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PLANT GROWTH PROMOTING POTENTIAL OF *PSEUDOMONAS* SP. SP0113 ISOLATED FROM POTABLE WATER FROM A CLOSED WATER WELL

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Abstract: The *Pseudomonas* sp. SP0113 strain from a partially closed aquatic environment was identified as a plant growth promoting bacterium (PGPB). Laboratory tests revealed that PS0113 has multiple plant growth promoting traits, including mineral phosphate solubilizing ability, ammonifying ability that increases nitrogen availability for plants via the root system, and phosphatase activity that plays an important role in organic phosphorus mineralization. Tricalcium phosphate ($Ca_3(PO_4)_2$) solubilizing ability was described as average (2-3 mm) after 7 days of incubation and as high (>3 mm) after 14 days of incubation. The analyzed bacterium was an antagonist of major crop pathogenic fungi. A high degree of pathogen growth inhibition was reported with regard to *Rhizoctonia solani* (38%), whereas the tested strain's ability to inhibit the growth of fungi of the genera *Fusarium* and *Microdochium nivalis* was somewhat lower at 20-29%. The bacterium proliferated in Roundup 360 SL solutions with concentrations of 0.1, 1 and 10 $mg \cdot ml^{-1}$.

Key words: PGPB; plant growth promoting bacterium; *Pseudomonas* sp.; biocontrol; glyphosate-tolerant bacterium

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INTRODUCTION

Soil is the habitat of bacteria and other organisms that establish close relationships and participate in the process of producing food for humans and animals. The functioning of agricultural ecosystems is influenced by numerous factors, including weather conditions, the physical properties and microbiological composition of soil, the use of agricultural chemicals, crop rotation schemes, presence of crop pathogens, pests and weeds, resistance to adverse biotic and abiotic factors (Allen et al., 2009; Doran and Zeiss, 2000; Fierer et al., 2007; Garbeva et al., 2008; Hinsinger et al., 2009).

Crop protection in the early stages of plant growth is one of the most important factors that

determine field emergence, healthy plant development and, consequently, the quality of the resulting agricultural produce. Biological control is an environmentally friendly method of plant protection, and the use of microbes improves yield and plant health (Figueiredo et al., 2010).

Bacteria that enhance plant growth and yield are referred to as plant growth promoting bacteria (PGPB) or plant growth promoting rhizobacteria (PGPR). They can produce phytohormones, increase seed vigor, improve field emergence, increase the availability of soil nutrients, enhance plant resistance to abiotic stress and induce defense mechanisms against biotic stress. Beneficial microorganisms can establish symbiotic relationships with the root system by colonizing the rhizosphere or the rhizoplane, but some of them can live in the soil environment without forming symbiotic associations with plants. PGPB exert positive effects on plant health and crop yield, and they can be used in biological crop protection (Ahemad and Kibret, 2014; Autoun and Kleopper, 2001; Beneduzi et al., 2012; Chen et al., 2006; Cummings, 2009; Czaban et al., 2007; Figueiredo et al., 2010; Yang et al., 2009; Mendes et al., 2012).

Among the various bacterial genera possessing traits characteristic of PGPR and which include *Bacillus*, *Pseudomonas*, *Azotobacter*, *Azospirillum*, *Acinetobacter*, *Enterobacter*, *Rhizobium* and *Bradyrhizobium* (Rodríguez and Fraga, 1999; Vessey, 2003), the γ-Proteobacteria deserve special attention. Members of this microbial class are highly effective in preventing phytopathogens from colonizing root systems. Much of the research into γ-Proteobacteria has been dedicated to bacteria of the genus *Pseudomonas*, which possess a high potential for fixing nitrogen, solubilizing phosphate, inhibiting the development of phytopathogens, producing phytohormones and colonizing root systems (Botelho and Mendonça-Hagler, 2006; Das et al., 2003; Thakker et al., 2013; Yu et al., 2011).

