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Plant growth promoting potential of *Bacillus, Azotobacter* and *Streptomyces* bacteria from nettle rhizospheric soil

Dragana Stamenov[1*], Simonida Đurić[1], Timea Hajnal Jafari[1], Dino Hasanagić[2]

[1]*University of Novi Sad, Faculty of Agriculture, University of Novi Sad, Trg Dositeja Obradovića 8, 21000 Novi Sad, Serbia*
[2]*Faculty of Natural Sciences and Mathematics, University of Banja Luka, Mladena Stojanovića 2, 78000 Banja Luka, Republic of Srpska*

*Corresponding author: dragana.stamenov@polj.uns.ac.rs

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A B S T R A C T

The significance of employing Plant Growth Promoting (PGP) microorganisms holds immense value in the cultivation of medicinal plants, where the attainment of high-quality plant biomass is indispensable. Therefore, it becomes imperative to isolate and identify a diverse array of microorganisms from the rhizosphere of various plants and assess their efficacy in enhancing the growth of medicinal plants. The primary aim of this research was to isolate and characterize bacteria belonging to the *Bacillus, Streptomyces*, and *Azotobacter* genera from the rhizosphere of nettle (*Urtica dioica* L.). Additionally, the study explored the influence of the bacterial isolates on the germination of oregano (*Origanum vulgare* L.) and marjoram (*Origanum majorana* L.) seeds. Isolation, physiological characterization (the growth of isolates at different temperatures, levels of acidity and concentrations of NaCl, and resistance of isolates to Cd and Pb), biochemical characterization (the production of lipase, amylase, pectinase, and cellulase), and PGP characterization of isolates were performed. The impact of isolates on seed germination was monitored under controlled conditions. The count of sprouted seeds was assessed at 7- and 10-day intervals. The results of this study reveal that the isolated rhizospheric bacteria of nettle have multiple physiological, biochemical and PGP properties. All isolates showed good PGP potential, but the isolates *Azotobacter* A1 and *Streptomyces* Ac1 stood out. The applied isolates had a positive effect on the seed germination of oregano and marjoram, the best effect being exhibited by *Bacillus* B2 and *Azotobacter* A1 on the seed germination of oregano and by *Bacillus* B1 and *Streptomyces* Ac1 on that of marjoram.

Keywords: medicinal herbs, PGP bacteria, inoculation, germination

И З В О Д

Значај употребе микроорганизама који подстичу раст биљака (ПГП) има огромну вредност у узгоју лековитог биља, где је неопходно постизање висококвалитетне биљне биомасе. Због тога постаје императив да се изолују и идентификују разноврсни микроорганизми из ризосфере различитих биљака и процени њихова ефикасност у побољшању раста лековитих биљака. Примарни циљ овог истраживања био је да се из ризосфере коприве (*Urtica dioica* L.) изолују и карактеришу бактерије из родова *Bacillus, Streptomyces* и *Azotobacter*. Поред тога, студија је истраживала утицај бактеријских изолата на клијање семена оригана (*Origanum vulgare* L.) и мајорана (*Origanum majorana* L.). Спроведена је изолација, физиолошка карактеризација (раст изолата на различитим температурама, нивоима киселости и концентрацијама NaCl, отпорност изолата на Cd и Pb), биохемијска карактеризација (производња липазе, амилазе, пектиназе, целулазе) и карактеризација ПГП својства изолата. Праћење ефеката примене изолата на клијавост семена оригана и мајорана је урађено у контролисаним условима. Број проклијалог семена мерен је након 7 и 10 дана. Резултати овог истраживања откривају да изоловане бактерије из ризосфере коприве имају вишеструка физиолошка, биохемијска и ПГП својства. У овом истраживању, сви изолати су показали добар ПГП потенцијал, а изолати који се издвајају су изолати *Azotobacter* A1 и *Streptomyces* Ac1. Примењени изолати су позитивно утицали на клијавост семена оригана и мајорана. Најбољи ефекат на клијавост семена оригана имали су изолати *Bacillus* B2 и *Azotobacter* A1. На клијавост семена мајорана најбољи ефекат имала је примена изолата *Bacillus* B1 и *Streptomyces* Ac1.

