattern

Soil samples were taken from rhizosphere of good growing maize (Qm), growing on extremely poor pseudogley soil and from non-rhizospheric soil (q) of the same locations on Vagan hill in Serbia.

Pseudomonas isolates were separated from other bacteria on nutrient agar (NA) medium containing chloramphenicol and ampicillin (100 μg/ml). All isolates were subcultured several times to obtaine pure cultures and tested for fluorescence on King B (KB) medium. For additional susceptibility patterns to antibiotics, isolates were grown on NA with addition of 10, 50 and 100μg/ml of gentamicin, 10, 50 and 100 μg/ml of tetracycline.

NA medium was supplemented with 4%NaCl for salt tolerance test and various pH (from 5 to 9) adjusted with 1M HCl or 1M NaOH for pH tolerance.

rep- PCR

Total genomic DNA was extracted using the method described by Ross et al. (2000). Screening of genodiversity was performed as recommended by Versalovic et al. (1994), using BOX type $(GTG)_5$ primer and ERIC 1R/ERIC 2 primer pair. PCR reactions were performed in 50μl reaction mixture using Dream Taq Green Polymerase (Fermentas, Lithuania). Amplifications were undertaken in a Eppendorf Master Cycler Personal (Germany) and

products were separated by gel electrophoresis on 1.5% agarose gel in 0.5x Tris borate EDTA buffer at 5V/cm. Similarity and clustering was based on the unweighted pair group arithmetic average-linkage algorithm using STATISTICA 7 software.

Production of extracellular enzymes

Cellulase and pectinase production was determined in M9 medium supplemented with yeast extract (1.2 g/l) and carboxy-methyl cellulose (10g/l) or pectin (4.8 g/l), respectively (Cattelan et al., 1999). After incubation at 28^0C during 6 days, cellulasa positive isolates were surrounded by clear halos. Pectinase activity of isolates was observed after 2 days of incubation: plates were flooded with 2M HCl and appearance of clear zones was verified. Protease production was determined using skim milk agar based on proteolytic activity, as described by Jha et al. (2008). 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 (Cappucino and Sherman, 2001). Production of phospholipases and lipases was assayed on basal medium supplemented with Tween 80 (1% w/v) and egg yolk, respectively (Lanyi, 1987) and production of opaque zones around colonies indicated lipolytic activity, since production of lecithinase (phospholipase) caused the zone of turbidity surrounding the colony. Chitinase activity was observed on Waksman medium (Minkwitz and Berg, 2001) supplemented with 0.2% colloidal chitin by clearing zone around bacterial colony.

Phosphate solubilization ability test

For solubilization assay, isolates were spot inoculated on Pikovskaya (1948) agar with 0.5% tricalcium phosphate [$Ca_3(PO_4)_2$]. Bacteria that induced clear zone around the colonies after 3 days of incubation at 28^0C were considered positive for production of phosphate solubilizing enzyme.

Production of siderophores and HCN

Siderophore production was estimated using the modified chrome azurol S (CAS) assay. Modification of methodology described by Schwyn and Neilands (1987) was reported by Milagres et al. (1999). For CAS assay in solid medium, plates were inoculated with 10µl of ~$5x10^6$ bacteria on the borderline between CAS and King B medium. The siderophores production was detected simultaneously on the other half-containing CAS-blue agar and intensity of production was measured by front of change color from blue to yellow-orange.

HCN production was tested on King B medium supplemented with glicine (4.4 g/l). After 4 days at 28^0C incubation of inverted plate with filter paper (impregnated with 0.5% picric acid and 2% sodium carbonate) placed on the lid and sealed with parafilm, production of cyanide was detected by discoloration of yellow filter paper to orange or brown (Ayyadurai et al., 2007).

