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*Original scientific paper*

# ANTIFUNGAL ACTIVITY AND GENETIC DIVERSITY OF SELECTED *Pseudomonas* spp. FROM MAIZE RHIZOSPHERE IN VOJVODINA

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Antibiotic production by plant-associated microorganisms represents an environmentally compatible method of disease control in agriculture. However, a vide application of bacterial strains needs careful selection and genetic characterization. In this investigation, selected *Pseudomonas* strains were characterized by rep-PCR methods using ERIC and $(GTG)_5$ primers, and partial 16S rDNA sequence analysis. None of strains produced

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homoserine lactones ($C_4$, $C_6$, $C_8$) as quorum sensing signal molecules. Very poor production of phenazines and no significant fungal inhibition was observed for PS4 and PS6 strains. High amount of phenazines were produced by *Pseudomonas* sp. strain PS2, which inhibited mycelial growth of 10 phytopatogenic fungi in percent of 25 (*Verticillium* sp.) to 65 (*Fusarium equiseti*). Genetic characterization of the *Pseudomonas* sp. PS2 and evaluation of phenazines production, as the main trait for growth inhibition of phytopathogenic fungi, will allow its application as a biosafe PGPR for field experiments of plant disease control.

*Key words:* antifungal activity, ERIC, $(GTG)_5$, PGPR, phenazines, *Pseudomonas*

## INTRODUCTION

Plant growth-promoting bacteria (PGPB) are commonly present in many environments (COMPANT *et al.,* 2005). PGPB colonizing the root surfaces and the closely adhering soil interface - rhizosphere, are plant growth-promoting rhizobacteria (PGPR) (KLOEPPER *et al.*, 1999). Many PGPR strains produce secondary antimicrobial metabolites and can alter the environmental conditions for the growth of other microorganisms (NEWTON and FRAY, 2004). Among the secondary metabolites a variety of antibiotics have been identified, including phenazine, pyoluteorin, pyrrolnitrin, tensin, amphisin, 2,4-diacetylphloroglucinol (DAPG), oomycin A, tropolone, and cyclic lipopeptides produced by different pseudomonads (RAAIJMAKERS *et al.*, 2002; NIELSEN *et al*., 2002; NIELSEN and SØRENSEN, 2003), macrolide antibiotic oligomycin A, produced by *Streptomyces* (KIM *et al*., 1999) and kanosamine and zwittermicin A, produced by *Bacillus* (COMPANT *et al.,* 2005). A general hypothesis regarding these antibiotic metabolites is that they have a competitive function by inhibiting the growth of other microorganisms. Ability of PGPR to suppress fungal growth by production of some of these antibiotics was used in biological control of phytopathogens (KLOEPPER *et al*., 1999; NIELSEN *et al*., 2002; NIELSEN and SØRENSEN, 2003; WELLER, 2007). The mechanisms of biocontrol include competition for an ecological niche or a substrate, production of inhibitory chemicals, and induction of systemic resistance (ISR) in host plants to a broad spectrum of pathogens (HAAS *et al*., 2000; BLOEMBERG and LUGTENBERG, 2001; GERHARDSON, 2002; HAAS *et al*., 2002).

In cultivation of medicinal and aromatic plants (MAPs), antibiotic production by plant-associated microorganisms represents an environmentally acceptable method of disease control. Use of synthetic fungicides for disease suppression is limited due to a low efficiency, phytotoxicity, development of resistance, long degradation periods, environmental pollution and side effects on human and animal health. Phytopathogenic fungi are responsible for several plant diseases in different medicinal plants and cause very important economic losses in Serbian plantation (PAVLOVIC, 2008). Indigenous population of PGPR may prevent or suppress plant disease caused by phytopathogenic fungi. In earlier work, we isolated dominant pseudomonads and selected three plant growth-promoting *Pseudomonas* isolates from the maize rhizosphere in Vojvodina, Serbia. In attempt

to characterize them before application, we choose rep-PCR method for this investigation. Interspersed repetitive DNA sequence elements (rep) present in the prokaryote genomes are very useful as primers sites for genomic DNA amplification and yield complex genomic fingerprints suitable for estimation of genetic diversity. Enterobacterial Repetitive Intergenic Consensus (ERIC) sequence and BOX element are located in distinct, intergenic positions all around the chromosome (DE BRUIJN, 1992). The genetic differences between PGP *Pseudomonas* were already estimated using this method (BOTELHO, 2001; RANGARAJAN *et al.*, 2001; LANDA *et al.*, 2002; JOSIC *et al.*, 2012a).