Soil enzymatic activity reflects the overall activity of soil-dwelling microorganisms. Enzyme activities can be used as indicators of soil fertility and agrobiocenotic balance, reflecting the yield potential of crops. The major enzymes produced by bacteria are dehydrogenases, phosphatases, urease, catalase, proteases and cellulases. An important role is played by nitrifying, ammonifying and nitrogen-fixing bacteria, which are responsible for making nitrogen available to plants and reducing external nitrogen supply. Cultivation practices and the use of fertilizers and crop protection chemicals disturb the soil equilibrium. Impoverishment of soil microbial communities decreases enzyme activity, impairs nutrient absorption by roots, decreases resistance to pathogens and lowers soil quality (Barabasz et al., 2002; Cummings, 2009; Fernandez et. al., 2009; Das et al., 2011; Duke et al., 2012).

The aim of this study was to determine whether bacteria isolated from non-agricultural environments can promote plant growth and development, and contribute to improving soil conditions. The *Pseudomonas* sp. SP0113 strain was analyzed to evaluate its adaptability to adverse environmental conditions, including low

Table 1. Essential morphological characteristics.

Basic morphological parameters	Result
Colony color	Creamy white
Colony shape	Circular, convex
Gram staining	Negative
Catalase	Positive
Oxidase	Positive
Colony growth rate at 28°C	High
Colony growth rate at 22°C	Average
Colony growth rate at 10°C	Average
Colony growth rate at 4°C	Low

temperature and resistance to phosphonate fungicides. Further research is needed to determine which properties support the survival of PGPB.

MATERIALS AND METHODS

Materials

The tested strain was isolated from samples of potable water collected from a closed water well. The well is situated in northeastern Poland, and it is partially closed off from external influences. The strain was isolated by the serial dilution method, and it was incubated on Pseudomonas-Selective Agar (PSA, MERCK, Germany) at 28°C for 24 h. The isolated strain was fixed in a 2:3 mixture of glycerol and Tryptic Soy Broth (TSB, MERCK, Germany) and stored at -86°C for further analysis.

Phenotypic characteristics of the strain

Bacterial growth potential was estimated in cultures incubated at 28, 22, 10 and 4°C on Tryptic Soy Agar (TSA, MERCK, Germany) for 24 h (Table 1). The resulting colony was visually compared at the tested temperature against the optimal temperature of 28°C. The experiment was performed in two replications.

The phenotypic traits of the evaluated strain were determined with the use of API® 20NE and API® ZYM tests (Biomerieux, France) that were performed in accordance with the manufacturer's instructions. Both tests were incubated at 28°C. API® 20NE was incubated for 24 h, and API® ZYM for 4 h 30 min. Bacteria were also identified by Gram staining.

The bacterium's ability to convert organic nitrogen to ammonia nitrogen was determined by adding 1 ml of Nessler's reagent to the 48-h bacterial culture in 1 ml of peptone water. Brown staining relative to control (peptone water) was a positive result. The experiment was conducted in two replications.

The bacterium's TCP solubilizing ability was determined by the disc diffusion test on the NBRIB (National Botanical Research Institute's phosphate growth) medium (Nautiyal, 1999). 10 µl of the bacterial suspension with the concentration of $5 \cdot 10^8$ cells was applied to a sterile filter-paper disc. Bacteria were cultured at 28°C for 14 days. The zones of clearance around the discs were measured after 3, 5, 7 and 14 days. The experiment was performed in five replications.

DNA extraction from pure bacterial culture

Genetic material was isolated by the method proposed by El-Lathy et al. (2009) with modifications. An overnight bacterial culture in 1.5-ml test tubes containing 1 ml of TSB was centrifuged (6 000 rpm, 10 min), the supernatant was removed, and 300 µl of TE buffer (1 M Tris pH 8, 0.5 M EDTA pH 8) containing proteinase K (0.2 $mg \cdot ml^{-1}$) was added to the pellet. The sample was incubated at 37°C for 1 h and then at 90°C for 10 min. The lysate was left to stand at room temperature for 1 h, and then centrifuged (12000 rpm, 10 min). The supernatant was transferred to a clean test tube, and the quality of genetic material was determined in a photometer (BioPhotometer Plus, Eppendorf, Germany) before further analyses.