Production of IAA

IAA production was tested according to standard procedure (Glickmann and Dessaux, 1995). For quantitative analysis, 10µl of each bacterial suspension (standardized to OD_{600} of 0.625) was inoculated in liquid King B medium amend with different concentration of tryptophan (0; 2.5 and 5 mM). After 24h and 48h of incubation at standard temperature, the supernatant was mixed (1:2 v/v) with Salkowski reagent (2% 0.5M ferric chloride in 35% perchloric acid) and intensity of pink color was measured at 530 nm for IAA production and standard curve of IAA and absorbance at 600 nm for concentration of isolates. All results for IAA are standardized to $OD_{600} = 1$.

Fungal inhibition bioassays

Screening of isolates for *in vitro* antagonism towards seven fungal pathogens (*Alternaria alternata*, *Dreschlera tetramera*, *Diaporte Eres Complex*, *Sclerotinia sclerotiorum*, *Fusarium verticillioides,*

F. sporotrichioides and *F. equiseti*) was obtained by standard coinoculation techniques on Waksman agar (Minkwitz and Berg, 2001). Inhibitions of mycelium growth by pseudomonads isolates with antifungal activity were observed on Waksman medium according Wolf et al. (2002).

All experiments were performed in triplicate and repeated twice, while fungal inhibition bioassays were performed in four replicate and repeated three times.

Plant pathogenicity test

Pathogenicity of isolates was tested *ex vivo* following the protocol of Moragrega et al. (2003). Young leaves of lilac were collected the same day that inoculation was performed and dipped in 1% active sodium hypochlorite solution for 5 minutes. Detached sterile leaves were inoculated with a 10 µl drop of the bacterial suspension (OD_{600} =0.625) on the midrib of the leaf. The same concentration of bacteria was inoculated (by injection) in surface sterilized bean pods. Sterile distilled water was used as negative control. Inoculated bean pods and lilac leaves were incubated 4 days at 24^0C in controlled environment chamber. Five-severity index levels scale (0 to 4) was applied to quantify the intensity of infections. Three replicates of five bean pods and lilac leaves per replicate were used per test, and tests were repeated twice.

Results

Isolation of pseudomonads from rhizosphere and IAR pattern

Bacterial isolates indigenously growing on maize rhizospheric soil were examined and 67 different bacterial isolates resistant to ampicillin and chloramphenicol (100µg/ml) were isolated. Five different groups with representative isolates and their frequency (Table 1) were obtained based on rep-PCR patterns and susceptibility to additional antibiotic as IAR (Intrinsic Antibiotic Resistance) patterns. All strains were fluorescent on King B medium and identified as *Pseudomonas* sp.

rep- PCR

Genotypic analysis was performed by rep-PCR. ERIC patterns (Figure 1a) and BOX patterns obtained with $(GTG)_5$ primer (Figure 1b) revealed the highest level of similarity between Q4m and Q20m (71%) and formed one cluster which was 43% similar to the other cluster (Figure 2). Isolates q4 and q5 formed sub cluster with 68% similarity and formed the second cluster with q3 (56% similarity).

Table 1
Salt and pH tolerance and Intrinsic Antibiotic Resistance of *Pseudomonas* isolates

Isolates	frequency [a]	King B fluorescence [b]	4% NaCl [c]	pH 5,5 -8,5 [c]	IAR (Intrinsic Antibiotic Resistance) [c] concentration (µg/ml)							
					Chl	Amp	Gen			Tet		
					100	100	10	50	100	10	50	100
Q4m	19 (32.2)	++	+	+	+	+	+	±	-	+	-	-
Q20m	12 (20.3)	+++	-	+	+	+	+	-	-	+	+	+
q3	9 (15.2)	+++	+	+	+	+	±	-	-	+	±	-
q4	11 (18.6)	+	-	-	+	+	+	±	-	±	-	-
q5	8 (13.5)	++	+	+	±	+	±	-	-	+	±	-

[a] number (and percent) of isolates with the same IAR and rep-PCR pattern

[b] intensity of fluorescence on King B medium after 24h of cultivation: ± low, + moderate, ++ strong, +++ very strong fluorescence; [c] isolate growth: (-) negative; (±) moderate; (+) positive

Production of extracellular enzymes

All isolates produced phospholipase (turbidity zone surrounding colony) and protease (clear halos on skim milk agar) (Table 2). None of the isolates produced amylase, celulase and pectinase. Isolate Q20m did not produce urease. Gelatinase activity was detected in two isolates by liquefied solid gelatin. Chitinase was produced only by isolate q5 growing on Waksman medium supplemented with colloidal chitin.

