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BIOSTIMULATORY POTENTIAL OF MICROORGANISMS FROM ROSEMARY (*ROSMARINUS OFFICINALIS* L.) RHIZOSPHERIC SOIL

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SUMMARY

The objective of the present paper was to isolate microorganisms (*Pseudomonas* sp., *Bacillus* sp. and *Azotobacter* sp.) from the rhizospheric soil of rosemary (*Rosmarinus officinalis* L.) and investigate their biostimulatory (plant growth-promoting – PGP) and biocontrol potential. The bacteria isolated from the rhizosphere of rosemary included 15 bacteria of the genus *Pseudomonas*, 20 of the genus *Bacillus*, and 11 of the genus *Azotobacter*. Based on the morphological characteristics of colonies and cells, representative isolates of each genus were chosen (marked as *Pseudomonas* sp. P42, P43, P44; for *Bacillus* isolates B83, B84, B85, B92, B93; and for *Azotobacter* isolates A15 and A16) for different physiological and biochemical examination. The study included in vitro screening of the bacterial isolates for their PGP and biocontrol properties. *Pseudomonas* isolates showed the ability to live at low temperature (10°C) and high pH (9), and to use different sources of carbon. All *Pseudomonas* isolates produced lipase, siderophore, hydrogen cyanide, and utilized organic and inorganic phosphorus, while only isolate P42 produced amylase, pectinase and cellulase. Only *Bacillus* isolates could grow at 45 °C (all *Bacillus* isolates), pH 5 (isolates B83), and on a medium containing NaCl 5 and 7% (all isolates). Isolates denoted as B83 and B93 produced lipase, amylase, and pectinase. All isolates had the ability to solubilize phosphate, produce siderophores (except B85) and hydrogen cyanide, while only two isolates (B84 and B85) produced IAA. *Azotobacter* isolates had the optimal growth at 37°C and minimal growth on a medium with pH 6 and 9. All *Azotobacter* isolates used all carbohydrates as a source of carbon and produced lipase, amylase, and hydrogen cyanide. The best result in suppressing the growth of pathogenic fungi *Fusarium oxysporum* was achieved by using isolate B92. Application of isolate B83 led to the greatest growth suppression of *Sclerotinium sclerotiorum*.

Key words: medicinal plants, plant-growth promoting rhizobacteria, siderophore, IAA

INTRODUCTION

The rhizosphere is a thin area around the root, the zone of touching between root and soil (Narula et al., 2009). Diversification of rhizospheric microorganisms is related to the root exudates, biochemical reactions, and soil respiration. Different compounds secreted by the plant root can increase or suppress the growth and activity of rhizospheric microorganisms. On the other hand, microorganisms colonizing the rhizosphere show different influences on the plants. They can be positive, negative, and neutral (Whipps, 2001). Microorganisms that show positive effect on plants – through diverse mechanisms, such as nitrogen fixation, solubilization of phosphorus, production of vitamins, phytohormones, antibiotics, enzymes, siderophores, etc. – are designated as Plant-Growth-Promoting Rhizobacteria (PGPR). Application of PGPR enhances plant growth and development and increases crops of many plants.

Investigating microorganisms from the rhizosphere of medicinal plants is very significant as these microorganisms have great impact on the plant growth and on the quality of the medicinal products (Bafana & Lohiya, 2013). Therefore, due to growing interest in the production of medicinal herbs, there has been increasing interest in studying

the diversity and characteristics of microorganisms isolated from the rhizosphere of medicinal plants. Bafana & Lohiya (2013) isolated 120 isolates of endophytes and rhizospheric microorganisms from the root and rhizosphere of *Origanum vulgare*. Kumar et al. (2012) isolated 123 morphologically different bacteria from the rhizospheric soil and roots of medicinal plant *Ajuga bracteosa*. Zhao et al. (2013) investigated the microbial diversity of the rhizospheric soils of many medicinal plants and found a total of 50 strains belonging to the following 7 genera: *Myxococcus*, *Corallococcus*, *Cystobacter*, *Archangium*, *Stigmatella*, *Chondromyces*, and *Pyxidicoccus*, while *Myxococcus* and *Corallococcus* were the dominant genera. Also, the same authors analyzed the actinobacterial diversity in the rhizosphere of seven medicinal plant species and identified 18 actinobacterial genera (Zhao et al., 2012).

