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ISOLATION AND CHARACTERIZATION OF PLANT GROWTH PROMOTING BACTERIA FOR POTENTIAL BIOREMEDIATION

SUMMARY

Modern agriculture is based on mineral fertilizers and use of synthetic chemicals in plant protection. Plant growth promoting bacteria (PGPB) represent a promising alternative to chemicals employed in agricultural practice. The aim of this study was to isolate and characterize PGP strains with the ability to promote plant growth from three different locations in the city of Zagreb. Phenotypic characterization of isolates included testing the tolerance to unfavorable soil conditions as well as intrinsic antibiotic resistance, biochemical characterization and screening for plant growth promoting properties. Twenty-four isolates were isolated. 28 % isolates were positive for the phosphate solubilization test, while IAA was produced by all isolates. Half of all isolates had the ability to synthesize lytic enzymes and exopolysaccharides. Some differences were found between isolates regarding their phenotypic characteristic, especially resistance to low pH and high temperatures.

Keywords: plant growth promoting bacteria, auxine production, phosphates solubilization, bioremediation, sustainable agriculture, ecological characterization

INTRODUCTION

Agricultural practice which relies on increased utilization of chemicals leads to a reduction or a complete loss of indigenous and beneficial microorganisms in the soil (Khatoon *et al*., 2020). Plant growth-promoting bacteria (PGPB) have attracted attention for decades for their ability to improve plant development and resistance to environmental stress conditions (Probanza *et al*., 2002). Mechanisms by which bacteria affect plant growth differ between species and strains (Castro *et al*., 2009). Plant growth-stimulating bacteria promote plant growth by direct mechanisms by increasing the availability of nutrients or synthesizing phytohormones (Gouda *et al*., 2018). They also

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participate in defensive metabolic processes in plants by suppressing the growth of pathogens and promoting systemic plant resistance (Jeyanthi and Kanimozhi, 2018). The application of PGP bacteria also improves the resistance of plants to stressful environmental conditions such as drought, soil salinity and the presence of heavy metals (Brown and Saa, 2015). In recent years, biocontrol agents based on PGPB have proven to be effective ecological solutions, since chemical pesticides have many harmful effects on the environment. Major concern in regard to food safety is their persistence in the soil, while biopesticides, based on PGPB have numerous advantages such as biodegradability, self-sustainability, economic profitability, ease of handling and safety for use (Beneduzi *et al*., 2012).

Rapid industrialization and modernization throughout the world has led to environmental pollution with significant amounts of toxic waste. Various physical, chemical and biological methods are used to remove soil pollution. In relation to the physico-chemical method of remediation, bioremediation has a great advantage. It is cost-effective, suitable for the environment, can completely decompose organic pollutants and has no negative impact on native flora and fauna. PGPB contribute in bioremediation of polluted soils by stimulating the resistance and growth of plants through the synthesis of various compounds such as siderophores, indole-3-acetic acid and antibiotics or through the stimulation of certain metabolic pathways (nitrogen fixation and absorption of nitrogen, phosphorus, magnesium and other nutrients) (Ipek *et al*., 2019).

The objective of the present study was to isolate and characterize plant growth promoting rhizobacteria (PGPR) found in the rhizosphere zone of plants growing in the soils of different type of use in Zagreb city area. It is assumed that those bacteria possess some direct and indirect mechanisms of plant growth promotion, and that they differ in their phenotypic and ecological characteristics.

MATERIAL AND METHODS

Sample collection

Soil samples were collected in the month of March 2022, from three different locations in the city of Zagreb. From the agricultural area of the Faculty of Agriculture (45° 82′ 56.36″ N, 16° 03′ 31.80″ E), Maksimir forest (45° 82′ 91.17″ N, 16° 02′ 82.61″ E) and polluted meadow in Maksimir (45° 82′ 77.40″ N, 16° 02′ 84.28″ E). Rhizosphere soil was collected at a depth of 30 cm and placed into a sterile plastic bag and transported to the laboratory for the microbiological analysis.

Bacterial isolation

For isolation of rhizosphere bacteria, 1.0 gram of the soil was mixed in 9.0 ml of saline solution (0.9 % NaCl) (w/v). Soil suspension were serially diluted (10-1 to 10-6) respectively. 100 µl of supernatant from each dilution of rhizosphere soil, solutions were transferred into Nutient agar (Sherpa *et al*., 2021) and incubated five days at the 30 °C. The purified bacteria cultures were

identified based on their morphology (Vincent, 1970) and screened for different PGP traits, abiotic stress tolerance and antibiotic resistance.

