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Selection of indigenous fluorescent pseudomonad isolates from maize rhizospheric soil in Vojvodina as possible PGPR

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Abstract

Among 268 bacterial isolates obtained from maize rhizospheric soil from Novi Sad, Vojvodina, Serbia, 59 were fluorescent pseudomonads. According to intrinsic antibiotic resistance (IAR) and heavy metal tolerance (HMT) patterns, the six representative isolates were selected and their diversity was assessed. Lytic enzyme activity (protease, chitinase, lipase, phospolipase, cellulase, gellatinase, pectinase, urease), plant-growth promoting treats (P-solubilization, siderophores, HCN and IAA production) and plant pathogenicity estimation (on lilac leaf, apple and bean pods) resulted in selection of three isolates (PS2, PS4, PS6) with the outlook for agriculture application. Antifungal activity was estimated on dual culture with eight phytopathogenic fungi (Curvularia lunata, Fusarium semitectum, Fusarium equiseti from Salvia officinalis L., F.equiseti from Matricaria chamomilla L., Myrothecium verrucaria, Verticillium sp., Diaporte eres complex and Sclerotinia sclerotiorum) isolated from medicinal plants in Serbia. Isolate PS2 showed hyphal deformation of all investigated fungi and effective inhibition of mycelial growth of 7 out of 8 phytopatogenic fungi, partly due to production of chitinases, siderpohores and lytic enzymes. Abundant production of IAA (14 to 37 mM) and siderophores, phosphate solubilization and especially fungal growth inhibition make it suitable for further investigation, field trials and possible application in maize cultivation as biocontrol agent.

Key words: *Pseudomonas*, plant-growth promoting rhizobacteria, antifungal activity, biocontrol agents

Introduction

Since pollution caused by pesticides used for crop protection increases and pathogen resistance to have steadily been developing, biological control of plant diseases has been recognized as ecologically significant approach in modern agriculture [1].

It is well known that mixture of biocontrol agents with different mechanisms of diseases suppression could improve biological control of crop plants against soil-borne pathogens [2,3]. Isolation and characterization of the new rhizobacteria with plant-growth promoting properties represent the crucial steps for creation of compatible bacterial mixture to increase biocontrol performances of microbial inoculum for agriculture field applications.

Indigenous soil microflora comprises both beneficial and deleterious microorganisms that influence the plant growth and development, managing soil and plant health, and affecting the crop productivity in general. The plant beneficial microorganisms that colonize the root surface and the closely adhering soil interface were termed plant-growth promoting

rhizobacteria (PGPR) [4]. Roots exudates, which composition is mainly affected by stage of plant development, are nutrient sources for rhizosphere microorganisms, stimulating their proliferation and enzyme activity, and increasing their competition as well [5]. Released root metabolites create different microniches and consequently have pronounced selective and promoting effect for certain bacterial genotypes within each microsites [6,7]. Despite inhabiting different niches, rhizosphere-associated bacteria share some mechanisms that improve plant growth and/or protect them from soil-borne deleterious organisms.

Among rhizosphere habitants, strains from genus *Pseudomonas* are widely recognized as PGPR bacteria [8]. Fluorescent pseudomonads are often considered as predominant bacteria in rhizosphere [9] and certain fluorescent pseudomonads have received particular attention as potent biofertilizing and biocontrol agents [10,11]. They possess diverse metabolic abilities that enable them to utilize a wide range of organic compounds while occupying different ecological niches. Direct promotion of plant growth entails either production of the phytohormones such as auxins, cytokinins, gibberellins, and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase affecting the root morphogenesis and increasing absorptive surface, or improvement of nutrient availability through asymbiotic N_2 fixation and phosphate solubilization [12,13]. Indirectly, the plant-growth promotion is related to the biological control of detrimental microorganisms and plant pathogens through the aggressive colonization of root environment, the production of a broad spectrum of extracellular lytic enzymes, siderophores, diverse antibiotics, hydrogen cyanide, or by activation of plant defense-responses [14,15,16].