The aim of this study was to investigate the genetic diversity of the selected soil bacterial strains using rep-PCR methods, estimate production of phenazines and to test their antimicrobial activity against fifteen pathogenic fungi originated from medicinal plants.

## MATERIALS AND METHODS

**DNA manipulations.** Bacterial isolates were grown in liquid King B medium, at 28ºC for 24h. Cell suspension was adjusted to $A_{600nm} \approx 0.625$ by sterile distilled water. Total DNA was extracted after cells suspensions were boiled for 10 min, immediately cooled on ice, centrifuged and the supernatants were kept at -20$^{o}$C or used for the PCR assay.

**rep-PCR**. Amplifications were performed in Eppendorf MasterCycler personal (Germany) with the temperature profile as reported by DE BRUIJN (1992). For the $(GTG)_5$ primer the cycles used were: an initial denaturation at 95$^{o}$C for 7 min followed by 35 cycles of a three-step PCR program (94$^{o}$C for 1 min, 52$^{o}$C for 1 min and 65$^{o}$C for 8 min) with a final extension at 65$^{o}$C for 16 min. ERIC1R/2 primer set were used for additional fingerprinting under following amplification conditions: initial denaturation at 95°C for 3 min, 35 cycles of 94°C for 1 min, 53°C for 1 min, and 65°C for 8 min with the final extension at 65°C for 16 min. PCR products were visualised by electrophoresis on 1.5% (w/v) agarose gels in 0,5 Tris-borate-EDTA buffer for 8h at 3 V/cm, stained with ethidium bromide and photographed under UV transillumination. BOX and ERIC PCR fingerprint results were converted to binary form and cluster analysis of strains were done with the program STATISTICA 7.

**PCR amplification of bacterial 16S rRNA gene**. The 16S rRNA gene was amplified by using universal bacterial primers fDl and rD1 (WEISBURG *et al.*, 1991). PCR amplification was carried out in a 50 µl reaction volume using Green Taq Dream master mix (Fermentas, Lithuania) with 1µl of template DNA and 0.1 µmol of each primers fD1 and rD1. DNA amplification was performed with the following temperature profile: an initial denaturation at 95°C for 4 min, followed by 35 cycles of denaturation (1 min at 94°C), annealing (1 min at 58°C), and extension (2 min at 72°C); with a final extension at 72°C for 5 min. Amplified DNA was examined by electrophoresis for 2h at 5V/cm in 1,2% (w/v) agarose gel with GeneRuler DNA Ladder mix SM0331 (Fermentas, Lithuania).

To determine the species identity, sequences of bacterial isolates were compared with the published sequences on the NCBI GenBank database using the algorithm

BLAST. The 16S rDNA sequences of bacterial strains are deposited under accession numbers JQ818423, JQ818424 and JQ818424.

**Production of phenazines and quorum sensing signal molecules.** Overnight culture of bacterial isolates grown in King B liquid medium were subjected for phenazine and 2-OH-phenazine extraction following the method of CHANCEY *et al.* (1999). Quantification of phenazine and 2-OH-phenazine were performed by the UV light spectroscopy measuring absorbance at 367 nm and 468 nm, respectively (MADDULA *et al.* 2008).

Production of N-acyl-homoserinelactone (AHLs) as quorum sensing (QS) signal molecules by maize-rhizoshere isolates was tested by using a AHLs-sensor strain *Chromobacterium violaceum*, as described by PORITSANOS *et al*. (2006). Briefly, bacterial isolate and *Chromobacterium violaceum* were inoculated on LB medium as an opposite lines at distance of 3 mm, and incubated for four days at 25ºC. A purple pigment production by *Chromobacterium violaceum* indicated on no long chain ACHLs production.