Phosphate solubilization ability test

Phosphate solubilization assay showed that four isolates induced clear zone on Pikovskaya agar supplemented with insoluble tricalcium phosphate (Table 3).

Production of siderophores and HCN

All investigated isolates were grown on the middle of the plate containing King B medium, but three isolates produced siderophores and changed the color of CAS solid medium from blue to orange. Production of siderophores were measured by color front change after 3 days of cultivation and ranged from 8.2 (q5) to 8.6 mm/day (Q4m). Three isolates changed color of yellow filter paper to orange caused by production of cyanide (Table 3).

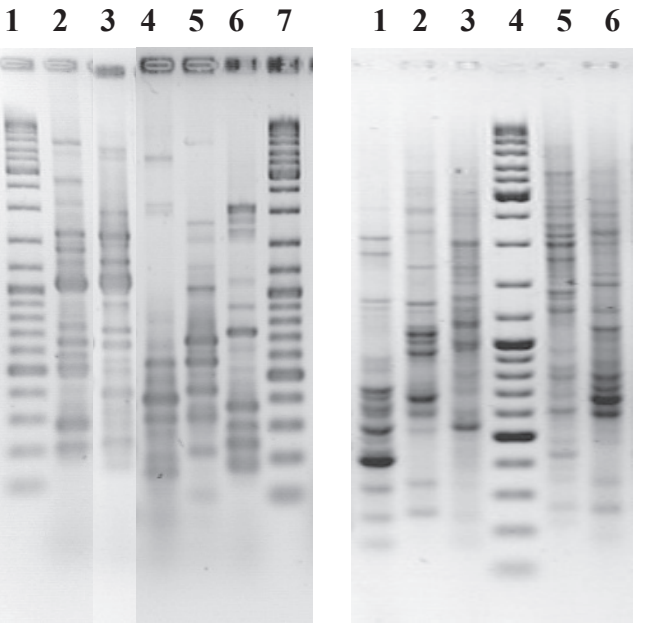

Fig. 1. rep-PCR of indigenous *Pseudomonas* sp. isolates from Serbian pseudogley soil on Vagan hill. a) ERIC patterns of representative isolates: lane 1. Marker; lane 2. Q4m; lane 3. Q20 m; lane 4. q3; lane 5. q4; lane 6. q5; lane 7. Marker; b) BOX patterns obtained by $(GTG)_5$ primer: lane 1. q3; lane 2. q4; lane 3. q5; lane 4. Marker; lane 5. Q4m; lane 6. Q20m. Marker: GeneRuler DNA Ladder mix SM0331 (Fermentas, Lithuania)