Occurrence of fluorescent *Pseudomonas* characterized by antimicrobial compounds production was evidenced in the environments of some important agriculture crop plants, and their abundance and prevalence in suppressive soils have been demonstrated [17,18]. Considerable amount of genetic variation among indigenous soil fluorescent pseudomonads plays a crucial role in the rhizosphere competence, plant development stimulation, biocontrol abilities and ability to overcome a stress in plant environments. The analysis of their genetic diversity and biochemical characterization creates a new approach in strains determination that are superior as individual or as compatible mixture for application in the modern agriculture systems. Therefore, investigation of functional diversity of bacterial community from rhizosphere has great practical importance, with application of bacterial strains as potent biofertilizing and biocontrol agents leading to improved crop productivity.

The aim of this investigation was to select indigenous *Pseudomonas* isolates from maize rhizosphere as possible PGPR and evaluate their plant pathogenicity and antagonistic effect on growth of different phytopathogenic fungi during co-cultivation experiments.

Materials and Methods

Isolation of fluorescent pseudomonad strains. The root-adhering soil sample was collected from 30 days old plants (Zea mays cv "NSSC640" produced in Institute of Field and Vegetable Crops) grown in carbonate chernozem type of soil (pH 8.39, organic matter percentage 2.06%, calcium carbonate percentage 13.61%, phosphorus 168.4 ppm, potassium 175,4 ppm, the water porosity $2.23 \cdot 10^{-3}$, the total porosity percentage 55.01%). A soil suspension was obtained by shaking 10 g of soil sample having root with tightly adhered soil in 90 ml of 0.1 M $MgSO4 \cdot 7H_2O$ buffer for 10 min at 180 rpm on a rotary shaker. Hundred microliters of the suspension was spread onto King B agar [19] and incubated at 25ºC for 48h. Occurrence of fluorescent pseudomonads was examined under UV light (356 nm). All

fluorescent colonies were picked up and recultivated a few times on KB agar to obtain pure cultures. These colonies were collected and cryopreserved at - 20ºC in 40% glycerol.

Taxonomic characterization of isolated strains was performed on the basis of tests for Gram staining, cytochrome oxidase, catalase, fermentation/utilization of glucose, lactose and sucrose, utilization of citrate and ability to degrade urea [20]. Fluorescent, Gram negative, both glucose and lactose non-fermenting isolates were evaluated for intrinsic antibiotic resistance (IAR) using different concentration of four antibiotics (ampicillin, tetracycline, gentamycin and chloramphenicol) and for heavy metal tolerance (HMT) using different concentrations of four heavy metal (Hg, Zn, Mo and Cd). According to IAR and HMT patterns, the six representative isolates were selected for further investigations.

***In vitro* assay of enzymatic activities**

In vitro assay of chitinolytic activity was assayed on the agar plates by method described by Chernin [21]. Semiminimal medium composed of synthetic media and nutrient broth (3:1) was supplemented with 0.2% colloidal chitin, and solidified with 1.5% agar. Colloidal chitin was prepared according to Kavino et al [22]. To investigate influence of different sugar occurrence on production of chitinolytic enzymes, semiminimal medium was modified by replacing Nutrient broth with Waksman [23] and Yeast mannitol medium [24]. Appearance of zone of clearance around bacterial colony indicated on chitinolytic activity.

***In vitro* assay of cellulolytic activity.** The cellulase activity of strains was tested on M9 medium [25] supplemented with yeast extract (0.12% w/v) and carboxyl-methylcellulose (CMC) (1% w/v) [26]. Isolates surrounded by clear halo after 8 days of incubation at 28ºC were considered as positive for cellulase production.

***In vitro* assay of pectinolytic activity.** The pectinase production was determined by protocol described by Cattelan et al.[26]. Bacterial isolates inoculated on M9 medium [25] amended with pectin (4.8 g l^{-1}) were incubated at 28ºC for two days and subsequently flooded by 2M HCl. The appearance of clear halo around colonies indicated on pectinase production.

***In vitro* assay of lipolytic and phospholipolytic activities.** Production of lipases was assayed on basal medium (bacto peptone 10 g l^{-1}, NaCl 5 g l^{-1}, $CaCl_2 \cdot H_2O$ 0.1 g l^{-1}, and agar 9 g l^{-1}) supplemented with Tween 80 (1% w/v) [27]. After 7 days of incubation at 26ºC, production of opaque zones around colonies indicated on lipolytic activity. Production of lecithinase (phospholipase) was assayed on basal media (tripton 5 g l^{-1}, yeast extract 2.5 g l^{-1}, glucose 1 g l^{-1}, agar 15 g l^{-1}) supplemented with egg yolk. After incubation at 30ºC up to 48h occurrence of the zone of turbidity surrounding colony in medium due to fats releasing indicated on lecithinase activity. The lipolytic activity could be observed on this medium additionally, by appearance of oblique lightening an iridescent sheen due to fatty acids releasing. To confirm free fatty acids released by lipolytic activity, plates were flooded with saturated $CuSO_4$ solution, and dry at 37ºC for 20 min [28]. Appearance of blue-greenish color at surface around colony indicated on lipolytic activity.