***In vitro* antifungal activity.** The bacterial isolates were screened for their ability to inhibit growth of 15 medical plant pathogenic fungi: *Alternaria alternata, Dreschlera tetramera, Curvularia lunata, Diaporte eres complex, Fusarium semitectum, Fusarium sporotrichioides, Fusarium equiseti* from *Salvia officinalis* L., *Fusarium equiseti* from *Matricaria chamomilla* L., *Myrothecium verrucaria, Verticillium* sp., *Fusarium oxysporum, Fusarium verticillioides, Fusarium proliferatum, Fusarium solani, and Sclerotinia sclerotiorum* (PAVLOVIC, 2008). The growth of a fungal colony (6 mm plugs placed in the midle of plate with Waksman agar) was determined after 7 days at 25ºC, depending on the growth speed of the fungus. Control treatments were the fungal inoculums used alone, since the bacterial inoculation were placed 30 mm from fungal inoculum. The assay was performed four times for each bacterial isolates. The mycelium lenght was measured towards and away from bacterial inoculum. The percentage of fungal growth inhibition was calculated as described by ZARRIN *et al.* (2009): % Inhibition = [(R-r)/R] x 100, where r is the lenght of the fungal colony opposite the bacterial colony and R is the maximum radius of the fungal colony away from the bacterial colony.

## RESULTS

**rep-PCR based genotyping and 16S rRNA gene amplification.** Genomic fingerprints were obtained by amplification with BOX or enterobacterial repetitive intergenic consensus sequence (ERIC) primers. BOX fingerprints by $(GTG)_5$ primer were used as a first step of genodiversity observation (Figure 1a). ERIC-PCR confirmed differences in repetitive elements dispersion (Figure 1b). rep-PCR amplifications of the strains yielded complex genomic fingerprints consisting amplicons ranging from 100 to 3000 bp in size. Cluster analysis of genomic fingerprint patterns separate PS6 strain from PS2 and PS4 and showed 67.8 % of diversity. Strains PS2 and PS4 showed 59% patterns dissimilarity (Figure 2).

16S rDNA gene sequences of 1500 bp were obtained from each strain by PCR amplification with the conserved eubacterial primers fD1 and rD1 defined by

WEISBURG *et al.* (1991). Partial sequencing of the PCR products from the selected strains confirmed high level of genetic diversity.

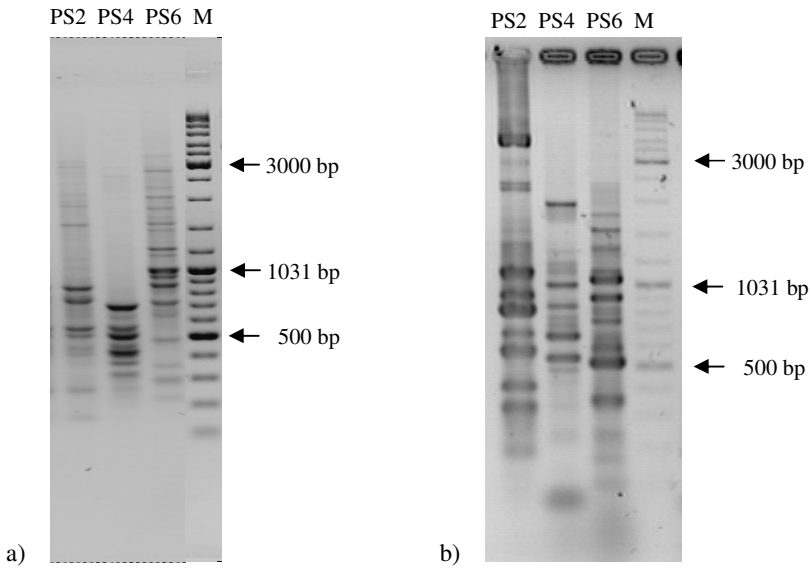


Figure 1. Genomic DNA fingerprint patterns generated by a) $(GTG)_5$ and b) ERIC PCR amplifications