Кључне речи: лековито биље, ПГП бактерије, инокулација, клијавост

1. Introduction

The rhizosphere is a highly dynamic system in which soil microorganisms and plant roots form a unity. The diversity of microorganisms in the rhizosphere is directly influenced by the plant species, root characteristics and exudates, plant age, physicochemical properties of the soil, and anthropogenic influences (Gusain and Bhandari, 2019).

The interaction between rhizospheric microorganisms and plants can be beneficial, neutral, or harmful. Rhizospheric microorganisms that have a positive impact on plant growth and development are called Plant Growth Promoting Rhizobacteria (PGPR). It has been proven that these microorganisms enhance

plant nutrient and hormone supply, participate in plant defense against phytopathogens, and enhance plant resistance to adverse abiotic factors and stress. Therefore, they have a significant impact on the quantity and quality of agricultural crop yields (Etesami and Beattie, 2017; Miskoska Milevska et al., 2020; Avdović et al., 2023).

To date, it has been determined that bacteria of various genera belong to PGPR. The most significant bacterial genera are: *Aeromonas, Agrobacterium, Arthrobacter, Azospirillum, Azotobacter, Bacillus, Beijerinckia, Bradyrhizobium, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Frankia, Klebsiella, Mesorhizobium, Mycobacterium, Phyllobacterium, Proteus, Pseudomonas, Rhizobium, Serratia, Staphylococcus, Streptomyces, Xanthomonas* (Berg, 2009). The most important fungi include *Acaulospor, Alternaria, Arbuscular mycorrhizal fungi, Aspergillus, Fusarium, Gigaspora, Glomus, Penicillium, Podospora, Scutellospora, Trichoderma, Variovovax* (Berg, 2009).

In medicinal plant production, the use of PGPR has become crucial because agricultural chemicals, including various pesticides (herbicides, insecticides, and fungicides) and fertilizers, are no longer acceptable (Malik et al., 2011). For this reason, scientists have become increasingly interested in studying the diversity of microorganisms isolated from the rhizosphere of medicinal plants and their potential use in the production of medicinal plants. Worldwide research results indicate that the rhizosphere of medicinal plants is highly specific (Bafana and Lohiya, 2013). Due to the production of various secondary metabolites, the rhizosphere of medicinal plants harbors diverse bacteria with multiple PGP properties that benefit plant growth (Zhang et al., 2013). Zhao et al. (2013) investigated the microbial diversity of the rhizospheric soil of many medicinal plants and found a total of 50 strains classified into 7 genera: *Myxococcus, Corallococcus, Cystobacter, Archangium, Stigmatella, Chondromyces, Pyxidicoccus,* with dominant genera being *Myxococcus* and *Corallococcus*. The same author analyzed the diversity of actinomycetes in the rhizosphere of seven medicinal plant species and found 18 dominant genera (Zhao et al., 2012).

Bacteria isolated from the rhizosphere of medicinal plants have a high capacity to be used as growth-promoting agents and biocontrol agents for many plant species. Many studies have demonstrated that rhizospheric bacteria of medicinal plants exhibit multiple PGP traits (Meena et al., 2023). Up to now, it has been established that microorganisms can be very successfully used in the production of medicinal plants because they enhance plant resistance to drought, high and low temperatures, and soil salinity levels (Singh et al., 2011; Jahanian et al., 2012; Ghorbanpour et al., 2013).

Bearing the above in mind, the aim of this research was the isolation and characterization of *Bacillus, Streptomyces*, and *Azotobacter* bacteria from the rhizosphere of nettle (*Urtica dioica* L.) and the investigation of their impact on the germination of oregano(*Origanum vulgare* L.) and marjoram (*Origanum majorana* L.) seeds.

2. Materials and methods

Under laboratory conditions, microorganisms from the genera *Bacillus, Streptomyces*, and *Azotobacter* were isolated from the rhizosphere of nettle (*Urtica dioica* L.).