Plant-growth promoting activities

Screening for inorganic phosphorus solubilization. Bacterial ability to solubilize sparingly soluble $Ca_3(PO_4)_2$ was assayed on Pikovskaya [29] medium containing yeast extract 0.5 g l^{-1}, glucose 10 g l^{-1}, $Ca_3(PO_4)_2$ 5 g l^{-1}, $(NH_4)_2SO_4$ 0.5 g l^{-1}, KCl 0.2 g l^{-1}, $MgSO_4 \cdot 7H_2O$ 0.1 g l^{-1}, $MnSO_4 \cdot H_2O$ 0.0001 g l^{-1}, $FeSO_4 \cdot 7H_2O$ 0.0001 g l^{-1} and agar 15 g l^{-1}. After 5 days of incubation, phosphate solubilization was verified by clear halo appearance around colonies. Diameters of bacterial colony and zone of clearance was measured and according to their values the relative efficacy of phosphate solubilization was evaluated [30].

Siderophores production. Bacterial ability to produce a iron-chelating molecules, the siderophores, was assayed on chrom-azurol S (CAS) medium [31] by protocol of Milagres et al [32]. Petri dishes were filled with CAS medium, and a half of this media was replaced with

King B agar. Bacterial inoculum was spread on King B medium as near as to border line with CAS medium and incubated at 28ºC for 5 days. The medium discoloration from blue to orange indicated siderophores production and diameter of discoloration was measured.

***In vitro* HCN production assay.** Bacterial isolates were inoculated on HCN induction medium (Tryptic Soy Broth 30 g l^{-1}, Glycine 4.4 g l^{-1}, agar 15 g l^{-1}) [36] as a line on small plates (5.5 cm of diameter). Inoculated plates with disk of Whatman paper previously dipped in HCN revealing solution (0.5% picric acid and 2% Na_2CO_3) on lid of Petri dish were tightly sealed with parafilm and incubated at 28 ºC for 4 days. Development of orange-brown color of the paper indicated on HCN synthesis ability.

***In vitro* fungal growth inhibition assay.** Bacterial isolates were screened for their ability to inhibit growth of 8 medical plant pathogenic fungi: *Curvularia lunata, Fusarium semitectum, Fusarium equiseti* from *Salvia officinalis* L., *F. equiseti* from *Matricaria chamomilla* L., *Myrothecium verrucaria, Verticillium* sp., *Diaporte eres complex and Sclerotinia sclerotiorum* [37]. Fungi were grown on PDA medium at 25ºC for 3 or 7 days. Six mm-plugs were cut out from periphery of fungal colonies and transffered to Waksman agar inoculated with bacterial inoculum. In control treatman the fungal inoculum was used alone. The growth of fungal colony was determined after 7 days at 25ºC, depending on the growth speed of the fungus. The assay was performed four times for each bacterial isolates.

Plant pathogenicity test

The pathogenicity tests were performed according Moragrega et al. [33] by *ex vivo* methods. Pathogenic effects were observed after bacterial inoculation of immature fruits or bean pods and detached young leaves in the same phenological stage. Before inoculation, leaves, bean pods and fruits were disinfected with sodium hypochlorite (1% active hypochlorite), rinsed in sterile distilled water and inoculated with 10 µl inocula containing $5 \cdot 10^6$ bacterial cells. Sterile distilled water was used as negative controls. After incubation on 25^0C for 3-5 days in controlled environment chamber, the pathogenicity indexes were assessed.

Quantitative estimation of indole-3-acetic acid (IAA)

Investigation of indol-3-acetic acid (IAA) production by bacterial isolates was performed according to the method of Glickman and Dessieux [34]. Briefly, bacterial isolates were inoculated on KB broth supplemented with 2.5 and 5 mM L-tryptophan (as precursor of IAA). IAA was assayed by colorimetric method using Salkowski reagent [35] having the formulation of 2 % 0.5 M $FeCl_3$ in 35 % $HClO_4$. Development of pink color was assayed with spectrophotometer at 530 nm. Concentration of produced IAA was determined from a standard curve of IAA (1 - 50 µg ml^{-1}).