The results of several studies confirmed that a significant number of isolated bacteria promote the growth and development of medicinal plants when associated with the plant roots (Whang et al., 2014; Koeberl et al., 2013; Bafana & Lohiya, 2013). Application of *Streptomyces pactum* (Act12) improved the soil microbial activity, which increased the resistance and root activity of ginseng plant and enhanced the harvest and its quality (Zhang et al., 2013). *Rhizobium meliloti*, isolated from medicinal plant *Mucuna pruriens*, produced siderophores and thus promoted the plant growth (Arora et al., 2001). According to the results of the mentioned investigations, as well as many others, it can be concluded that isolation, characterization, and application of rhizospheric microorganisms during cultivation of medicinal plants is important for improving the medicinal values and yield of medicinal plants.

Rosemary (*Rosmarinus officinalis* L.) is a famous and worldwide used medicinal and aromatic plant species. Rosemary grows under a wide range of climates, distinctive to Europe, Asia, and Africa, primarily in areas nearby the Mediterranean Sea (Pintore et al., 2002). It is well-known that its flowering tops and the leaves have bioactive substances such as flavonoids, phenolic acids, specifically rosmarinic acid, and essential oil (including pinene, camphene, cineole, borneol, and camphor) (Oluwatuyi et al., 2004). Due to its properties, rosemary oil has been extensively used for centuries as an ingredient in cosmetics, soaps, perfumes, deodorants, both for flavoring and for preservation of food products (Arnold et al., 1997). It is also used in treatments of various diseases, including depression and arthritic pains (Lakshmipathy et al., 2001).

In view of the above, the objective of the present study was selection of microorganisms (*Pseudomonas* sp., *Bacillus* sp. and *Azotobacter* sp.) from the rhizospheric soil of rosemary (*Rosmarinus officinalis* L.) and investigation of their biostimulatory plant growth-promoting (PGP) and biocontrol potential.

MATERIAL AND METHODS

Microorganisms were isolated from the rhizospheric soil of rosemary (*Rosmarinus officinalis* L.) (locality Novi Sad: Lat. 45.2493312, Long.19.8443008) from chernozem having the following characteristics: 1.67% CaCO₃; 5.20% humus; 0.25% N; 176.5 mg P₂O₅ in 100g of soil; 104.34 mg K₂O in 100g of soil; pH in H₂O 6.75; pH in KCl 5.75). A soil sample of 10 g was suspended in 90 ml of sterile 0.85% NaCl. The soil suspension was then diluted in three-fold steps in glass tubes to an end point dilution of 10⁻⁴. Hundred microliters of the dilutions (10¹ to 10⁻⁴) were spread on an appropriate selective media: King-B (King et al., 1954), Nutrient Agar (NA) (Shamseldin et al., 2008) and N-free medium (Jensen, 1987) for *Pseudomonas*, *Bacillus*, *Azotobacter* isolates (respectively) and incubated at 28°C for 3 days.

Biochemical and morphological characterization

Gram-negative, rod-shaped cells that synthesize pigment fluorescein were chosen as *Pseudomonas* isolates. The ability of fluorescein production was tested on Pseudomonas Flo agar (proteose pepton 20 g l⁻¹, bacto maltose 10 g l⁻¹, K₂HPO₄ 1.5 g l⁻¹, MgSO₄ 0.73 g l⁻¹, glycerol 10ml/l, agar 15 g l⁻¹).

Catalase and Gram-positive, rod-shaped cells that create endospores were selected as *Bacillus* isolates. Appearance of oxygen bubbles after putting a drop of 3% hydrogen peroxide on 48 h old bacterial colony was the proof of catalase activity.