Rhizosphere soil was collected during the summer, in August 2022, when nettle plants were in full flowering stage. The rhizosphere soil was sampled by picking 5 plants and separating the rhizospheric soil and roots from the rest of the plant in sterile plastic bags. Afterwards, the samples were transferred in a hand freezer to a regular freezer until further analyses.

Bacterial isolation was performed using agar medium for *Bacillus*, synthetic agar for *Streptomyces*, and a selective medium without nitrogen (F-medium) for *Azotobacter* species.

2.1. Physiological characterization of isolates

The growth of isolates at different temperatures (5°C, 28°C, 41°C), on media with different acidity (pH 5, 7, 9), and on media with different concentrations of NaCl (3%, 5%, 7%) was observed on appropriate nutrient media: *Bacillus* on nutrient agar, *Azotobacter* on F-medium, and *Streptomyces* on synthetic agar. After 48 hours of incubation, qualitative growth of isolates was observed and compared with the control. Complete absence of growth was marked with -, minimal growth with +, optimal with ++, and abundant growth with +++.

The diffusion method was used to test the resistance of microorganisms to solutions of the two heavy metals: cadmium (CdCl2) and lead (PbCl2), at different concentrations: 10^{-2} and 10^{-4} (mol/dm^3) (Kuffner et al., 2008). The size of the inhibition zone depends on the sensitivity of bacteria to heavy metals: (-) no inhibition zone, (+) inhibition zone 1–10 mm, (++) inhibition zone larger than 10 mm, and (*) growth stimulation.

2.2. Physiological characterization of isolates

The production of lipase was examined on the medium (peptone 10 g L^{-1}, NaCl 5 g L^{-1}, $CaCl_2 \cdot H_2O$ 0.1 g L^{-1}, agar 15 g L^{-1}) with added Tween 80 (Lanui, 1987). The incubation period was seven days at 26°C. Cloudy zones around the colony indicated lipolytic activity.

The ability of microorganisms to hydrolyze starch was determined using the agar plate method on starch agar (starch powder 10 g L^{-1}, 10 g L^{-1}, KH_2PO_4 0.5 g L^{-1}, K_2HPO_4 0.5 g L^{-1}, $MgSO_4$ x $7H_20$ 0.2 g L^{-1}, agar 15 g). Incubation was carried out at 28°C for 48 hours. Colonies were then flooded with iodine solution. An uncolored zone (hydrolysis zone) around the colony indicated starch hydrolysis by the microorganism.

The ability to produce pectinase was investigated by the agar plate method on pectin agar (K_2HPO_4 2 g L^{-1}, NaH_2PO_4 1 g L^{-1}, $MgSO_4$ x $7H_2O$ 0.5 g L^{-1}, NH_4NO_3 2 g L^{-1}, yeast extract 1g L^{-1}, pectin 10 g L^{-1}, agar 16 g L^{-1}). Incubation lasted 24 hours at 37°C, after which colonies were flooded with iodine solution. The appearance of uncolored zones around the colony indicated pectinase activity (Soares et al., 2001).

Cellulase production was examined on CMC agar (carboxymethyl-cellulose agar). The incubation period was seven days at 28°C. After incubation, Petri dishes were flooded with a Congo red solution (mgcm^{-3}

water). After fifteen minutes, Congo red was poured off, and Petri dishes were flooded with a 1M NaCl solution. Decolorized zones around colonies indicated the cellulase activity of microorganisms.

2.3. PGP characterization of the isolates

The production of IAA was tested using a medium rich in the amino acid tryptophan (tryptophan broth). The ability to produce the enzyme tryptophanase was demonstrated by the formation of a red ring after the addition of Kovac's reagent.

The ability to produce siderophores was determined using chrome azurol S agar (CAS) (Slimani et al., 2023).

The synthesis of hydrogen cyanide (HCN) was examined on a medium containing soy tripton 30 g L^{-1}, glycine 4.4 g L^{-1}, and agar 15 g L^{-1} (Frey-Klett et al., 2005). After inoculation, a Whatman disk previously soaked in a solution of 0.5% picric acid and 2% Na_2CO_3 was placed on the medium. The incubation period was 4 days at 28°C. The color change of the disk from yellow to orange-brown proved the ability of microorganisms to produce HCN.