Results and discussions

Isolation and characterization of bacterial isolates

The aim of current investigation was the screening and selection of indigenous fluorescent pseudomonads from maize rhizospheric soil with antagonistic activities against phytopathogenic fungi and prospective plant growth stimulating characteristics with purpose for further field application. Isolation of bacteria from innate of rhizosphere of the target crop is essential for successful identification of potential biocontrol and biofertlizing agents [38]. Among PGPR, soil-borne fluorescent pseudomonads represent ecologically important group of beneficial bacteria in agricultural management practices. Their effectiveness for the promotion of plant growth and the biocontrol of phytopathogens has been proved in numerous studies [39, 40].

A total of 268 bacterial isolates were obtained on King B medium from rhizosphere soil tightly adhered to maize roots. Out of 268 isolates, 59 isolates (22%) characterized as fluorescent, Gram-negative, tested positive for citochrom oxidase, were assumed to belong to fluorescent *Pseudomonas* spp. These isolates were evaluated for intrinsic antibiotic resistance (IAR) using different concentration of ampicilin, tetracycline, gentamicin, and chloramphenicol, as well as for heavy metal tolerance (HMT) to molibdenum, zink, cadmium, and mercury.

According to different IAR and HMT patterns, all fluorescent isolates were classified into six groups. Representative isolates of each groups denoted as PS1, PS2, PS3, PS4, PS5, and PS6 were examined for different biochemical properties. It has been determined that the most of isolates were sensitive to the highest applied concentrations of both antibiotics and heavy metals (table 1). The wider spectrum of antibiotic tolerance was found for PS2 and PS4 isolates, while PS5 isolate was sensitive to all tested concentrations, growing poorly on 50 μg/ml of gentamicin. All six isolates failed to grow at 100 μg/ml of gentamicin. Test for tolerance to different concentration of heavy metal revealed that the order of the metals toxicity for the most isolates was Hg > Cd > Mo > Zn.

Table 1. Intrinsic antibiotic resistance (IAR) and heavy metal tolerance (HMT) pattern of six representative fluorescent *Pseudomonas* isolates

Representative isolates	IAR-pattern										HMT-pattern							
	Antibiotics (μg/ml)										HM (μg/ml)							
	Amp			**Tet**			**Gen**		**Chl**		**Hg**		**Cd**		**Mo**		**Zn**	
	10	50	100	5	50	100	50	100	50	100	5	10	20	25	20	50	100	200
PS1	+	+	-	-	-	-	+	-	+	±	+	+	±	-	±	-	+	-
PS2	+	+	+	+	±	-	-	-	+	+	±	-	±	-	+	±	+	-
PS3	+	±	-	±	-	-	-	-	±	-	+	-	-	-	-	-	+	±
PS4	+	+	±	+	+	±	-	-	+	±	±	-	±	-	±	-	±	-
PS5	-	-	-	-	-	-	±	-	-	-	+	-	+	±	-	-	+	±
PS6	+	±	-	±	-	-	±	-	±	-	+	±	±	-	-	-	±	-

Amp – ampicillin; Tet – tetracycline; Gen – gentamicin; Chl – chloramphenicol; + growth; - growth absent; ± the weak growth.

Enzymatic and plant-growth promoting activities

Proteolytic enzyme productions was detected as formation of a clear zone around cells on skim milk agar medium for four strains – PS1, PS3, PS4, and PS6, while gellatinolytic activity was not observed (table 2). Production of hydrolytic enzymes involved in pectin degradation, a component of plant cell wall, and in cellulose degradation, a component of not just plant cell wall but also of some fungi from Oomycota, was not observed. Three strains had ability for urea degradation by urease production and releasing of ammonium ions into medium.

Siderophore production was detected only for three strains of fluorescent pseudomonads, PS2, PS4, and PS6. Last two strains induced an appearance of wide range orange zone (≥ 20 mm) on CAS medium.