PCR amplification

The isolate was identified as belonging to the genus *Pseudomonas* by PCR amplification (Ps-PCR) with the use of Ps-for (5'-GGTCTGAGAGGATGATCAGT-3') and Ps-rev (5'-TTAGCTCCACCTCGCGGC-3') primers (Widmer et al., 1998).

Table 2. Essential biochemical properties.

	Biochemical properties	Substrate	Result*
ENZYME ACTIVITY	Alkaline phosphatase	2-naphthtyl phosphate	+
	Acid phosphatase	2-naphthtyl phosphate	+
	Esterase (C 4)	2-naphhthyl butyrate	+
	Ester lipase (C 8)	2-naphthyl hexanoate	+
	Lipase (C 14)	2-naphthyl myristate	-
	Leucine arylamidase	L-leucyl-2-naphthyl amide	+
	Valine arylamidase	L-valine-2-naphthyl amide	d
	Cystine arylamidase	L-cysteine-2-2-naphthyl amide	d
	Trypsin	N-benzoyl-DL-arginin-2-napthyl amide	-
	α-chymotrypsin	N-glutaryl-phenylalanine-2-naphthyl amide	-
	Naphthol-AS-BI phosphohydrolase	naphthol-AS-BI-phosphate	+
	α-galactosidase	6-Br-2-naphhtyl-α-D-galactopyranose	-
	β- galactosidase	2-naphthyl-β-D-glucopyranose	+/w
	β- galactosidase	4-nitrophenyl-β-D-galactoside	+
	β-glucuronidase	naphthol-AS-BI-β-D-glucuronide	-
	α-glucosidase	2-naphthyl-α-D-glucopyranose	w/-
	β- glucosidase	6-Br-2-naphthyl-β-glucopyranose	+
	β- glucosidase	Esculin	+
	N-acetyl-β-glucosaminidase	1-naphthyl-N-acetyl-β-glycosyl amide	-
	α-mannosidase	6-Br-2-naphthyl-α-mannopyranosidase	w/-
	α-fructosidase	2-naphthyl-α-L-fructopyranose	-
	Arginine dihydrolase	L-arginine	+
	Urease	Urea	-
	protease	Gelatin	-
ASSIMILATION	D-glucose	D-glucose	+
	L-arabinose	L-arabinose	-
	D-mannose	D-mannose	-
	D-mannitol	D-mannitol	+
	N-acetyl glucosamine	N-acetyl glucosamine	+
	D-maltose	D-maltose	+
	potassium gluconate	potassium gluconate	+
	decanoic acid	decanoic acid	w/-
	adipic acid	adipic acid	-
	malic acid	malic acid	+/w
	trisodium citrate	trisodium citrate	+
	phenylacetic acid	phenylacetic acid	-
Reduction of nitrate to nitrite		Sodium nitrate	+
Reduction of nitrite to nitrogen		sodium nitrate	-
Indole production		L-Tryptophan	-
Glucose fermentation		D-Glucose	+

* + – positive result, w – weak positive result, - – negative result

PCR was carried out in a reaction volume of 50 µl containing 1 X PCR buffer (A&A Biotechnology, Poland), 200 nM of each deoxynucleotide (A&A Biotechnology, Poland), 5 mg·ml^{-1} of bovine serum albumin (New England Biolabs, USA), 200 nM of each primer (Genomed, Poland), 4 µl of lysate containing DNA, and 1 U of Run DNA polymerase (A&A Biotechnology, Poland). The reaction began with initial denaturation at 95°C for 3 min, incubation at 80°C during which polymerase was added, followed by 40 cycles: denaturation at 94°C for 1 min, primer annealing at 65°C for 1 min, and elongation at 72°C for 1 min. Final elongation was carried out at 72°C for 10 min.