The capacity to break down organic phosphorus compounds was assessed using Menkin's adapted Rodin's agar medium. The isolates were subjected to scrutiny for their proficiency in mobilizing inorganic phosphates on Pikovskaya's agar medium. Following a 5-day incubation period at 28°C, the emergence of clear zones surrounding the colonies served as an indicator of the microorganisms' capability to dissolve phosphates.

2.4. Examination of the effect of isolates on seed germination

The impact of isolates on seed germination was studied using oregano (*Origanum vulgare* L.) and marjoram (*Origanum majorana* L.) seeds. The experiment was conducted under laboratory conditions. Twenty seeds inoculated with the appropriate bacterial inoculate (the seeds were submerged for 30 minutes in bacterial inocula, titer 10^9 CFU ml^{-1}) were placed on filter paper and put in a thermostat at 22 °C for germination. The seeds in the control were submerged for 30 minutes in an appropriate sterile nutrient broth medium. The germination rate was recorded after 7 and 10 days.

2.5. Statistical analysis

Statistical analysis was performed using Statistics 13.3 software (TIBCO Software Inc.), and the significance of differences between applied treatments was determined using Fisher's LSD test.

3. Results and discussions

In this study, five bacteria were isolated from the rhizosphere of nettle (*Urtica dioica* L.): three belonging to the genus *Bacillus* (Bac1, Bac2, Bac3), one to the genus *Azotobacter* (A1), and one to the actinomycete genus *Streptomyces* (Ac1). Colonies were isolated based on morphological characteristics and subcultured several times on appropriate media to obtain pure cultures.

The results of the examination of physiological characteristics of isolates are presented in Table 1. It was found that all isolates of the genus *Bacillus* exhibited optimal growth at a temperature of 28°C, while at 41°C, either complete absence of growth or minimal growth (in the case of Bac3 isolate) was observed. At 5°C, minimal growth was observed only in the case of the Bac2 isolate, while Bac1 and Bac3 did not grow. Optimal colony growth for *Azotobacter* isolates was observed at 28°C, while minimal growth was observed at 5°C and 41°C. For *Streptomyces* isolates, complete growth inhibition was observed at 5°C, while minimal growth was determined at 28°C and 41°C.

On media with pH values of 5, 7, and 9, optimal growth was observed for all isolates of the genus *Bacillus*. In this study, it was noted that on media with pH values of 7 and 9, optimal colony growth of *Azotobacter* isolates occurred, while minimal growth was observed at pH 5. For *Streptomyces* isolates, minimal colony growth was observed on media with pH 5, while optimal growth was observed at pH 7.

On media with the addition of 3% NaCl, all isolates exhibited optimal or minimal growth, while on the same medium with 5% and 7% NaCl, there was an absence of isolates growth, except for Bac3, where minimal growth was observed.

Cadmium did not affect the growth of *Bacillus, Streptomyces* and *Azotobacter* isolates. In contrast, lead solutions caused growth inhibition for *Bacillus* and Ac1 isolates, which was indicated by the appearance of inhibition zones up to 10 mm. For A1, the application of lead in these concentrations led to the stimulation of isolate growth.

Table 1.
Physiological characteristics of the isolates

Isolates	Temperature[a] (°C)			pH[a]			NaCl[a] (%)			Heavy metals[b]			
	5	28	41	5	7	9	3	5	7	Cd		Pb	
										10^{-2}	10^{-4}	10^{-2}	10^{-4}
B1	–	++	–	++	++	++	++	–	–	–	–	+	+
B2	+	++	–	++	++	++	++	–	–	–	–	+	+
B3	–	++	+	++	++	++	++	+	+	–	–	+	+
A1	+	++	+	+	++	++	+	–	–	–	–	*	*
Ac1	–	+	+	+	++	–	+	–	–	–	–	+	+