Fluorescent pseudomonads produce different siderophores type, which are efficient at dissolving minerals, and release Fe improving its bioavailability as well [41]. In present work we detected three siderophore-producing isolates where two of them produced higher level of

siderophores and/or more efficient ones. Previous investigation indicated that the rhizosphere soil-associated bacteria could exert particular impact on plant iron nutrition. Crowley et al. [42] demonstrated significantly increased Fe-uptake rate of maize by rhizosphere microorganisms in comparison with axenic maize cultures, while Sharma and Johri [43] detected increasing of shoot and root length as well as dry weights of maize seedlings inoculated with siderophore-producing strains. Also, siderophore are known to induce systemic resistance in plants. *Pseudomonas fluorescens* WCS374 induced resistance of radish against Fusarium wilt [44], while a pyoverdine-negative mutant of *Pseudomonas fluorescens* strain CHA0 lost partly its ability to induce systemic resistance against tobacco necrosis virus [45]. Additionally, siderophore mediated competition for iron was shown to be major mechanism of antagonistic activity of fluorescent pseudomonads in the control of some parasitic fungi, limiting iron availability to them [46]. Some microorganisms have also been shown to take iron directly from naturally occurring iron-binding acids. Such "functional siderophores" include citrate, additionally found to be constitutive element of siderophore of some bacteria [47].

As chelating agent, siderophores participate in heavy metals and different ions acquisition. Previous studies showed involvement of a siderophore in acquisition of cupper, cobalt, chrome, molybdenum, lead and zinc [48]. On the other hand, it is well known that diverse inorganic pesticides contain mercury, lead and cadminum, which do not degrade readily and persist a long time in soil. In our investigation the representative isolates originated from maize rhizosphere exerted different abilities to grow on diverse heavy-metal concentrations, including mercury and cadmium. These results strongly suggest on possibility for utilization of efficient siderophore-producing microorganisms to protect and stimulate plant growth in soil polluted with pesticides and agrochemicals.

Three of six investigated isolates (PS2, PS4 and PS6) exerted ability for phosphate solubilization on Pikovskaya medium with different efficacy, using glucose as a carbon source (table 2). Strain PS6 has the highest degree of efficacy for solubilization, while PS4 exerted a weak activity. The phosphate-solubilizing activity characterizes the microorganisms with ability to produce and release metabolites such as organic acids that chelate the cations bound to phosphate (mainly calcium), converting them into soluble forms [49]. Solubilization of different form of phosphates and improvement of its availability presents very important treat of plant-associated bacteria since possible increasing of mass and productivity of agriculture plants.

Five bacterial isolates have been characterized by ability to produce indole-3-acetic acid (IAA) growing in medium without addition of tryptophane. Isolates PS2, PS4 and PS6, showed good IAA production, since isolates PS1 and PS5 were poor producer of IAA.

Selection of isolates with potential inhibition of fungal growth was performed in dual culture and eight phytopathogenic fungi known for detrimental effects on medicinal plants [36] were used. None of the bacteria tested improved growth of phytopathogenic fungi. Isolate PS2 showed inhibition of 7 out of 8 investigated phythopathogenic fungi, PS4 showed inhibition of growth *Diaporte eres complex* and isolate PS6 growth inhibition of *Fusarium equiseti* isolated from *Matricaria chamomilla* L. The most significant changes in mycelial growth rates was achieved by PS2 isolate against *Curvularia lunata* and *Fusarium equisetii* isolated from sage (*Salvia officinalis*). Effect on *Sclerotinia sclerotiorum* was diminished inducing fungal hifa deformation and growth suppresion at margin with bacterial culture.

Table 2. Lytic enzymes production and PGP traits of six representative fluorescent *Pseudomonas* spp. isolates from rhizosphere of maize (*Zea mays* NSSC640)

Bacterial isolate	Lytic enzyme production[a]					PGP treats				Plant pathogenicity		
	Protease	Gellatinase	Cellulase	Pectinase	Urease	IAA production[b]	Siderophore[c]	P-solubilization[d]	Antifungal activity[e]	lilac leaf	apple	bean pods
PS1	+	-	-	-	-	±	-	-	0	3	4	4
PS2	-	-	-	-	+	++	++	+++	7	1	0	0
PS3	+	-	-	-	+	-	-	-	0	3	3	3
PS4	±	-	-	-	+	+	+++	+	1	0	0	0
PS5	-	-	-	-	-	±	-	-	0	4	3	3
PS6	±	-	-	-	-	+	+++	+++	2	0	1	0

[a] Protease, gellatinase, cellulase, pectinase, urease activities were determined by plate assay. (+) hydrolysis; (-) no hydrolysis.
[b] IAA production in medium without added tryptophane
[c] Siderophore activity on CAS medium: + represents 1-5 mm wide of orange zone; ++ represents 5-20 mm wide of orange zone; +++ represents ≥ 20 mm wide of orange zone.
[d] Efficacy of phosphate solubilization evaluated according to halo diameter and colony diameter: + represents 1-4 mm/day; ++ represents 4-7 mm/day; +++ represents ≥ 7 mm/day
[e] Number of fungal pathogen (of total 8 tested) affected by bacterial isolates in *in vitro* bioassay.