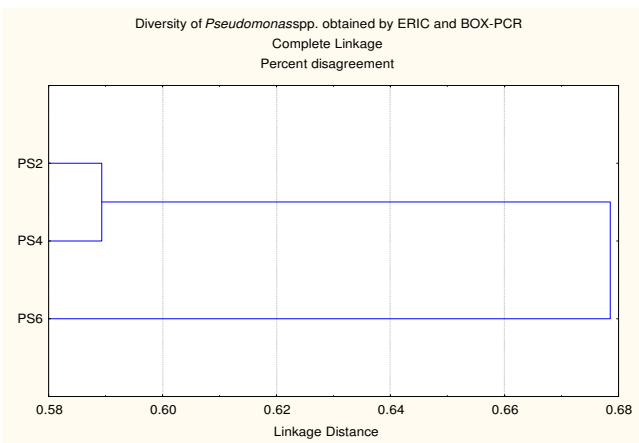


Figure 2. Cluster analysis of genomic fingerprint patterns generated by $(GTG)_5$ and ERIC PCR amplifications of whole-cell genomic DNA

**Production of phenazines and quorum sensing signal molecules.** The production of phenazines was evaluated by measuring absorbance of phenazine-carboxylic acid (PCA) at 367 nm and 2-hydroxi-phenazine-carboxylic acid (2-OH-PCA) at 468 nm. Although the three strains were producing both antibiotics, 2-OH-PCA was produced predominantly from each of them. The Strain PS2 synthesized significantly more amount phenazines (both PCA and 2-OH-PCA) (Table 1). *In vitro* production of the signal molecules was evaluated in a plate with *Chromobacterium violaceum* CV026 as an indicator strain. The results showed that none of the strains was able to produce exogenous $C_4$, $C_6$ or $C_8$ homoserine lactones as quorum sensing signal molecules.

*Table 1. Production of acyl-homoserinelactones and phenazines by Pseudomonas spp. strains from maize rhizosphere*

| | | Absorbance of | |
|---|---|---|---|
| Strain | AHLs | PCA[a] | 2-OH-PCA[b] |
| PS2 | - | 0.160 ± 0.016 B[c] | 0.194 ± 0.010 B |
| PS4 | - | 0.019 ± 0.002 A | 0.042 ± 0.006 A |
| PS6 | - | 0.007 ± 0.002 A | 0.029 ± 0.004 A |

[a] Determination of phenazine-carboxylic acid (PCA) production at absorbance at 367 nm.

[b] Determination of production of 2-hydroxyl-phenazine-carboxylic acid (2-OH-PCA) at absorbance at 468 nm

[c]values in the columns marked wiht the same letter are not statistically significant according to the Duncan's test (P=0,05)

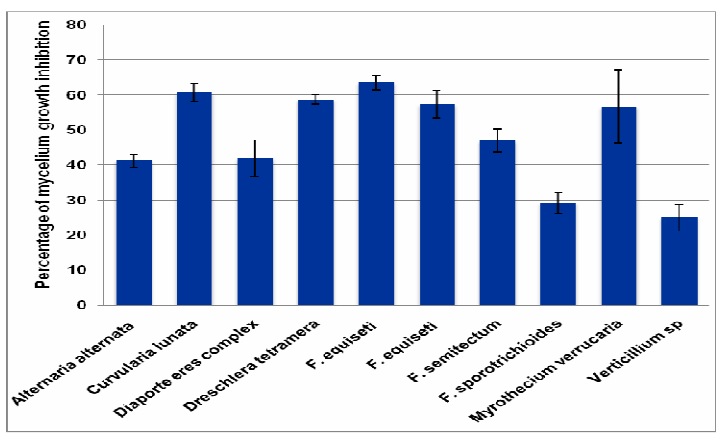


Figure 3. *In vitro* bioaasay of fluorescent *Pseudomonas* strain PS2 against phytopathogenic fungi isolated from medical plants

