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# Impact of PGPR Seed Priming on Germination, Yield, and Metabolite Profile of Basil (*Ocimum basilicum* L.) and Sage (*Salvia officinalis* L.)

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## Abstract

This study evaluated the potential of plant growth-promoting rhizobacteria (PGPR) from the genera *Bacillus* and *Actinomycetes* to enhance growth and secondary metabolite production in basil (*Ocimum basilicum* L.) and sage (*Salvia officinalis* L.). Six isolates obtained from the rhizosphere of *Urtica dioica*, including three *Bacillus* spp. (Bac1-Bac3) and three actinomycetes (Act1-Act3), were morphologically and biochemically characterized and screened for physiological tolerance and plant growth-promoting (PGP) traits, identifying two isolates with strong PGP potential. The selected strains were further evaluated in *in vitro* seed inoculation assays and pot experiments under controlled conditions. Plant yield parameters were assessed alongside biochemical analyses of plant material including total phenolics, rosmarinic acid content, phenylalanine ammonia-lyase activity, and antioxidant capacity. Inoculation with selected strains significantly improved early seedling performance, including germination (Act1 up to 117% in sage), root elongation (Act1 up to 200%), and shoot elongation (Bac3 up to 175%). After six weeks, Act1 maximized sage fresh biomass (77.6%), while Bac3 enhanced basil dry matter (60% increase). Beyond growth promotion, PGPR treatments significantly increased phenolic content, rosmarinic acid concentration, phenylalanine ammonia-lyase activity, and antioxidant capacity (lower $IC_{50}$ values), indicating activation of the phenylpropanoid pathway. Molecular identification (16S rRNA sequencing) revealed Bac3 as *Bacillus pretiosus* (97.95% identity) and Act1 as *Streptomyces ardesiacus* (97.73% identity).

**Keywords:** *Streptomyces, Bacillus,* siderophore, rosmarinic acid, antioxidant

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## Impacto del *priming* de semillas con PGPR sobre la germinación, el rendimiento y el perfil de metabolitos de la albahaca (*Ocimum basilicum* L.) y la salvia (*Salvia officinalis* L.)

### Resumen

Este estudio evaluó el potencial de rizobacterias promotoras del crecimiento vegetal (PGPR) de los géneros *Bacillus* y *Actinomycetes* para mejorar el crecimiento y la producción de metabolitos secundarios en albahaca (*Ocimum basilicum* L.) y salvia (*Salvia officinalis* L.). Seis aislados obtenidos de la rizosfera de *Urtica dioica*, incluidos tres *Bacillus* spp. (Bac1-Bac3) y tres actinomicetos (Act1-Act3), fueron caracterizados morfológica y bioquímicamente y evaluados por su tolerancia fisiológica y sus características promotoras del crecimiento vegetal. Dos aislados mostraron un fuerte potencial PGP y fueron seleccionados para ensayos de inoculación de semillas *in vitro* y experimentos en maceta bajo condiciones controladas. La inoculación mejoró significativamente el desarrollo temprano de las plántulas, aumentando la germinación (Act1 hasta 117 % en salvia), la elongación de las raíces (Act1 hasta 200 %) y la elongación de los brotes (Bac3 hasta 175 %). Después de seis semanas, Act1 incrementó la biomasa fresca de la salvia en 77,6 %, mientras que Bac3 aumentó la materia seca de la albahaca en 60 %. Los tratamientos también incrementaron significativamente el contenido de fenoles, la concentración de ácido rosmarínico, la actividad de la fenilalanina amonio-liasa y la capacidad antioxidante, indicando una activación de la vía de los fenilpropanoides. La identificación molecular mediante secuenciación del ARNr 16S identificó Bac3 como *Bacillus pretiosus* (97,95 % de identidad) y Act1 como *Streptomyces ardesiacus* (97,73 % de identidad).

**Palabras clave:** *Streptomyces*, *Bacillus*, sideróforo, ácido rosmarínico, antioxidante

## Impacto do priming de sementes com PGPR na germinação, no rendimento e no perfil de metabólitos do manjericão (*Ocimum basilicum* L.) e da sálvia (*Salvia officinalis* L.)