Plant pathogenicity test

Plant pathogenicity of maize rhizosphere isolates was tested by *ex vivo* methods based on inoculation on immature fruits (apple), bean pods and detached young leaves (lilac). Following Moragrega et al. [33] severity index scheme, five index levels were used (0-4). According to evaluation of the detrimental effects appearing as necrosis expanding through the midvein and additional veins in leaves or necrotic area of 5-10 mm on fruits or bean pods, it has been observed that isolate PS3 induced symptoms of index level 3. The necrosis degree of more than 50% leaf surface and necrotic area higher than 10mm diameter on fruits provoked by PS1 and PS5 isolates has been scored as index 4, and it seemed that PS1 strain had pronounced detrimental effects.

Selection of potential plant-growth promoting rhizobacterial (PGPR) strains from maize rhizosphere

According to potential for phosphate solubilization, antifungal activities, ability for siderophore and indoleacetic acid production, supporting by non-plant pathogenicity effects, three isolates of fluorescent *Pseudomonas* spp. (PS2, PS4 and PS6) from maize rhizosphere were selected for further investigation. Additional biochemical characterization was performed and secondary metabolites production was evaluated (table 3).

Isolates were tested for *in vitro* production of chitinase on different medium composition. Hydrolysis of chitin, both fungal and insect integument cell wall polymer, was observed for PS4 and particularlly for PS2 isolate, but only on medium supplemented with Waksman agar where glucose is dominant ingredients. On the other hand, all strains failed to exert chitinolytic activity on other media supplemented with Nutrition broth and Yeast-mannitol broth, containing mainly proteins and polyol mannitol, respectively. For lipases production, isolates were assayed on Tween 80 as substrate as well as on egg-yolk. Isolates PS2 and PS4 were exerted lipolytic activity on both or solely on EYA, respectively, while phospholipases (lecithinases) production was absent. Furthermore, it has been shown that PS6 isolate possesses both lipolytic and phospholypolitic activities.

Ability for hydrogen cyanide synthesis was observed only for isolate PS6 which was unable to utilize citrate, while other two citrate-utilizing isolates of fluorescent pseudomonads did not produce HCN on medium with glycine. Hydrogen cyanide synthesis observed for isolate PS6 showed no significant effects on fungal growth inhibition, which is in disagreement to earlier reports [15]. The increased production of HCN by the efficient strain of *P. fluorescens* contributed to effective inhibition of mycelial growth of *Rhizoctonia solani in vitro* [39] and appears to be a major factor in control of soil-borne disease by *Pseudomonas fluorescens* CHA0 [15].

Table 3. Characterization of selected fluorescent *Pseudomonas* spp. from rhizosphere of maize with potential PGP activities and Indole-3-acetic acid (IAA) production at different incubation period and tryptophan concentration

Bacterial isolate	HCN production	Citrate	Lytic enzyme production					Indole-3-acetic acid production (mM)					
			Tween 80[a]	Lipase[b]	Lecithinase[c]	Chitinase activity[d] on		0 mM L-tryptophan		2,5 mM L-tryptophan		5 mM L-tryptophan	
						YMA, NB	WA	24h	48h	24h	48h	24h	48h
PS2	-	+	+	+	-	-	+	6.53 ± 0.38	14.18 ± 0.28	9.58 ± 0.3	32.51 ± 0.36	9.08 ± 0.28	36.90 ± 0.33
PS4	-	+	-	+	-	-	+	2.67 ± 0.41	4.54 ± 0.24	4.85 ± 0.17	8.44 ± 0.42	11.55 ± 0.29	12.80 ± 0.24
PS6	+	-	+	-	+	-	-	3.41 ± 0.19	5.21 ± 0.28	7.21 ± 0.21	9.03 ± 0.23	14.75 ± 0.29	15.44 ± 0.92

[a] Lipase activity determination on Tween 80 as substrate.
[b] Determination of lipolytic activity on Egg-yolk agar (EYA) medium.
[c] Determination of phospholipolytic (lecithinase) activity on Egg-yolk agar (EYA) medium.
[d] Chitinase activities on medium with Nutrienth broth (NB), Waksman agar (WA) and Yeast-mannitol agar (YMA).