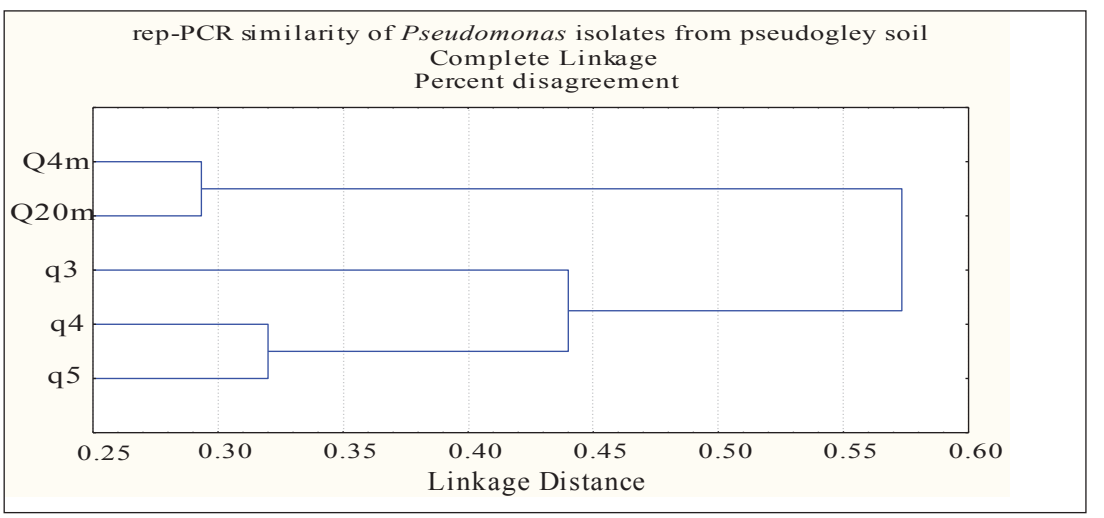

Fig. 2. Similarity of representative indigenous *Pseudomonas* sp. isolates from Serbian pseudoglay soil on Vagan hill obtained by rep-PCR (ERIC and BOX primers)

Table 2
Enzymatic activity of *Pseudomonas* isolates

Isolate	Enzymatic activity[b]					
	protease, phospholipase	urease	gelatinase	pectinase, celulase, amylase -	lipase	hitinase
Q4m	+	+	-	-	+	-
Q20m	+	-	-	-	+	-
q3	+	+	+	-	+	-
q4	+	+	-	-	+	-
q5	+	+	+	-	-	+

[b] enzymatic activity: (-) negative; (±) moderate; (+) positive

IAA production

All isolates showed production of IAA in KB medium without addition of tryptophan. Less than 4µg/ml of IAA after 24h and 48h of cultivation was produced by isolates Q20m, q3 q4 and q5, isolate Q4m produced more then 5µg/ml IAA. Influences of two tryptophan concentrations on tested isolates are shown in Table 3.

Fungal inhibition bioassays

The growth of seven phytopathogenic fungi: *Alternaria alternata*, *F. verticillioides, F. sporotrichioides, F. equiseti, Sclerotinia sclerotiorum, Dreschlera tetramera* and *Diaporte eres complex* (DEC) was tested in combination with selected bacteria. Isolates Q4m, Q20m and q5 inhibited growth of DEC. Low inhibition ability of Q4m and q3 isolates, represented by zones smaller then 5 mm, were observed for *Alternaria alternata* and *Dreschlera tetramera* growth, as shown in Table 4.

Plant pathogenicity test

Two different *Pseudomonas* sp. were isolated from root surface of maize - Q4m and Q20m, and no necrosis effect on lilac leaves or bean pods were observed (Table 4). Isolates from non-rhizospheric soil q3, q4 and q5 were pathogenic according to Moragrega et al. (2003) pathogenicity test.

Discussion

Population of fluorescent pseudomonads in plant rhizosphere is very abundant in agriculture (cultivated) soils and often led to plant growth promoting and biocontrol activity. We detected different populations of pseudomonads in rhizosfere of maize plant, also in bulk soil near the investigated rhizospheres. However, the extremely poor pseudogley soil on the old magmatic hill Vagan in Serbia, with rural land usage, showed low diversi-

Table 3
Main PGPR trait analysis of *Pseudomonas* isolates

Isolates	P- solubilization[a]	Siderophores production[b]	phenazine	HCN production	IAA production. µg/ml					
					without tripthophane		2.5 mM tripthophane		5 mM tripthophane	
					24h	48h	24h	48h	24h	48h
Q4m	+	3+	+	+	7.23±0.20	8.61±0.13	10.01±0.23	13.48±0.17	15.84±0.29	26.09±0.20
Q20m	+	–	+	+	3.59±0.11	4.06±0.19	5.91±0.22	7.14±0.15	12.20±0.22	11.77±0.14
q3	+	3+	+	+	1.45±0.18	3.07±0.11	5.28±0.16	4.60±0.14	12.75±0.27	7.44±0.16
q4	–	–	-	-	4.57±0.21	1.29±0.10	4.89±0.22	2.69±0.19	6.20±0.19	4.79±0.12
q5	+	3+	-	-	4.13±0.07	2.77±0.17	5.94±0.27	3.86±0.24	9.33±0.22	5.61±0.33