Isolates which were stated as Gram-negative, coccoid-shaped cells that synthesize large quantities of capsular slime and could grow on N-free medium were selected as *Azotobacter* isolates.

Utilization of carbon sources (lactose, fructose, glucose, sacharosa, galactosa) was tested using Hugh-Leifson medium (pepton 2g, K₂HPO₄ 0.3g, NaCl 5g, carbon source 10g, brom-thymol blue 0.03g, agar 3g) with different carbon sources (glucose, fructose, lactose, sacharose, xylose). In the case of a positive reaction, the colour of the medium around the isolates colony changed from greenish to yellow.

Bacterial isolates screened for abiotic stress tolerance activities

Pseudomonas, *Bacillus* and *Azotobacter* isolates were incubated at different temperatures (3, 13, 37 and 45°C), pH levels (4, 5, 6, 9), and salt concentrations (5 and 7%) for 4 days, on the following growth media: King-B, Nutrient

agar, and N-free medium, respectively. The colony width of each isolate was measured and compared to a colony of the same isolate grown under the optimal conditions (at 28 °C, pH 7 and salt concentration 3%). Based on this comparison, the isolate growth was described as: - absence of growth; + minimal growth; ++ optimal growth; +++ intense growth.

In vitro screening of bacterial isolates for their plant growth promoting traits

Bacterial ability to solubilize sparingly soluble $\text{Ca}_3(\text{PO}_4)_2$ was examined on Pikovskaya (1948) medium. Phosphate solubilization was confirmed by a transparent zone around the isolates colonies.

The ability of mineralization of phosphorus organic compounds was assayed on Menkina medium, modified by Rodina (Menkina, 1961). Appearance of a clear zone around the colony indicates the ability of the isolate to dissolve phosphate.

Investigation of indol-3-acetic acid (IAA) production by bacterial isolates was observed using the method developed by Etesami et al. (2015).

Isolates capability to produce siderophores was examined on chrom-azurool S (CAS) medium (Milagres et al., 1999).

In vitro screening of bacterial isolates for their biocontrol properties

The ability to produce lipase was tested using a medium (pepton 10g l^{-1} , NaCl 5g l^{-1} , $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ 0.1g l^{-1} , agar 15g l^{-1}) with Tween 80. The existence of a clear area around the isolates colony was the proof of lipolytic activity.

Hydrolysis of starch was performed by flooding iodine on the colonies developed on starch agar. Amylase activity was detected by a discoloured zone around the isolates colony.

The ability to produce pectinase was tested on a medium with pectin (Soriano et al., 2000). The incubation lasted 24 h at 28°C, after which the grown colonies were overflowed with lugol. The presence of unstained zones around the colony was the evidence of pectinase activity.

Cellulase activity was tested on CMC agar (carboxy methyl cellulose agar). A transparent zone around the colony was the confirmation of cellulase activity.

To estimate hydrogen cyanide (HCN) production, we used the method by Frey-Klett et al. (2005).

Suppressive influence of the isolates on the growth of pathogenic fungi *Sclerotinium sclerotiorum* and *Fusarium oxysporum* (both from the collection of the Laboratory of Microbiology, Department of Field and Vegetable Crops, Faculty of Agriculture, Novi Sad) was investigated using the method by Toure et al. (2004). The percentage of growth inhibition (I) was expressed by the formula: $I (\%) = (C - T) / C \times 100$ (C – mycelial length in the control Petri plates; T – mycelial length in the experimental Petri plates).

RESULTS AND DISCUSSION

The bacteria isolated from the rhizosphere of rosemary included 15 isolates of the genus *Pseudomonas*, 20 of the genus *Bacillus* and 11 of the genus *Azotobacter*. According to the morphological description of the colonies and cells, representative isolates of each genus were chosen for physiological and biochemical examination. Isolates belonging to the same genus were selected so that they clearly differed in color and/or structure and edge of the colony, as well as in cell size. The isolates were marked as *Pseudomonas* sp. P42, P43, and P44, for *Bacillus* isolates B83, B84, B85, B92, B93, and for *Azotobacter* isolates A15 and A16.