In vitro IAA production assay

Among selected PS2, PS4 and PS6 isolates IAA production was detected without presence of L-tryptophan into medium. Higher amounts of IAA were yielded for all isolates in media with tryptophan amendment after 24h and 48h of incubation then without tryptophan. Also, higher amount of IAA was produced after 48h then 24h of incubation (table 3). Significantly higher amount of indoleacetic acid was constantly detected for isolate PS2 then other two isolates, except in presence of 5 mg/ml tryptophan after 24h of incubation. Interestingly, PS2 produced higher amount of IAA after 48h without of tryptophan, then PS4 after 24h and 48h in presence of 2.5 mM and 5 mM of tryptophan, and PS6 after 24 h and 48 h in presence of 2.5 mM of tryptophan. After 48h of incubation, in media with both tryptophan concentrations, this isolates yielded almost three times higher amount of IAA then PS4 and PS6 isolates.

Beneficial effect of rhizosphere microorganisms could be realized via phytohormons production also, particularly by auxins. Although indole-3-acetic acid is a plant auxin hormone and until recently have been considered with no apparent function in bacterial cells [40], many studies shown that production of IAA is wide-spread among plant-associated bacteria. It is well known to have important role in root morphogenesis by increasing root proliferation as well as in plant development. All three bacterial isolates PS2, PS4 and PS6 presented as IAA-producing bacteria produced higher amount of IAA with increasing of tryptophan concentration. Isolate PS2 formed markedly high amount of IAA (more then 30 µg/ml) at both tryptophan concentrations after 48h. In the rhizosphere tryptophan can originate from root exudates or from decomposition of root and microbial cells. The amount

of tryptophan in plant root exudates can vary strongly among plant species [50] and is considered to be rather low, but bacteria exhibit high affinity for tryptophan making them as good scavengers. Interestingly, all three selected *Pseudomonas* isolates had ability to produce IAA without tryptophan supplementation. Isolate PS2 produced higher amount of IAA on tryptophan-independent manner then both other isolates at each of tryptophan concentration. It has been reported previously on tryptophan-independent pathway of IAA production for *Azospirillum brasilense* [51]. Level of generated IAA by isolate PS2 was comparable to those presented in other reports. Margues et all. [52] reported levels of 15 mg l^{-1} for *Ralstonia* spp. isolated from *Zea mays* rhizosphere.

Isolate PS2 showed effective inhibition of mycelial growth of 7 out of 8 phytopatogenic fungi, partly due to production of chitinases, siderpohores and lytic enzymes. Production of IAA and phosphate solubilization in adition to fungal growth inhibition makes it suitable for further investigation for application in maize cultivation. Several reports evidenced the crucial role of fluorescent *Pseudomonas* spp. in soil suppressive to fusarium wilt [2] and take-all disease caused by the phytopathogen fungus *Gaeumanomyces graminis* var. *tritici* [11]. Strains of *Pseudomonas fluorescens*, UTPF16 and UTPF26, which were shown to reduce damage caused by *Rhizoctonia solani* on bean and to improve the growth of the infected plants was also shown to directly stimulate growth of healthy plants in a disease-free environment [53].

Conclusions

Our investigation confirmed occurrence of different fluorescent *Pseudomonas* spp., indigenous soil bacteria in maize rhizosphere. Isolate PS2, with antagonistic activities against a wide range of phytopathogenic fungi and with functional properties distinctive for plant growth promoting rhizobacteria may represent precious biological alternative for harmful pesticides and chemical fertilizers application in agriculture fields due to crucial role of rhizobacteria to plant health maintenance and soil fertility. Further analysis with maize seedlings and field trials are necessary for true evaluation of the influence of this isolate on maize plants development in natural environments. These results have practical relevance, since potentially effective bacterial strain PS2 was selected and could be tested for use in agriculture for enhancement of plant growth and suppression of diseases in maize crop.

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