### Resumo

Este estudo avaliou o potencial de rizobactérias promotoras do crescimento vegetal (PGPR) dos géneros *Bacillus* e *Actinomycetes* para melhorar o crescimento e a produção de metabolitos secundários em manjericão (*Ocimum basilicum* L.) e sálvia (*Salvia officinalis* L.). Seis isolados obtidos da rizosfera de *Urtica dioica*, incluindo três *Bacillus* spp. (Bac1–Bac3) e três actinomicetos (Act1–Act3), foram caracterizados morfológica e bioquimicamente e avaliados quanto à tolerância fisiológica e às características promotoras do crescimento vegetal. Dois isolados apresentaram forte potencial PGP e foram selecionados para ensaios de inoculação de sementes in vitro e experimentos em vasos sob condições controladas. A inoculação melhorou significativamente o desenvolvimento inicial das plântulas, aumentando a germinação (Act1 até 117% na sálvia), o alongamento das raízes (Act1 até 200%) e o alongamento dos rebentos (Bac3 até 175%). Após seis semanas, Act1 aumentou a biomassa fresca da sálvia em 77,6%, enquanto Bac3 aumentou a matéria seca do manjericão em 60%. Os tratamentos também aumentaram significativamente o teor de fenóis, a concentração de ácido rosmarínico, a atividade da fenilalanina amónia-liase e a capacidade antioxidante, indicando a ativação da via dos fenilpropanoides. A identificação molecular por sequenciação do ARNr 16S identificou Bac3 como *Bacillus pretiosus* (97,95% de identidade) e Act1 como *Streptomyces ardesiacus* (97,73% de identidade).

**Palavras-chave:** *Streptomyces*, *Bacillus*, sideróforo, ácido rosmarínico, antioxidante

## 1. Introduction

Since ancient times, plants have been utilized in medicine and continue to be used today. Most medicinal plant material is collected from wild plants; however, in recent years, due to the growing demand, a significant number of medicinal plant species have been cultivated worldwide to obtain valuable substances for use in medicine and pharmacy (Kinghorn & Seo, 1996).

Considering that medicinal plants are used for therapeutic purposes, the cultivation method of these plants should ensure the production of high-quality and healthy plant material, free from any polluting substances. According to the World Health Organization, good agricultural practice (GAP) guidelines outline key factors to consider in medicinal plant cultivation, including site selection, climate, soil characteristics, irrigation and drainage, plant maintenance and protection, environmental conditions, and social impact (World Health Organization, 2003). These guidelines also emphasize minimizing the use of agricultural chemicals and fertilizers, recommending their application only when no effective alternatives are available. Malik et al. (2011) considered the use of agricultural chemicals, including various pesticides (herbicides, insecticides, and fungicides) and fertilizers, in the production of medicinal plants to be no longer acceptable.

The introduction of live plant growth-promoting rhizobacteria (PGPR) into the soil to achieve a higher and healthier plant yield aligns with the GAP (Mohanty et al., 2021). PGPR, a diverse group of microorganisms residing in the rhizosphere and in association with roots, is recognized for promoting plant growth and/or controlling plant diseases. Global research indicates the successful utilization of PGPR in the cultivation of medicinal plants, demonstrating enhanced resistance to environmental stressors such as drought, extreme temperatures, and varying soil salt levels (Kumar et al., 2022). Furthermore, the application of microorganisms has been shown to positively influence the production of biologically active substances in medicinal plants (Rizvi et al., 2022).

The mechanisms by which PGPR enhance plant growth are not yet fully understood; however, their beneficial effects are widely attributed to the combined action of direct and indirect mechanisms (Khoso et al., 2024). Direct mechanisms involve phytohormone production, including indole-3-acetic acid, gibberellins, and cytokinins, as well as modulation of ethylene levels, thereby regulating plant cell division, elongation, and overall development (Egamberdiyeva, 2007). PGPR also improve plant nutrient acquisition through asymbiotic nitrogen fixation (Salantur et al., 2006), siderophore-mediated iron mobilization and pathogen suppression (Shao et al., 2024), and solubilization of inorganic phosphates and other mineral nutrients, increasing their bioavailability to plants (Timofeeva et al., 2022).

Indirect mechanisms are primarily associated with biocontrol and induction of plant defense responses. These include the production of antimicrobial compounds (e.g., antibiotics and antifungal metabolites) and extracellular lytic enzymes that degrade fungal cell walls (Ahmad et al., 2008). In addition, PGPR suppress phytopathogens through competitive exclusion in the rhizosphere, iron sequestration via high-affinity siderophores, and induction of systemic resistance, thereby enhancing plant tolerance to biotic stress (Sarkar et al., 2025).