***In vitro* antifungal activity.** For a further characterization of a selected strain PS2 with potential for plant growth promotion, dual culture were performed using fifteen phytopathogenic fungi known for their detrimental effects on medical plants. None of the bacteria tested improved growth of the phytopathogenic fungi. Pseudomonas PS2 strain was induced hifal deformation and growth suppresion at margin with bacteria on *Fusarium oxysporum, F. verticillioides, F. proliferatum, F. solani, and Sclerotinia sclerotiorum.* The valuable percentage of mycelia growth inhibition of ten phytopathogenic fungi were observed only for PS2 strain (Figure 3). *Pseudomonas* PS4 strain caused hyphal deformation of *Diaporte eres complex* near to bacterial colonies. Growth of *Dreschlera tetramera* and *F. equiseti* were inhibited by PS6 strain less then 10%.

## DISCUSSION

Three distinct genotypes of investigated *Pseudomonas* strains were differentiated by amplification of ERIC and BOX elements. Strains PS2 and PS4 clustered separately from PS6 strain, but all strains showed very high dissimilarity level (59-67.8 %). Differences in phenotypic and plant growth promoting properties of investigated strains in previous investigation (DJURIC *et al.*, 2011) are in agreement with high genetic diversity obtained in this work. Pseudomonad strains from soybean rhizosphere were analysed by BOX and ERIC - PCR (BOTELHO, 2001) and similar profiles were observed to the *Pseudomonas fluorescens* strain 2-79 which is phenazine-producer. To assess the genotypic diversity of *Pseudmonas* collection from wheat rhizosphere, MCSPADDEN GARDENER *et al.* (2000) compared their ERIC and BOX-PCR patterns to 4 previously described 2,4-DAPG producers. LANDA *et al*. (2002) reported the relationship between pea rhizosphere competence and genotype. Genetic profiles generated by BOX-PCR correlate very well with RFLP profiles of **phlD** genes and this relationship will allow a more directed selection of 2,4-DAPG-producing strains for use in biological control.

Fluorescent *Pseudomonas* has been suggested as potential biological control agent (HAS and DEFAGO, 2005) due to its ability to colonize rhizosphere and protect plants against a wide range of important agronomic fungal diseases such as damping-off of sugar beet (KUMAR *et al.*, 2002) and damping-off of cotton with *Rhizoctonia solani* as a causal agent (PAL *et al*., 2000). Fluorescent pseudomonad antagonizes all the reproductive phases of the *Phytophthora capsici*, the causal organism of foot rot disease (PAUL and SARMA, 2006) and exhibits strong antifungal activity against *Rhizoctonia solani* (REDDY *et al*., 2008), *R. bataticola* and *Fusarium oxysporum* found in rice and sugarcane rhizosphere (KUMAR *et al.*, 2002). *Pseudomonas* shows biocontrol potential against phytopathogenic fungi *in vivo* and *in vitro* conditions from chickpea rhizosphere (SARAF *et al.*, 2008). Fluorescent *Pseudomonas* suppresses plants fungal diseases mainly through the production of antifungal metabolites. We detected lytic enzymes and HCN production in addition to siderophores and IAA production in our previous investigation of different fluorescent *Pseudomonas* from maize rhizosphere (DJURIC *et al.*, 2011). In this study

we investigated selected strains (PS2, PS4 and PS6) for short chain ACHLs and phenazines production. None of the strains produced a short chain ACHLs, two strains were characterized as very poor producers of 2-hydroxil-phenazinecarboxylic acid and 1-phenazinecarboxylic acid, while strain PS2 produced high amount of both antibiotics. This strain produced higher amount of 2-OH-PCA than PCA. A greater antibiotic activity of 2-OH-PCA has been demonstrated against array of bacterial and fungal organisms in comparison to PCA (MADDULA *et al.*, 2008). PICARD *et al.* (2000) demonstrated that, in the field condition, maize is more sensitive to rhizosphere pathogens during the first stage of growth, which corresponds to the end of germination. They detected low density occurrence of an antibiotic DAPG (2,4-diacetylphloroglucinol) -producing population of *Pseudomonas* isolated from the maize rhizosphere during the first stage of maize growth, and highlighted the importance for releasing an effective bio-control agent during the first stage of maize development.