The reaction was carried out in the Mastercycler® Gradient thermal cycler (Eppendorf, Germany). The expected PCR products (approximately 990 kbp) were separated in 1.2% agarose gel in 1 X Tris-Acetate-EDTA buffer (TAE) at 100 V for 1 h.

The strain's antagonistic potential against phytopathogenic fungi

Mycelial growth inhibition was evaluated in the dual culture plate assay on PDA (A&A Biotechnology, Poland). Bacterial concentrations were determined spectrophotometrically (600 nm) at 5·10^{8} cfu·ml^{-1}, and 10 µl of the suspension were applied to the opposite edges of the plate (3 cm from the center), and a 5 mm disc with the mycelium was placed in the center. Sterile distilled water was the control. The plates were incubated at 28°C for 5 days. The bacterium's antagonistic potential against *Fusarium culmorum*, *F. graminearum*, *F. oxysporum*, *Monographella nivalis* and *Rhizoctonia solani* was determined.

Evaluation of the strain's sensitivity to glyphosate in the Roundup 360 SL herbicide

Liquid mineral salt medium (MSM) containing 2.2 g of Na_2HPO_4, 1.4 g of KH_2PO_4, 0.6 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.05 g of NaCl, 0.02 g of $CaCl_2$, 0.02 g of yeast extract, 1.0 g of glucose in 1 l of water, with the pH of 7.0 ± 0.2, was used. The medium was sterilized and combined with a filter-sterilized 20% glucose solution. The medium was used to prepare dilutions of the Roundup 360 SL herbicide (MSM-glyphosate) to obtain initial glyphosate concentrations of 10, 1 and 0.1 mg·ml^{-1}. One ml of concentrated bacterial suspension (final OD_{600} in control A=0.7) was added to 250 ml Erlenmeyer flasks containing 99 ml of MSM-glyphosate. The flasks were shaken at 180 rpm for 48 h. Optical density was measured immediately after inoculation of the MSM-glyphosate medium and after every 12 h in 100 ml samples collected from each experimental variant. Measurements were performed in a biological spectrophotometer (BioPhotometer Plus, Eppendorf, Germany). Bacteria were exposed to the herbicide in two replications, and optical density was measured twice in each replication. MSM without Roundup 360 SL, diluted with sterile demineralized water, was the control.

Calculations

TCP solubilizing ability was determined based on the size of clearance zones, and was evaluated according to the following scale: lack of ability (no zone), weak (<1 mm), average (1-3 mm) and strong (>3 mm). The results for each of the three analyzed zones were averaged and presented in the range of 0.0 (no effect) to 3.0 (strong effect). The strain's antagonistic potential against pathogenic fungi was calculated according to the formula proposed by Ashwini and Srividya (2013): % inhibition = [1-(mycelial growth in the presence of bacteria mycelial growth in control^{-1})]·100.

Data were processed by ANOVA in the Statistica 10 application. The results were presented in graphical form in SigmaPlot 12 software.

RESULTS

Morphological characteristics

The strain was identified by Ps-PCR as *Pseudomonas* sp. It was labeled as *Pseudomonas* sp. SP0113 (Fig. 1a.). According to the results of the API® 20NE test, there was a high probability that the isolate belonged to the species of *Pseudomonas luteola* (formerly *Chryseomonas luteola*).

A morphological analysis of colony growth revealed differences that were determined by temperature and incubation time. Bacteria formed circular colonies with a convex surface (Table 1, Fig. 1b.). The highest colony growth rate was noted at 28°C. The average colony size was estimated at 3 mm. Young colonies were colorless or milky white. After 24 h of incubation at 28°C, colonies became creamy white, and after 48 h, they turned yellow. Incubation at lower temperatures decreased colony staining intensity. At 22°C and 10°C, the colonies were smaller (approximately 2 mm in diameter), translucent or milky white. At 4°C, translucent colonies were 1-2 mm in diameter, and their counts were lower than those noted at the remaining temperatures.