Among medicinal plants, basil (*Ocimum basilicum* L.) and sage (*Salvia officinalis* L.) have a significant place. Both aforementioned plants, belonging to the Lamiaceae family, are cultivated in the Mediterranean region and possess significant economic value, having been utilized since ancient times as spices, teas, or traditional medicines. These species are rich in essential oils and phenolic compounds, particularly rosmarinic acid, which contribute to their antioxidant, antimicrobial, anticancer, anti-inflammatory, antidiabetic, antipyretic, and antiarthritic activities (Sharma et al., 2019; Zhan et al., 2020). Phenolic compounds are also widely studied for their role in plant defense mechanisms against biotic and abiotic stresses, including pathogens, drought, salinity, and UV radiation (Jovičić et al., 2017; Kumar et al., 2020; Popović et al., 2017). Under stress conditions, phenolic compounds act as potent antioxidants, protecting plant cells from the harmful effects of accumulated reactive oxygen species (ROS), which can cause oxidative damage to proteins, lipids, and nucleic acids. Their biosynthesis is primarily regulated by the phenylpropanoid pathway, where phenylalanine ammonia lyase (PAL) catalyzes the initial and key step –the deamination of L-phenylalanine to trans-cinnamic acid, a precursor for various phenolic metabolites. Popović et al. (2017) reported that in response to environmental stress, the rapid upregulation of genes in this pathway leads to increased activity of enzymes and accumulation of phenolic compounds.

Considering the significance of food safety and the dual roles of basil and sage as fresh spices and essential ingredients for various medicines and cosmetics, cultivating these herbs requires innovative technologies. Based on the available evidence, this study is grounded on the hypothesis that the application of selected plant growth-promoting rhizobacteria (PGPR) belonging to the genera *Bacillus* and *Actinomycetes* may positively influence plant growth and development, as well as the accumulation of bioactive metabolites in basil (*Ocimum basilicum* L.) and sage (*Salvia officinalis* L.). Accordingly, the aim of this study was to isolate PGPR strains from genera *Bacillus* and *Actinomycetes* and evaluate their effects on germination, growth, and metabolite profiles in basil and sage.

## 2. Materials and Methods

Microorganisms were isolated from the rhizosphere of nettle (*Urtica dioica* L.) growing on the slopes of Mount Manjača, Bosnia and Herzegovina (44º 34' 48" N, 17º 0' 23" E, 760 m.a.s.l.). Serial dilutions were plated on Starch Casein Agar (SCA; HiMedia) for *Actinomycetes* isolates, and Nutrient Agar (NA; HiMedia) for *Bacillus* spp. Morphological characterization was conducted using light microscopy (Motic BA210) and identification followed standard morphological and biochemical methods per *Bergey's Manual of Determinative Bacteriology* (Holt et al., 1994).

### 2.1 Physiological Characterization

The selected isolates were incubated on NA and SCA media under different temperatures (5 °C and 41 °C), pH (4.5 and 9), and salinity (3%, 5%, 7%) for 48 or 96 h, depending on the isolate. Colony diameters were compared with the control (28 °C, pH 7, 0.9% NaCl), and growth was categorized as: – no growth; + minimal; ++ optimal; +++ intense (Benson, 2002).

### 2.2 PGP Characterization

The isolates were screened for various plant growth-promoting (PGP) traits. Indole-3-acetic acid (IAA) and siderophore production were determined according to Etesami et al. (2015) and Patel et al. (2018), respectively. Hydrogen cyanide (HCN) production was evaluated on glycine-supplemented tryptic soy agar (tryptic soy broth, 30.0 g/L; glycine, 4.4 g/L; and agar, 15.0 g/L). A color change of picric acid-impregnated filter paper from yellow to brown indicated HCN formation.

The ability of isolates to solubilize sparingly soluble $Ca_3(PO_4)_2$ was tested on tricalcium phosphate medium containing (g/L): glucose, 10.0; $Ca_3(PO_4)_2$, 5.0; $(NH_4)_2SO_4$, 0.5; NaCl, 0.2; $MgSO_4 \cdot 7H_2O$, 0.1; KCl, 0.2; $MnSO_4 \cdot H_2O$, 0.002; $FeSO_4 \cdot 7H_2O$, 0.002; yeast extract, 0.5; and agar, 18.0. Phosphate solubilization was confirmed by the formation of a clear zone around colonies (Tao et al., 2008).

Mineralization of organic phosphorus compounds was evaluated on a medium composed of (g/L): peptone, 10.0; NaCl, 5.0; beef extract, 10.0; one fresh egg yolk; and agar, 18.0. The formation of a transparent halo around colonies indicated phosphate mineralization.