[a] phosphate solubilization zones (mm/day): – no solubilization; 1+: 1 to 3; 2+: 3 to 5; 3+: more then 5;
[b] siderophores production and front colour change (mm/day): – no production: 1+: 1 to 4; 2+: 4 to 8; 3+: more than 8.

ty of pseudomonads. Representative rhizospheric isolates (Q4m and Q20m) formed separate cluster which showed only 43% similarity with non-rhizospheric isolates. Isolate q3 was 44% distinct from other non-rhizospheric isolates.

Previous studies revealed that plant rhizospheric pseudomonads in Serbia exhibited high enzymatic activity (Djuric et al., in press). In this study, protease and phospholipase activity was observed in all isolates, while amylase, pectinase and cellulase were not detected. Chitinase activity was showed only by phytopathogenic isolate q5. Urease activity was not detected in Q20m isolate and lipase activity was not detected in q5 isolate. Gelatinase activity was shown by two phytopathogenic isolates – q3 and q5. The maize rhizospheric isolates Q4m and Q20m exhibited less enzymatic activity then phytopathogenic isolates from pseudogley soil. The functional characterization and genetic variability of fluorescent pseudomonads in rhizosphere soils has been reported by Ayyadurai et al. (2007) and included *Pseudomonas pseudoalcaligenes*, *P. fluorescens*, *P. putida*, and *S. maltophilia*. These antagonistic bacteria exhibited production of one or more antifungal metabolites (hydrogen cyanide, pyrrolnitrin, phenazine, pyoluteorin) and cell-wall-degrading enzyme (protease, pectinase, cellulase, chitinase).

Siderophore-producing bacteria promote plant growth indirectly, by sequestering the limited iron in the rhizosphere and reducing its availability for the growth of pathogens. In the present investigation, isolates q3 and high-phytopatogenic isolate q5 from non-rhizospheric soil and Q4m isolate from maize rhizosphere showed very good production of siderophores, with more than 8 mm/day front activity. Similar results for siderophore activity zones (6 mm/day) Poritsanos et al. (2006) reported for *P. chlororaphis* PA23 wild type strain under similar condition.

The beneficial effects of PGPR by production of phytohormones that promote root development and proliferation have been reported by Weller and Cook (1986), Patten and Glick (1996). All isolates in our investigation produce IAA ranging from 1.45 to 26.09 μg/ml. Stimulatory effect of higher

Table 4
Plant pathogenicity of *Pseudomonas* isolates and antifungal activity against phytopathogenic fungi

Antifungal activity against:[a]	Isolate				
	Q4m	Q20m	q3	q4	q5
Alternaria alternata	±	-	±	-	-
Dreschlera tetramera	-	±	±	-	±
Sclerotinia sclerotiorum	-	-	-	-	-
Fusarium sporotrichioides	-	-	-	-	-
F. verticillioides	-	-	±	-	-
F. equiseti	-	-	-	-	-
Diaporte eres complex	+	+	-	-	+
Plant pathogenicity [b]					
lilac leaves	0	0	3	2	4
been pods	0	0	3	2	3

[a] antifungal activity: (-) no growth inhibition ; (±) growth inhibition zone less than 5mm; (+) growth inhibition zone 5-15 mm; (++) growth inhibition zone more than 15 mm;