Temperature is one of the most frequent abiotic stress factors influencing plant growth very strongly and adversely (Wang et al., 2003). PGP bacteria that have a high tolerance to diverse abiotic stresses, such as extreme temperature, salinity and pH, can be used as a good bioresource for improving plant productivity in adverse conditions for plant growth (Prasad et al., 2019). *Bacillus* and *Azotobacter* isolates could grow at 37°C, while all *Pseudomonas* isolates and one *Bacillus* isolate (B85) developed well grown colonies at 10°C (Tab. 1). Only *Bacillus* isolates (except one, B84) could grow at high temperature (45°C).

Table 1. Growth of isolates at different temperature, pH, NaCl levels, and with various source of carbon

Isolates	Temperature (°C)				pH				NaCl (%)			Source of carbon			
	3	10	37	45	4	5	6	9	5	7	L ^b	F	G	S	Ga
P42	- ^a	++	-	-	-	-	++	++	-	-	-	-	+	+	-
P43	-	++	-	-	-	-	++	++	-	-	-	-	+	-	+
P44	-	++	-	-	-	-	++	++	-	-	-	-	+	-	+
B83	-	-	++	+	-	++	++	++	+	++	-	+	+	+	-
B84	-	-	++	-	-	+	++	++	+	+	-	+	+	+	-
B85	-	++	+	+	-	+	++	++	++	+	-	+	+	+	+
B92	-	-	++	++	-	+	++	+	-	+	-	+	+	+	+
B93	-	-	++	++	-	+	++	+	-	-	-	+	+	+	-

A15	-	-	++	-	-	-	+	+	-	-	+	+	+	+	+
A16	-	-	++	-	-	-	+	+	-	-	+	+	+	+	+

Legend: ^a - absence of growth; + minimal growth; ++ optimal growth; +++ intense growth; ^b L- lactose; F- fructose; G- glucose; S- sacharose; Ga- galactose

Soil pH is another limiting factor for plant growth but also for PGPR activities (Stamenov et al., 2011). In the present study, the optimum pH for the vast majority of *Pseudomonas* and *Bacillus* isolates was 6 and 9. *Azotobacter* isolates could also grow in the same range, but in both cases minimal growth was detected for both *Azotobacter* isolates. Only *Bacillus* isolates could grow at pH 5, and it was minimal growth, except isolate B83, where optimal growth was determined. Isolate B83 tolerated a wide range of pH, indicating its ability to survive in the extreme condition of the soil.

Salinity is also a very common abiotic stress factor that negatively affects plant growth and yield (Nakashima & Yamaguchi-Shinozaki, 2006). The results of our study showed that only *Bacillus* isolates could tolerate salinity, which indicates their ability to survive in saline surroundings. *Bacillus* isolates (except isolate B93) could grow on media with 5 and 7% of NaCl.

The isolates differed in terms of use of carbohydrate as a carbon source. The isolates utilized different carbohydrate including lactose, fructose, glucose, sacharose, and galactose. Isolates of genus *Azotobacter* are the only isolates that used all carbohydrates, while *Pseudomonas* isolates used only two.

All isolates were tasted for different hydrolytic enzymes activities (Tab. 2). According to the results of this investigation, 80% of the isolates were positive for lipase production, 60% for amylase, 40% for pectinase and 30% for cellulase production.

Table 2. Enzymatic activity, production of IAA, siderophores, hydrogen cyanide and phosphate utilization

Isolates	Lytic enzyme production ^a				Plant growth promoting traits				
	Lipase	Amyl	Pectinase	Cellulase	IAA ^b	Siderophore ^c	HCN ^d	Phosphorus ^e	
								organic	inorganic
P42	+	+	+	+	+	++	+	++	+
P43	+	-	-	-	+	++	+	++	+
P44	+	-	-	-	-	+	+	++	+
B83	+	+	+	+	-	++	++	-	-
B84	-	-	-	-	+	+	++	+	-
B85	-	-	+	-	+	-	+	++	-
B92	+	+	-	-	-	+++	+	-	-
B93	+	+	+	-	-	+	++	+	+
A15	+	+	-	+	-	-	++	-	+
A16	+	+	-	-	-	-	++	+++	-