Biochemical characteristics

Basic catalase and oxidase tests produced positive results (Fig. 1c., 1d.), and the Gram staining produced a negative result, which is characteristic of PGPB of the genus *Pseudomonas* (Nathan et al., 2013). Biochemical tests revealed the activity of alkaline phosphatase, acid phosphatase and other enzymes metabolizing phosphorus compounds, indicating that the analyzed bacterium enhanced the availability of organic phosphorus for plant roots (Caldwell, 2005). The process can take place in acidic soils and in soils where liming leads to a periodic increase in pH. The analyzed strain was able to degrade and assimilate several substrates. It demonstrated esterase, acrylamidase, phosphohydrolase, β-galactosidase, β-glucosidase and dihydrolase activity, and it was characterized by denitrifying and glucose fermenting abilities (Table 2). Due to its broad enzymatic activity and assimilation efficiency, the analyzed strain can use substrates from various sources and survive in diverse habitats, which makes it an effective competitor of plant pathogens.

Denitrifying and TCP solubilizing abilities

The bacterium reduced nitrates to nitrites and converted organic nitrogen into ammonia nitrogen, a form of nitrogen that is most readily available to the roots (Table 2, Fig. 2.). Denitrification, aminization and ammonification are positive processes that make nitrogen compounds available for plant uptake via the root system. The analyzed strain produced organic acids that increased the availability of mineral sources of phosphorus, such as calcium phosphate $Ca_3(PO_4)_2$ (Fig. 2.). The bacterium's TCP solubilizing ability increased steadily from an average level on incubation day 3 to a high level on day 14 (Fig. 3.).

Antagonism against pathogenic fungi

The analyzed strain inhibited the growth of all five tested species of phytopathogenic fungi. It exerted the highest inhibitory effect on *Rhizoctonia solani* (by about 38%). The growth of the remaining fungi was inhibited by 20-29% (Fig. 4.).

Bacterial proliferation in an environment containing glyphosate

The *Pseudomonas* sp. SP0113 strain responded to glyphosate exposure by increasing bacterial bio-

mass. The greatest increase was noted after the first 12 h of incubation. The greatest amount of biomass, which was higher than in control, was produced in response to the glyphosate dose of 0.1 mg·ml^{-1}, whereas the increase in the number of bacterial cells under exposure to the glyphosate dose of 1 mg·ml^{-1} was comparable to control. The highest glyphosate concentrations inhibited bacterial proliferation already after the application of the suspension at the beginning of the experiment, but a similarly high increase in biomass was observed. The decrease in the bacterial growth rate in response to the highest glyphosate dose could be attributed to a lower pH than in the remaining treatments due to higher herbicide concentrations (Fig. 5.).

DISCUSSION

Numerous representatives of the genus *Pseudomonas* and other rod-shaped bacteria are non-pathogenic rhizobacteria, many of which