Lipase activity was determined on a medium containing (g/L): peptone, 10.0; NaCl, 5.0; $CaCl_2 \cdot H_2O$, 0.1; and agar, 15.0, supplemented with Tween 80. The appearance of a turbid zone around the colonies indicated lipolytic activity.

Amylase activity was assessed on starch agar (g/L): starch, 10.0; $KH_2PO_4$, 0.5; $K_2HPO_4$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; and agar, 15.0. Pectinase activity was tested on pectin agar (g $L^{-1}$): $NaNO_3$, 1.0; KCl, 1.0; $K_2HPO_4$, 1.0; $MgSO_4$, 0.5; yeast extract, 0.5; pectin, 10.0; and agar, 20.0. After incubation at 28 °C for 48-96 h (depending

on the isolate), plates were flooded with Lugol's solution, and the appearance of a clear zone around colonies indicated enzyme activity (Nimisha et al., 2019).

Protease activity ($H_2S$ production) was determined on deep-peptone-iron agar containing (g/L): peptone, 15.0; proteose peptone, 5.0; ammonium ferric citrate, 0.5; sodium glycerophosphate, 1.0; sodium thiosulfate, 0.08; and agar, 15.0. A color change from orange to black indicated positive activity.

Cellulase production was evaluated using carboxymethyl cellulose (CMC) agar containing (g/L): CMC, 10.0; $MgSO_4 \cdot 7H_2O$, 0.2; $KNO_3$, 0.75; $K_2HPO_4$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.02; $CaCl_2$, 0.04; yeast extract, 2.0; D-glucose, 1.0; and agar, 15.0. After incubation, the plates were overlaid with Congo red solution (1.0 mg/mL $H_2O$) for 15 min, rinsed with 1 mol $L^{-1}$ NaCl, and the presence of a clear halo indicated cellulose degradation (Ferbiyanto et al., 2015).

Urease activity was tested on Christensen medium containing (g/L): peptone, 1.0; glucose, 1.0; NaCl, 5.0; $KH_2PO_4$, 2.0; phenol red (1.6%), 0.75 mL/L; urea solution (20%), 100.0 mL/L; and agar, 16.0. The appearance of a red color after incubation indicated urea hydrolysis.

All media were adjusted to pH 7.0 using 1 mol/L NaOH or HCl and sterilized by autoclaving at 120 °C for 20 min.

### 2.3 Examination of the Effect of Isolates on Seed Germination and Initial Growth

The effect of bacterial isolates on the seed germination of basil (*Ocimum basilicum* L.) and sage (*Salvia officinalis* L.) was evaluated *in vitro*. Selected bacterial isolates were streaked on appropriate growth media (NA and SCA) and incubated at 28 ºC for 48 or 96 hours, depending on the isolate. Two full loops of bacterial colonies from each isolate were then transferred to Erlenmeyer flask containing 25 mL of the appropriate liquid nutrient medium (Nutrient broth for *Bacillus* isolates and Starch Casein broth for *Actinomycetes* isolates) and incubated at 28 ºC with continuous shaking at 130 rpm for 48 h or 96 hours, depending on the isolate. After the incubation period, the bacterial cultures were centrifuged at 3500 rpm for 15 minutes. The supernatant was discarded, and the cell pellet was resuspended in sterile deionized water. The bacterial suspension from each isolate was adjusted to a concentration of $10^9$ CFU/mL (colony-forming units per milliliter). Before inoculation, seeds were first surface-disinfected by soaking in 70% ethanol for 2 minutes and then rinsed with 200 mL of sterile deionized water. A total of 100 seeds of each plant were inoculated by immersing them in a bacterial suspension for 30 minutes. The selected soaking durations were sufficient for isolates colonization on basil and sage seeds without adversely affecting germination rates. Meanwhile, untreated seeds soaked in sterile deionized water served as controls. Following inoculation, 20 seeds were placed on filter paper in a Petri dish, with a total of 100 seeds distributed across five Petri dishes per isolate. The Petri dishes were transferred into a thermostat set at 25 ºC in the dark to simulate soil sowing conditions. To maintain moisture, 1 mL of sterile deionized water was applied to the filter paper daily. After seven days, the germination rate, shoot, and root length were recorded. The vigor index was calculated using the formula: VI = (mean root length + mean shoot length) × germination %.