Legend: ^a Lipase, amylase (Amyl), pectinase, cellulase activities: + hydrolysis; - no hydrolysis; ^b Production of indole-acetic acid: - not detected; + IAA production; ^c Production of siderophores; width of orange zone : + 0-10 mm; ++ ≥ 10 mm; - no zone; ^d Production of HCN; + HCN production, - not detected; ^e Mineralization of phosphorus organic compounds and phosphate solubilization- evaluated according to zone diameter: + represents 4mm/day (weak activity); ++ represents 5-8mm/day (moderate activity) +++ ≥8mm/day (high activity)

Hydrolytic enzymes have a crucial role in maintaining soil fertility. These enzymes hydrolyze complex compounds, such as polysaccharides, proteins, and urea into simpler forms, improving in this way soil fertility (Pidwirny, 2006).

Besides, lipase and cellulose-producing bacteria can act as biocontrol agents (Kaur et al., 2005). It is well-known that hydrolytic enzymes can cause lyses and death of pathogenic microbes, especially fungi, in the close vicinity of plants (Beneduzi et al., 2012). In this way, microorganisms which have the ability to produce hydrolytic enzymes act as agents for prevention of plant diseases. Microorganisms that produce one or more lytic enzymes are found to have biocontrol ability against a range of plant pathogenic fungi and bacteria and to improve plant growth (Gomes et al., 2001). The results of this study indicate that isolates P42 and B83, having the ability to produce all examined lytic enzymes (Tab. 2), could be further examined as potential biological control agents of plant pathogens.

One of the most physiologically active auxin is indole-3-acetic acid (IAA). IAA has a very important role in stimulation of root elongation and proliferation of root hairs and lateral roots (Davies, 2010). Also, it has a stabilizing effect on plants under untoward environmental conditions (Bianco et al., 2009). Many microorganisms produce IAA which can be used by plants. In this study, it was found that four isolates (P42, P43, B84, and B85), out of the total of ten isolates, were able to produce this phytohormone.

An important trait of some microorganisms is production of siderophores (Shameer & Prasad, 2018). Production of siderophores may directly and indirectly influence plant growth. According to Gupta et al. (2007), siderophore producing microorganisms suppressed many plant pathogens, especially pathogenic fungi. In this study, medium discoloration from blue to orange indicated the ability of the isolates to produce siderophores. All isolates were screened for siderophores production. It was found that three isolates did not produce siderophores (B85, A15, and A16), while the isolate denoted as B92 had the widest orange zone and was found to be a good producer of siderophores.

One of the well-known mechanisms of biocontrol is through volatile compounds (Sarangi et al., 2010). According to Datta et al. (2011), HCN production has a beneficial effect on plants. In this investigation, all isolates had the ability to produce HCN. This means that *Pseudomonas* isolates and *Bacillus* isolates denoted as B83, B84, B92, and B93, having siderophore and HCN production ability, could be efficiently used in control of phytopathogenic fungi as an adequate alternative to chemical agents.

Phosphorus solubilizing bacteria can easily convert phosphorus compounds into forms that can be quickly absorbed by plants (Wen et al., 2019). The isolates were checked for their ability to solubilise phosphates and to do mineralization of phosphorus organic compounds. Based on the width of the transparent zone around the colony, the isolates were classified into three groups: weak, moderate and high activity (Tab. 2). In our study, out of 10 analyzed isolates, 7 isolates had the ability to do mineralization of phosphorus organic compounds, while 5 isolates were able to solubilize phosphate. All *Pseudomonas* isolates exerted the activity of solubilization and mineralization of phosphorus compounds. Isolate A16 had the highest degree of efficacy for mineralization, while isolate B93 had the weakest.