[b] plant patogenicity: 0- no infection; 1- necrosis limited to the inoculation point; 2- necrosis of the leaf midvein or necrotic area less than 5 mm diameter on bean pods; 3- necrosis of midvein and additional veins in leaves or necrotic area of 5-10 mm on bean pods; 4- necrosos of more than 50% leaf surface or necrotic area higher than 10 mm diameter on bean pods

applied concentration of tryptophan was observed after 24h in all selected isolates and ranged from 9.33 for q5 to 15.84 μg/ml for Q4m. Zarrin et al. (2009) reported production of 5.5 μg/ml IAA in presence of 1mg/l tryptophan for *Pseudomonas* WPR-61 in investigation of PGPR isolates against *Rhizioctonia solani* in wheat. Karnwal (2009) also tested *Pseudomonas* strains – *P. fluorescens* AK1 and *P. aeruginosa* AK2, for their ability to produce indoleacetic acid in pure culture in the absence and presence of L-tryptophan at 50- 500 μg/ml. It was also found that indole production increased with increasing of tryptophan concentration (from 0.2 to 9.3 μg/ml). Isolate Q4m produced 7.23 μg/ml IAA in medium without the presence of tryptophan and very high amount in presence of 2.5 and 5mM tryptophan – 10 and 15.84 μg/ml IAA after 24h of cultivation and 13.48 and 26.09 μg/ml after 48h of cultivation. Isolate Q4m showed better results than those reported by Zarrin (2009) and Karnwal (2009).

Solubilization of insoluble phosphorous to accessible forms (orthophosphate) promotes plant growth and increase yield (Tao et al., 2008; Yazdani et al., 2009). Many fluorescent pseudomonas have been reported as phosphate solubilizers: *P. chlororaphis*, *P. savastanoi*, *P. pickettii* (Cattelan et al., 1999), *P. fluorescens* EM85 (Dey et al., 2004), *P. plecoglossicida*, *P. mosselii*, *P. aeruginosa* (Jha et al., 2008), *P. trivalis*, *P. poae*, (Poonghuzhali et al., 2008). In our investigation four *Pseudomonas* isolates showed good phosphate solubilization ability, but only nonpathogenic isolates (Q4m and Q20m) can be taken into consideration for application as PGPR.

Diverse rhizobacteria (*Bacillus, Pseudomonas, Azotobacter, Azospirillum, Cryseobacterium*, etc.) suppressed growth of fungal mycelium by differencial mechanisms such as production of siderophores (Audenaert et al., 2002; Compant et al., 2005), antibiotics (Ayyadurai et al., 2007; Jha et al, 2008), hydrolytic enzymes (Pragash et al., 2009) and plant growth promoting hormones such as IAA and phosphatase (Zarrin et al., 2009; Zehnder et al., 1999). All plant-pathogenic non-rhizobacterial isolates showed low antifungal potential in our investigations. Mycelial growth of *Dreschlera tetramera* was stopped near isolates Q20m, q3 and q5, while the growth of *Alternaria alternata* was stopped near to Q4m and q3. Clear antagonistic activity was shown by isolates Q4m, Q20m, and q5 against *Diaporte eres complex* and formed zones of inhibition larger than 8 mm. Jha et al. (2008) reported that pseudomonad strains *P. plecoglossicida*, *P. mosselii*, *P. aeruginosa* formed very large inhibition zones, from 10 to 36 mm, against 13 phytopathogenic fungi. Seven different strains of fungi were used in our investigation; therefore, the size of the inhibition zones formed by Q4m, Q20m and q5 isolates is not comparable with their results.

Conclusion

Examination of indigenous PGP pseudomonads diversity, in maize rhizospheric soil and in non-rhizospheric soil, on specific locations (old magmatic hill Vagan) with rural land use was performed for the first time in Serbia. Low diversity of indigenous pseudomonads was confirmed by rep-PCR and IAR patterns. Isolate Q4m, effective in releasing P from inorganic complexes through solubilization, with the ability to produce high amounts of IAA, siderophores and to grow at high salt concentrations and different pH (from 5.5 to 8.5), has high potential for application. Estimation of ecological and economic significance of this isolate needs a further investigation in greenhouse and field conditions.

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