[a] complete absence of growth -; minimal growth +; optimal ++; abundant growth +++
[b] without zone of inhibition -; + 1–10 mm zone; ++ zone greater than 10 mm; * growth stimulation

Similarly, Joo et al. (2007) determined that *Bacillus* isolates exhibited optimal growth on media with pH 6–9 at a temperature of 28°C. In the study by Karagoz et al. (2012), isolates of this genus had optimal growth on media with NaCl concentrations of 5% and 7%. Several authors have mentioned that the prevalence of the *Azotobacter* genus in acidic soil is weak, and its presence is often difficult to ascertain (Milicic, 2009). The more favorable conditions for the intensive development of *Azotobacter* include a neutral environment, sufficient moisture, organic matter, and an adequate amount of physiologically active substances, especially phosphorus (Aquilanti et al., 2004). Our study results align with Abbas's findings (2006), who identified the optimal growth temperature for actinomycetes as 25–28°C. In the work of Moncheva et al. (2002), optimal growth of actinomycetes was observed on media containing lower percentages of NaCl (1.5%, 3%, and 5%), while no growth of actinomycetes was found on media with 7%, 15%, and 20% NaCl.

The results of the examination of biochemical characteristics of isolates are presented in Table 2. It was found that all isolates of the genus *Bacillus* have the ability to produce lipase, while amylase production was observed only in the case of the Bac2 isolate. Pectinolytic activity was not proven for any isolates of this genus.

Table 2.
Enzymatic activity of the isolates

Isolates	Lypase[a]	Amylase	Pectinase	Cellulase
B1	+	–	–	+
B2	+	+	–	+
B3	+	–	–	+
A1	+	+	+	+
Ac1	+	+	+	+
Percentage of positive isolates	100	60	40	100

[a] (+) positive reaction / produces; (-) negative reaction / does not produce

The isolate of the *Azotobacter* genus demonstrated the ability to produce extracellular lipases, as well as the capability for starch hydrolysis. Additionally, pectinolytic and cellulolytic activities were identified. For *Streptomyces* isolates, the ability to produce lipases, starch hydrolysis, as well as enzymes such as pectinase and cellulase were observed.

Hydrolytic enzymes are pivotal in sustaining soil fertility as they contribute to the degradation of complex compounds like polysaccharides, proteins, and urea. This breakdown process transforms these complex substances into simpler forms, ultimately enhancing the overall fertility of the soil (Pidwirny, 2006). Moreover, hydrolytic enzymes are known to cause paralysis and death in pathogenic microorganisms, especially fungi (Beneduzi et al., 2012). Thus, microorganisms capable of producing hydrolytic enzymes, one or more, may have applications in combating various plant pathogenic fungi and bacteria, as well as enhancing plant growth (Gomes et al., 2001). In this study, isolates A1 and Ac1 produced the highest number of examined enzymes, indicating that these isolates could be further investigated as potential bioagents for controlling phytopathogens.

One of the most important PGP characteristics is the ability to produce IAA (indole-3-acetic acid), a hormone from the auxin group that controls numerous physiological processes in plants, including cell elongation, tissue differentiation, and responses to light, gravity, and environmental stress conditions (Gupta et al., 2015). In this research, IAA production was observed only in the case of the Bac3 isolate, while other isolates did not exhibit the production of this hormone (Table 3).

Table 3.
Plant growth promoting properties of the isolates

Isolates	IAA[a]	Siderophores	HCN	Mineralization of phosphorus	Solubilization of phosphates
B1	–	–	+	+	–
B2	–	+	+	–	–
B3	+	–	+	+	–
A1	–	+	+	+	–
Ac1	–	–	+	–	+
Percentage of positive isolates	20	40	100	60	20

[a] (+) positive reaction / produces/ performs decomposition; (-) negative reaction / does not produce/ does not perform decomposition

One of the ways in which plants and bacteria acquire iron is through the production of low molecular weight molecules with a high affinity for Fe+3, known as siderophores (Souza et al., 2015). Among the tested isolates, siderophore production was observed only in the case of isolates B2 and A1. In contrast to these results, in the study by Minut et al. (2022), where the ability to produce siderophores in bacteria of the *Bacillus* and *Azotobacter* genera was also examined, all *Bacillus* isolates produced siderophores, while *Azotobacter* isolates did not.