Screening for in vitro plant growth promoting characteristics

Phosphate Solubilization

The inorganic phosphate solubilization activity of bacterial isolates was determined using Pikovaskaya's medium. The cultures were inoculated on the Pikovskay's medium plates and incubated at 30 °C for 7 days. The appearances of the clear zone around inoculated colonies indicated the solubilization of inorganic phosphate (Tsegaye *et al*., 2019).

Production of indole acetic acid (IAA)

IAA production was detected as described by Sherpa et al. (2021). Bacterial cultures were grown on Luria Bertani broth amended with 100 mg/l tryptophan as the precursor of IAA and incubated in a shaker (Biosan ES-20, Latvia) at 150 rpm at 30 °C five days in the dark followed by a change in color post addition of Salkowski reagent. The optical density (OD) was recorded at 530 nm using a UV-VIS spectrophotometer (Lambda EZ 210, Perkin Elmer, USA).

Screening bacterial isolates for hydrolytic enzyme production

Bacterial isolates were screened for their hydrolytic enzyme production like protease and amylase. Bacterial isolates were screened for their ability to produce protease onto skim milk agar or SMA (3 % v/v) medium. Bacterial isolates showing clear halo zone on skim milk agar was indicated a positive result for protease synthesis (Tsegaye *et al*., 2019).

Amylase production by bacterial isolates was determined using starch agar as described in Mir *et al.* (2021) and incubated at 30 °C for 48 h. At the end of the incubation period, the plates were flooded with iodine solution. Iodine reacts with starch to form a blue color compound. Hence the colorless zone surrounding colonies indicates the production of amylase.

Exopolysaccharide production

Exopolysaccharide (EPS) producing activity of the bacterial isolates was done according to the method described by Jain i sur. (2016). Formation of precipitation was considered as positive result for EPS production.

Ecological characterization

PGPB were examined for their tolerance to salt on Nutrient Agar supplemented with 1, 2 and 3 % (w/v) NaCl (Shultana et al., 2020). Temperature tolerance was tested by incubating the inoculated plates at 4 °C, 37 °C and 42 °C (Ashraf *et al*., 2019). The ability of the isolates to grow in acidic or alkaline medium was tested by streaking each isolate on separate Petri plates on Nutient Agar with pH adjusted to 5, 6 and 9, as a method indicated by Fitriatin (2022).

Antibiotic resistance

The antibiotic sensitivity of PGPB was determined by disc diffusion method for the following antibiotics (ampicillin 10 μg/ml, streptomycin 10 μg/ml,

erythromycin 15 μg/ml and kanamycin 30 μg/ml) and incubated for five days at 30 °C. Isolates were considered resistant when growth occured and sensitive when no growth was detected.

RESULTS AND DISCUSSION

The results revealed that the number of bacteria differed depending on the sampling site. The highest number of bacteria was found in the sample taken from the polluted meadow in Maksimir (SM), the analysis of which determined $\bar{x}$ (CFU/ml) of 5.33E+06, while the lowest number of microorganisms was determined in the forest soil of Maksimir (ŠM) with $\bar{x}$ (CFU/ml) of 4.00E+06 (table 1). The diversity and abundance of rhizobacteria can be influenced by many factors such as the type of soil, the presence of nutrients in the soil, soil moisture, agroclimatic conditions, plant species, interactions between plants and microorganisms, and the way the soil is used (Tsegaye *et al*., 2019).

Table 1. Colony-forming unit (CFU/g).

Sample designation	Colony-forming unit (CFU/g)				
	1st repetition	2nd repetition	3rd repetition	$\bar{x}$ (CFU/ml)	stDev
ŠM	3,50E+06	4,70E+06	3,80E+06	4,00E+06	6,24E+05
FP	5,90E+06	4,40E+06	5,30E+06	5,20E+06	7,55E+05
SM	6,30E+06	5,80E+06	3,90E+06	5,33E+06	1,27E+06
ŠM- Maksimir forest; FP- Agricultural area; SM- Polluted meadow in Maksimir					

After determining the CFU, 25 colonies were selected and included in further research. A morphological characterization of the isolates was conducted, showing that most of the isolates belong to Gram-negative rods.

After nitrogen, phosphorus is the second main limiting nutrient in agricultural production. It is present in the soil in relatively high amounts, but in a form which is unavailable to plants, so they cannot meet their needs for phosphorus (Kishore *et al*., 2015). PGPB release organic acids and thus reduce the pH of the rhizosphere, and as a result, there is a transition of insoluble phosphorus into soluble forms available to plants (Adedeji *et al*., 2020). This research determined that only 28 % of the isolates have the ability to dissolve phosphate. One of the possible reasons may be the fact that only 20-40 % of phosphorus solubilizers can be grown in laboratory conditions (Naseem *et al*., 2018). González *et al*. (2021) conducted research on PGP bacteria that were isolated from soil with elevated mercury concentration and proved that only 7.5 % of them have the ability to dissolve phosphate. On the other hand, a higher percentage of phosphorus solubilizers (28.2 %) was observed in environmental conditions with absence of heavy metals (Wang *et al*., 2020).