PGP microorganisms can indirectly enhance plant growth by suppressing pathogenic microorganisms, especially fungi that inhibit plant growth. The mechanisms by which they suppress pathogens are mostly through antibiosis, parasitism, and competition for food and territory in the vicinity of plant roots, and/or activation of host defence feedback. *Bacillus subtilis* and fluorescent pseudomonas strains are the most extensively used PGPR because of their ability to produce antibiotics, antifungal metabolites and their capability to release iron-chelating siderophores (Alina et al., 2015; Suresh et al., 2010). Our study examined the effect of the isolate application on growth of two plant pathogens: *Fusarium oxysporum* and *Sclerotinium sclerotiorum*. *F. oxysporum* is a typical soilborne pathogen. This fungus lives in soil in the form of a chlamydospore, and enters the plant root by hyphae, spreads through the root tissue, causing first yellowing and wilting, and then the plant death (Tsutomu, 2019). *S. sclerotiorum* has the potential to cause disease in more than 400 plant species (Bolton et al., 2006). Therefore, due to the economic factors, as many of the plants are affected by *F. oxysporum* and *S. sclerotiorum*, developing effective disease management strategies is very important and urgent. In this paper it was observed that *Pseudomonas* and *Azotobacter* isolates have no negative impact on the growth of phytopathogenic fungi. On the other hand, *Bacillus* isolates showed strong repressive effect on the growth of used fungi (Fig. 1).

Fig.1. Antagonistic activity of *Bacillus* isolates against *F. oxysporum* and *S. sclerotiorum*

In the center of the Petri plates is agar plug of *F. oxysporum* (a, c, e, g, h) and *Sclerotinium* sp. (b, d, f); (j) shows the control variant; on both sides of each Petri-plates is *Bacillus* isolate; the fungi clear zones around *Bacillus* isolate indicate an inhibitory effect of the bacterial isolate on the growth of *F. oxysporum* and *Sclerotinium* sp.

Isolates B83, B84 and B93 had an inhibitory effect on *S. sclerotiorum* and *F. oxysporum*, while isolates denoted as B85 and B92 had such effect only on *F. oxysporum* (Tab. 3).

Table 3. Suppressive influence of the isolates on mycelial growth of the pathogenic fungi

Isolates	<i>F. oxysporum</i>	<i>Sclerotinium</i> sp.
	Growth inhibition rate (%)	
P42	-	-
P43	-	-
P44	-	-
B83	51.1	88.9
B84	55.5	77.8
B85	53.3	-
B92	66.7	-
B93	60.0	73.33
A15	-	-
A16	-	-

Legend: - Inhibitory effect of the bacterial isolate on fungi growth was not detected

The best result in suppressing the growth of pathogenic fungi *F. oxysporum* was achieved by using isolate B92. In this case, the growth of *F. oxysporum* was 66.7% less in the experimental Petri plates compared with the growth of the fungi in the control variant. Application of isolate B83 led to the greatest suppression of the growth of pathogenic fungus *S. sclerotiorum*. Surprisingly, although isolate P42 showed the ability to produce all examined lytic enzymes, the inhibitory effect of this bacterial isolate on *F. oxysporum* and *S. sclerotiorum* growth was not detected. The results obtained here referring to in vitro sensitivity of phytopathogenic fungi to *Bacillus* isolates are in line with the findings published by other authors (Matar et al., 2009; Jayaprakashvel et al., 2019).

CONCLUSION

Identification of bacterial isolates with great PGP and biocontrol properties has great importance as it provides a good starting point for formulating preparations that could adequately replace chemical substances. In this study, the selected rhizospheric isolates of rosemary (*Rosmarinus officinalis* L.) showed different abiotic stress tolerance abilities and had multiple plant growth-promoting (PGP) properties. Isolate *Pseudomonas* sp. P42 and *Bacillus* isolates marked as B83, B84 and B93 showed a physiological profile that allows them to be classified as plant growth-promoting bacteria. The results of this study suggest that rhizospheric isolates of rosemary can be tested for use as microbiological formulations in agriculture, for promoting plant growth and/or biological control of plant diseases.

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