HCN production, as a secondary metabolite that inhibits ATP synthesis and leads to the death of pathogenic microorganisms, was observed in all isolates examined in this study. According to Datta et al. (2011), the production of HCN by microorganisms has a favorable impact on plants.

Phosphorus is an essential nutrient for plants, a component of nucleic acids, phospholipids, and ATP, and a key element in many metabolic and biochemical pathways, such as biological nitrogen fixation and photosynthesis (Khan et al., 2007). The ability to mineralize organic phosphorus compounds and thus supply plants with this essential nutrient was determined in 60% of the examined isolates, specifically in B1, B3, and A1, while the rest of the isolates did not exhibit this capability. The ability of isolates to solubilize inorganic phosphates was observed only in one isolate, Ac1. Similar results were obtained by Azzawi and Kamal (2022), who also found the ability to mineralize and solubilize phosphorus compounds in isolates of *Azotobacter* and bacteria of the *Bacillus* genus.

Seven days after oregano seed inoculation, a statistically significant increase in germination was observed in all variants compared to the control (Table 4).

Table 4.
Effects of the isolates on seed germination of oregano and marjoram

Isolates	7 days				10 days			
	Oregano		Marjoram		Oregano		Marjoram	
	No.[a]	%	No.	%	No.	%	No.	%
B1	6,7d	33,5	3,6b	18	12cd	60	6,3c	31,5
B2	10,3b	51,5	1,6c	8	14b	70	5cd	25
B3	8,6c	43	1,6c	8	13bcd	65	4,3d	21,5
A1	7,7cd	38,5	1,6c	8	13,7bc	68,5	4,6cd	23
Ac1	10,6b	53	6a	30	11,7d	58,5	9,3a	46,5
Control	1,6a	8	1c	5	7,7a	38,5	2b	10

[a] Number of germinated seeds
Values in the same row followed by different letters indicate significant differences ($p < 0.05$) between the means.

The best result was achieved using isolates B2 (51.5%) and Ac1 (53%). The germination rate of the control was only 8%. The application of the tested isolates to marjoram seeds resulted in a statistically significant increase in germination only in the case of isolates B1 and Ac1. In other variants, there was also an increase in germination, but this increase was not statistically significant.

Ten days after the seed inoculation of the tested medicinal plants, the number of germinated seeds significantly increased for all tested plants compared to the control. Oregano germination was most abundant with the application of isolates Bac2, Bac3, and A1. The highest germination percentage in marjoram was caused by inoculation with Act (46.5%).

The positive impact of bacterial inoculation on the germination and vitality index of medicinal plants was also observed by Lenin and Jayanthi (2012). Khaosaad et al. (2006) obtained similar results by inoculating oregano seeds with PGP bacteria, resulting in a positive effect on germination, shoot length, and plant biomass. Furthermore, our findings align with the research conducted by Shaukat et al. (2006). They observed a beneficial impact on both germination and seedling length through the introduction of Azotobacter into the rhizosphere of sunflower and wheat. Also, the positive effect of the application of microorganisms, especially those of the genera *Bacillus* and *Azotobacter*, on the germination and initial growth of different plants was confirmed by Arpanahi et al. (2019) and Mohammadi et al. (2018).

4. Conclusions

The results of this study reveal that the isolated rhizospheric bacteria of *Urtica dioica* L. have multiple physiological, biochemical and PGP properties.

All isolates showed good PGP potential, but the isolates A1 and Ac1 stood out.

The applied isolates had a positive effect on the seed germination of oregano and marjoram. The best effect was exhibited by *Bacillus* (B2) and *Azotobacter* (A1) isolates on the seed germination of oregano and by *Bacillus* (B1) and *Streptomyces* (Ac1) isolates on that of marjoram.

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Declaration of competing interests

The authors affirm that they do not possess any identifiable conflicting financial interests or personal connections that might have seemed to exert an impact on the research presented in this manuscript.

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