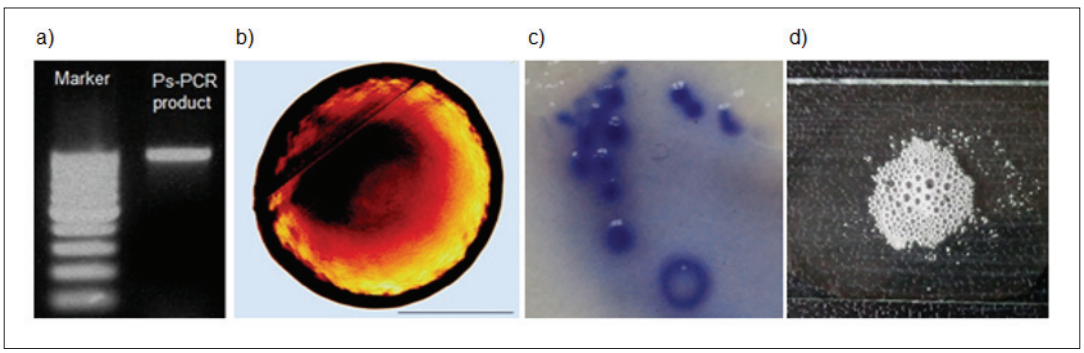

Fig. 1. (a) PCR amplification using primers specific for *Pseudomonas* spp. (Ps-PCR) (molecular weight marker was 100-1000 bp); (b) microscopic images of colonies, 1 mm scale; (c) cytochrome c peroxidase activity – the dark blue stain produced by 1% tetramethyl-p-phenylenediamine solution is a positive result; (d) catalase activity – foaming after the addition of 3% H_2O_2 solution is a positive result.

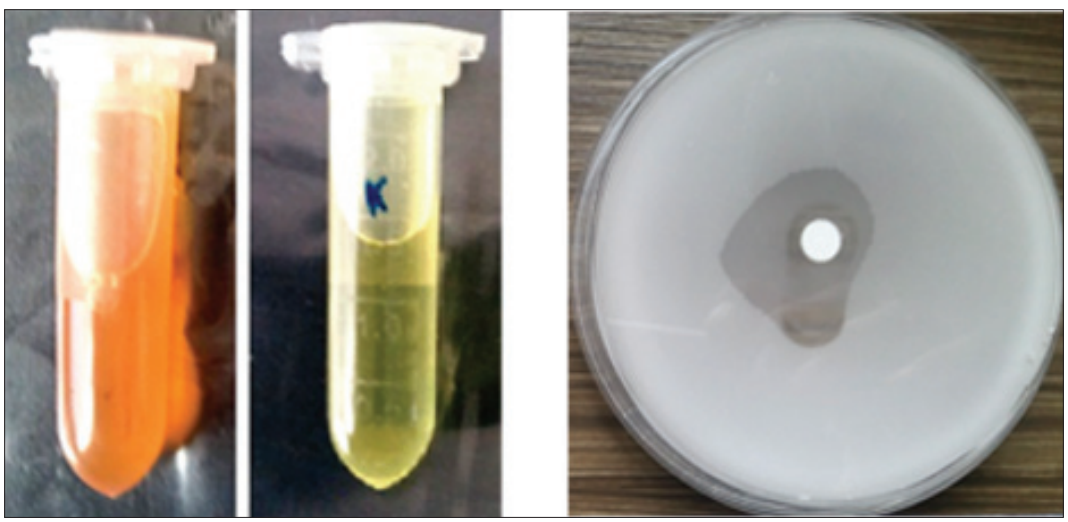

Fig. 2. Left: the tested strain's ability to convert organic nitrogen into plant-available nitrogen (NH_4^+); right: TCP solubilizing ability after 14 days of incubation.

are phosphorescent, whereas isolates of species such as *Pseudomonas fluorescens*, *P. putida*, *P. aeruginosa*, *P. aureofaciens*, *P. stutzeri* and *P. chlororaphis* are capable of inhibiting the growth of soil-borne pathogens (Botelho and Mendonça-Hagler, 2006; Jan et al., 2011). The most widespread bacterial mechanisms responsible for the inhibition of pathogen proliferation include the ability to colonize the root zone, production of antibiotics and enzymes that degrade pathogen cell walls, production of siderophores and hydrogen cyanide (Ahemad and Kibret, 2014; Jan et al., 2011; Shen et al., 2013). A wheat (*Triticum* spp.) inoculation study revealed that the AXMP7 strain of *P. putida* exerted positive effects on plants by producing stress-inhibiting enzymes, including catalase, ascorbate peroxidase (APX) and superoxide dismutase (SOD) (Ali et al., 2011). *Pseudomonas* sp. SP0113 was able to catalyze the decomposition of H_2O_2 and, consequently, produce catalase, which inhibits the production of reactive oxygen species in plants in response to environmental stressors.