*Pseudomonas* PS2 showed effective mycelial growth inhibition of 10 out of 15 phytopatogenic fungi. The most significant changes in the mycelial growth rates was achieved against *Fusarium equisetii* isolated from sage (*Salvia officinalis*) and *Curvularia lunata*, inducing the growth suppression of 64% and 61%, respectively. The least inhibitory effect was detected on the mycelium extension of *Fusarium sporotrichoides* (25%). Appreciable efficacy of *P. fluorescens* against *Alternaria tenuissima* (~83%) and *F. solani* (~79%) reported SEETHA RAMULU *et al.* (2010). Biocontrol of *Alternaria tenuissima* originated from *Ocimum basilicum* L. using indigenous *Pseudomonas* spp. were reported recently (JOŠIĆ *et al*., 2012b), showing that PS2 reduced 90% of disease incidence in greenhouse experiments with non-sterile soil. About 80% of the growth inhibition of *F. moniliformae* and *Colletotrichum acutatum* cause six days old culture filtrate of *P. fluorescens* MA-4 strain (MISHRA *et al*., 2011). As previously shown, the phenazines are redox-active antibiotics (DIETRIX *et al.* 2008) and may contribute to iron mobilization in soils in addition to siderophores (HERNANDEZ *et al*., 2004; WANG and NEWMAN, 2008).

## CONCLUSION

Selected *Pseudomonas* strains isolated from maize rhizosphere showed high genetic diversity on the basis of ERIC and $(GTG)_5$ PCRs, which is confirmed by 16S rDNA sequences analysis. Ability to produce IAA and siderophores, as detected earlier, and substantial production of phenazines are main characteristic of *Pseudomonas* PS2 strain. Growth inhibition grater then 50% was observed for *Curvularia lunata*, *Myrothecium verrucaria, Dreschlera tetramera* and *Fusarium equiseti* isolated from two medicinal plants -*Salvia officinalis* L. and *Matricaria chamomilla* L. Further field trials will test the competitiveness of PS2 strain with indigenous population, effects in biological control of phytopathogenic fungi and possible advantage based on detected PGP traits.

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# ANTIFUNGALNA AKTIVNOST I GENETIČKI DIVERZITET SELEKTOVANIH *Pseudomonas* spp. IZ RIZOSFERE KUKURUZA U VOJVODINI

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Ekološki prihvatljiv metod kontrole bolesti bilja u poljoprivredi obuhvata primenu rizosfernih mikroorganizama koji produkuju antibiotike. Široka primena bakterija zahteva njihovu pažljivu selekciju i genetičku karakterizaciju. Ova istraživanja obuhvatila su karakterizaciju selektovanih *Pseudomonas* sojeva na osnovu rep-PCR metode korišćenjem ERIC i $(GTG)_5$ prajmera, kao i parcijalne sekvence 16S rDNA. Nije detektovana produkcija acil-homoserin laktona ($C_4$, $C_6$, $C_8$) kao QS molekula. Sojevi PS4 i PS6 su vrlo oskudno produkovali fenazine i nisu značajno inhibirali rast fitopatogenih gljiva. Soj *Pseudomonas* sp. PS2 je obilno produkovao 2-OH-PCA i PCA i inhibirao rast micelija 10 fitopatogenih gljiva od 25% (*Verticillium* sp.) do 65% (*Fusarium equiseti*). Genetička karakterizacija soja *Pseudomonas* sp. PS2 i detekcija produkcije fenazina kao glavnog inhibitora rasta fitopatogenih gljiva omogućiće primenu ovog soja kao bezbednog PGP agensa za biološku kontrolu u budućim ogledima u polju.

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