Auxins, among them indole-3-acetic acid (IAA), are biologically active molecules involved in numerous physiological processes in plants. Several studies have shown that the growth rate of plants is significantly higher in those treated with PGPR-producing auxins compared to untreated plants (Mahmoud *et al.*, 2020). All tested isolates had the ability to produce IAA. The highest concentrations were recorded in isolates taken from a polluted meadow in Maksimir (SM8 69.9 µgml-1), while the lowest concentrations were recorded in isolates isolated from agricultural soil (FP10 16.5 µgml-1). Similar results were obtained by Robas *et al*. (2021) who reported the increased synthesis of IAA in the growth medium with an increase mercury concentration.

Table 2. Bacterial isolates screened for plant growth promoting traits.

Bacterial isolates		P-solubilization	IAA production (µg ml^{-1})	Lytic enzyme production		Production of EPS
				Protease	Amylase	
Maksimir forest	ŠM1	-	31,8	+	-	+
	ŠM2	-	23,1	-	-	-
	ŠM3	-	15,6	+	-	+
	ŠM4	+	20,78	-	-	-
	ŠM5	-	24,63	-	-	-
	ŠM6	-	30,3	+	-	+
Agricultural area	FP1	-	26,85	+	-	-
	FP2	+	24,83	+	+	+
	FP3	+	23,5	+	+	+
	FP4	+	50,78	-	+	+
	FP5	-	55,6	-	-	-
	FP6	-	58,9	+	+	+
	FP7	-	62,5	-	-	-
	FP8	-	31,4	+	+	+
	FP9	-	18,4	+	+	+
	FP10	-	16,5	-	-	-
Polluted meadow in Maksimir	SM1	-	53,4	-	+	+
	SM3	+	67.9	+	+	+
	SM4	+	68,4	+	+	+
	SM5	-	67,2	-	-	-
	SM6	+	61,7	+	+	+
	SM7	-	66,3	+	-	-
	SM8	-	69,9	+	+	+
	SM9	-	34,6	-	+	+
	SM10	-	38,9	+	-	-
*- = no production, +/- = weak production, + = high production						

One of the main mechanisms used by PGPB to suppress soil pathogens and thus protect the plant is the production of lytic enzymes. With their help, bacteria can break down the cell wall of pathogens. In this research, 60 % isolates

produced protease, while 48 % of them produced amylase. Exopolysaccharides are involved in soil microbial aggregation and surface attachment. They also maintain the optimal moisture level for plant growth in drought conditions by producing a biofilm on the roots (Khan and Bano, 2019). In saline soils, bacterial exopolysaccharides have the potential to bind cations (including Na+) and thus limit their uptake by plant and maintain the K+/Na+ balance (Morcillo and Manzanera, 2021). A summary of plant growth promoting traits of isolates from this study are shown in Table 2.

Table 3. Effect of NaCl concentration, pH and temperature on isolates

Bacterial Isolates		Abiotic stress tolerance								
		Temperature			Salt concentration NaCl (w/v)			pH		
		4°C	37°C	45°C	1	2	3	5	6	9
Maksimir forest	ŠM1	-	+	-	+	+	-	+	+	+
	ŠM2	-	+	-	+	+	+	+	+	+
	ŠM3	-	+	-	-	-	-	-	+	-
	ŠM4	-	+	-	+	+	+	+	+	+
	ŠM5	-	+	-	-	-	-	-	+	+/-
	ŠM6	-	+	-	-	-	-	-	+	+/-
Agricultural area	FP1	-	+	-	+	+	+	-	+	+/-
	FP2	-	+	+	+	+	-	+/-	+	+
	FP3	-	+	+	+	+/-	-	+/-	+	+
	FP4	-	+	-	+/-	-	-	-	+	+/-
	FP5	+	+	-	+	+	+	+	+	+
	FP6	-	+	-	+	-	-	+	+	+/-
	FP7	-	+	-	+/-	-	-	+	+	+/-
	FP8	-	+	+	+	+	-	+/-	+	+/-
	FP9	-	+	+	+	-	-	+	+	+/-
	FP10	-	+	-	+	+	+	+/-	+	+
Polluted meadow in Maksimir	SM1	-	+	-	-	-	-	+	+	+
	SM3	-	+	-	+	-	-	+/-	+	+
	SM4	-	+	-	+	+	-	+	+	+/-
	SM5	-	+	-	-	-	-	+	+	+
	SM6	-	+	-	+	-	-	+/-	+	+
	SM7	-	+	-	+	-	-	+/-	+	+/-
	SM8	-	+	-	+	+	-	+/-	+	+/-
	SM9	-	+	-	+	-	-	+/-	+	+/-
	SM10	-	+	-	+	-	-	+/-	+	+/-
*- = no production, +/- = weak production, + = high production										

PGPB are mostly mesophilic organisms. In order to test whether the obtained isolates are able to adapt to different temperatures, the growth of these isolates was tested at 37 °C, 45 °C and 4 °C.