Nutrient availability is a key determinant of yield potential and quality. Soil-dwelling microorganisms contribute to nutrient availability and element cycling in the ecosystem, organic matter decomposition, oxidation and reduction processes, solubilization of substances that are not readily available to plants, and storage of biogenic elements (Cummings, 2009; Motavalli et al., 2004; Rodríguez and Fraga, 1999). Nitrogen plays the key role in yield formation. PGPB that participate in nitrogen metabolism and fixation have a beneficial influence on plant growth. Soil-dwelling ammonifying bacteria convert organic nitrogen into ammonia nitrogen that is readily taken up by plants via the root system (Cummings, 2009; Witte, 2011).

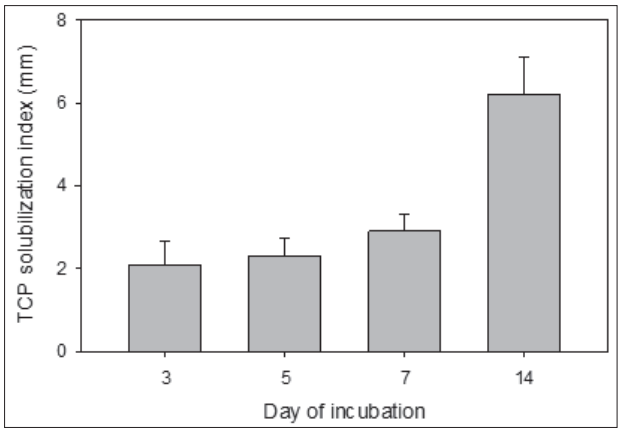

Fig. 3. TCP ($Ca_3(PO_4)_2$) solubilizing ability.

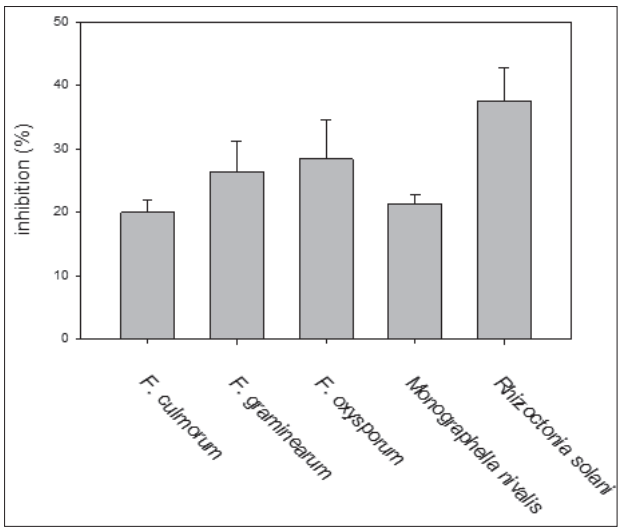

Fig. 4. The ability of the SP0113 strain to inhibit the growth of pathogenic fungi.

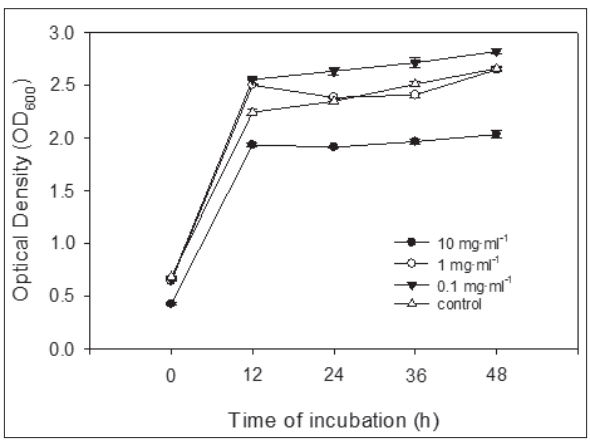

Fig. 5. Increase in bacterial biomass in the course of 48 h treatments with three different glyphosate concentrations

Phosphorus also plays a very important role in plant growth, yield formation and resistance to stress. It is a component of genetic material and an energy carrier in living organisms. Phosphorus concentrations in soil vary, subject to naturally occurring nutrient levels, and phosphorus often occurs in soil in water-insoluble, mineral form (Schachtman et al. 1998). Soil-dwelling bacteria produce organic acids that solubilize immobilized mineral phosphorus and make it available to the roots. The following genera of phosphate solubilizing bacteria occur in nature: *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Aereobacter*, *Flavobacterium*, *Erwinia* and *Arthrobacter*. Phosphatase-producing bacteria also release phosphorus from organic compounds (Caldwell, 2005; Chen et al., 2006; Šarapatka, 2003; Rodríguez and Fraga, 1999). The PGPR strain analyzed in this study has denitrification and ammonifying abilities that enhance nitrogen cycling and facilitate nitrogen uptake by plants via the root system. The evaluated strain also produced organic acids when cultured on a medium containing calcium triphosphate ($Ca_3(PO_4)_2$), which suggests that it is capable of solubilizing immobilized phosphorus. The observed activity of acid phosphatase, alkaline phosphatase and other enzymes participating in phosphorus metabolism indicates that the tested strain has the ability to convert organic phosphorus into plant-available phosphorus that is assimilated by the root system.

Like other species of the genus *Pseudomonas*, the evaluated strain inhibited the growth of plant pathogens of the genera *Fusarium* and *Rhizoctonia*, which makes it a suitable candidate for a biological control agent. Fungi of the genera *Fusarium* and *Rhizoctonia* cause significant losses in agricultural production. The tested pathogenic species affect cereals and grasses where they cause mainly seedling infections and stem-base diseases. The analyzed strain can be used as a biocontrol agent to effectively prevent the spread of pathogens in organic farms and public green areas where chemical control is not an option. In addition to dangerous *Fusarium* fungi and *Rhizoctonia solani* that cause root rot and seedling blight, the tested strain also inhibited the growth of *Monographella nivalis*, a fungus that infects cereals and grasses under snow cover in early spring (Gilbert and Haber, 2013; Hudec and Muchová, 2010; Pumphrey et al., 1987).

The analyzed strain was capable of growth at temperatures lower than the optimal temperature, even at 4°C. The SP0113 strain is suitable for biological control and growth enhancement in fall and spring when kernels and young seedlings are susceptible to infections and the negative effects of low temperature.

Pseudomonas sp. SP0113 is characterized by multiple plant growth promoting traits. Those traits have also been observed in non-rhizosphere PGPB. The *Pseudomonas* sp. OG strain isolated from seawater is capable of solubilizing mineral phosphorus, producing ammonia, IAA, siderophores, HCN and catalase. In a pot experiment, *Pseudomonas* sp. OG enhanced the growth of chickpeas (*Cicer arietinum* L.) and mung beans (*Vigna radiata*) by increasing the size of the aboveground parts of the plant, the roots and plant biomass (Thakker et al., 2013). Furthermore, antifungal antibiotics such as 2,4-diacetylphloroglucinol, phenazines, pyoluteorin and pyrrolnitrin are metabolites produced by *Pseudomonas* genera (Garbeva et al., 2004; Jošić et al., 2014; Ramette et al., 2011).

Microorganisms are the preferred biological control agents in organic farms. PGPB are also recommended for agricultural systems that rely on phosphonate herbicides, toxic compounds

that disrupt the soil balance, contribute to the spread of pathogens and exert negative effects on plant health (Duke et al., 2012; Fernandeza et al., 2009).

CONCLUSION

Non-rhizosphere PGPB can increase plant yield and improve plant health. Further research is needed, in particular under field conditions, to verify the analyzed strain's potential during interactions with plants, environmental conditions and pathogens. The evaluated strain can also be used on farms that rely on phosphonate herbicides to minimize their negative impact on the agricultural ecosystem.

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