Table 4. Antibiotic resistance of isolates

Bacterial isolates		Ampiciliin 10 µg/ml	Streptomycin 10 µg/ml	Erythromicin 15 µg/ml	Kanamycin 30 µg/ml
Maksimir forest	ŠM1	S	R	S	S
	ŠM2	R	S	S	S
	ŠM3	S	S	S	S
	ŠM4	R	R	R	R
	ŠM5	S	S	S	S
	ŠM6	S	S	S	S
Agricultural area	FP1	S	S	S	S
	FP2	S	S	R	S
	FP3	S	S	S	S
	FP4	S	S	S	S
	FP5	S	S	R	S
	FP6	S	S	S	S
	FP7	S	S	S	S
	FP8	S	S	S	S
	FP9	S	S	S	S
	FP10	S	S	S	S
Polluted meadow in Maksimir	SM1	S	S	R	S
	SM3	S	S	S	S
	SM4	S	S	S	S
	SM5	S	S	S	S
	SM6	S	S	S	S
	SM7	R	R	R	S
	SM8	S	S	S	S
	SM9	S	S	S	S
	SM10	S	S	S	S
*R - resistant, S - susceptible					

All isolates showed good growth at 37 °C. On the other hand, only the isolate from the agricultural area (FP5) had the ability to grow at 4 °C (Table 3). Tsegaye *et al*. (2019) obtained similar results by examining the ability of isolates to grow at different temperatures. They found that the majority of PGPB did grow at 4 °C and 50 °C, while all isolated bacteria grew well at 30 °C. Compared to bacteria, plants are much more sensitive to elevated salt concentrations in the soil. Therefore, PGPBs can promote plant growth and productivity under unfavorable conditions by producing various enzymatic and non-enzymatic components (Venkateswarlu *et al*., 2008). It should be noted that the ability to tolerate high salinity varies depending on the type of PGPB. This is also confirmed in the present research, where the growth of most tested isolates gradually decreased as the concentration of NaCl increased. At a concentration of 1 % NaCl, 72 % of the isolates grew, while at a concentration of 2%, 40 % of them grew. The fewest isolates grew on 3 % NaCl, only 20 % (Table 3). One of the main factors affecting direct and indirect mechanisms of PGPB is pH. An acidic soil reaction can negatively affect IAA production (Mohite, 2013).

In this research it was shown that all isolates grew well at pH 6 while at pH 5, 32 % of the isolates grew. In alkaline conditions (pH 9), good growth was recorded for 40 % of the isolates, while 52 % of them grew partially. From this it can be concluded that the examined isolates adapted very well to both alkaline and acidic conditions, regardless of the location from which they were isolated (table 3). A large number of soil microorganisms have the ability to synthesize antibiotics, which is why they can impair the growth and survival of PGPB. In order to protect their cells from the effects of antibiotics, some bacterial species have developed natural resistance to antibiotics. Many environmental factors can negatively affect antibiotic resistance (Naamala *et al.,* 2016). By checking the antibiotic resistance in this study, it was established that 80 % of the isolates were sensitive to all tested antibiotics (Table 4).

Most of the isolates were sensitive to kanamycin (96 %). Susceptibility to streptomycin and ampicillin was 88 %, while 80 % of the isolates were sensitive to erythromycin (Table 4). In a study conducted by Xia et al. (2020) antibiotic resistance of the PGPR species Bacillus xiamenensis PM14 was tested and it was found that this strain was resistant to erythromycin, ampicillin and streptomycin. Opposite results were obtained by Leite *et al*. (2018) who tested different strains of the genus Bradyrhizobium and found that most strains were susceptible to streptomycin. By comparing the research conducted, it can be determined that the resistance of PGPB to different types of antibiotics varies greatly between species and strains.

CONCLUSIONS

The presence of PGPB was found in all sampled soils and the number of bacteria differed depending on the sampling site. In the conducted research, strain SM4, which was isolated from a polluted meadow in Maksimir, stood out the most since it possessed most of the tested PGPR characteristics. As such, it has great potential for use in the bioremediation process as well as a promoter of plant growth and development. Treating plants with this strain could stimulate their growth and development, as well as protect against pathogens, which would give the opportunity to reduce the use of chemical protective agents.

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