### 2.4 Testing the Impact of Isolates on Plant Yield Parameters

In this study, 850 mL pots, each filled with Klasmann™ substrate up to 2 cm below the rim, were used. Plant seeds were first inoculated with selected isolate suspensions following the same procedure used to assess the effect of isolates on seed germination (as described above). A total of 25 seeds were sown per treatment (5 seeds per pot) in a completely randomized design with five replications. The same procedure was repeated for the control variant (seeds treated with water). Three weeks after seeding, a dosage of 10 mL booster PGPR ($10^9$ CFU/mL) was applied to the inoculated treatments. The plants were cultivated under controlled conditions at a room temperature of 25 ± 2 ºC, with a natural photoperiod of 16 hours of light and 8 hours of darkness

(day/night), for six weeks. Plants were irrigated twice weekly, maintaining soil moisture at approximately 70% of water-holding capacity (WHC). After the six-week cultivation period, various parameters, including stem and root length (cm), fresh and dry yield (g), and biochemical analysis of the plants were performed.

### 2.5 Testing the Impact of Bacterial Isolates on Biochemical Parameters in Plants

Fresh leaves (0.5 g) were homogenized with 4 ml 0.1 M phosphate buffer (pH 7.0) using a mortar and pestle. Then the extract was centrifuged at 5000 rpm for 20 minutes at 4 ºC (Boeco U-320R, Germany). The supernatant was used to determine soluble protein content and phenylalanine-ammonia-lyase (PAL) activity.

To determine total phenolic content (TPC), 1,1-diphenyl-2-picrylhydrazyl radical scavenging capacity (DPPH-RSC), and rosmarinic acid (RA) content, dried aerial parts of basil and sage were ground and used to prepare ethanol extracts. Modern ultrasonic extraction with an ethanol/water mixture (70/30, v/v) was used, as described by Jurić et al. (2024). Dry plant material (1 g) was mixed with 10 mL of solvent and then sonicated for 2 h at 40 ºC using an Elmasonic S 100 H ultrasonic bath (Elma Schmidbauer GmbH, Germany). The extracts were centrifuged at 5000 rpm for 15 minutes, after which the supernatant was collected for further analysis and stored at 4 ºC.

Soluble protein contents were estimated by the method of Bradford (1976) using bovine serum albumin as a standard.

PAL activity was measured as described by Şirin et al. (2016), using L-phenylalanine as the substrate in a reaction mixture containing 100 mM Tris-HCl buffer (pH 8.8) with 40 mM L-phenylalanine. After incubating the reaction mixture at 37 ºC for 30 min, the reaction was stopped by the addition of 4M HCl. The produced *trans*-cinnamic acid was measured spectrophotometrically by recording the absorbance at 290 nm using an Evolution 220 UV-Visible Spectrophotometer (Thermo Fisher Scientific Inc., USA). PAL activity was expressed in units corresponding to nmol of *trans*-cinnamic acid produced per hour per mg of protein.

The total phenolic content (TPC) was determined using the Folin-Ciocalteu method (Singleton et al., 1999) with some modifications. Briefly, 30 µL of ethanol extract (diluted 60 times) was added to 150 µl of Folin-Ciocalteu reagent (0.1 M). After ten minutes, 120 µL of $Na_2CO_3$ solution (75 g/L) was added. The mixture was then left to stand in the dark for one hour, after which absorbance was measured at 760 nm using a spectrophotometer (Biotek Epoch microplate reader, Agilent Technologies, USA). Results were expressed in milligrams of gallic acid equivalent (GAE) per gram of dried plant, determined using a gallic acid standard curve (12.5-100 µg/mL).

Antioxidant activity was measured using DPPH-RSC, as described by Brand-Williams et al. (1995), with slight modifications. Briefly, 180 µL freshly prepared DPPH solution (90 µM) was mixed with 20 µL of extract (serial double dilution: 5-0.3125 mg/mL), and discoloration was measured at 515 nm after 1 h. DPPH-RSC of the extracts was calculated using the following equation:

$$\text{DPPH-RSC (\%)} = (Ac-As)/ Ac \times 100$$

where: Ac is absorbance of control; As is absorbance of extract.

The antioxidant activity was expressed as $IC_{50}$ value, which was determined graphically using Origin software by plotting the percentage of DPPH-RSC against the sample concentration. The $IC_{50}$ value represents the concentration of extract which is necessary to decrease the initial DPPH concentration by 50%.

The concentration of rosmarinic acid in the plant extracts was analyzed using a high-performance liquid chromatograph (Nexera X2, Shimadzu, Japan) equipped with a Luna C18 column (150 × 2 mm, 3µm) and C18 guard column (4 mm × 2 mm i.d., 3µm) (Phenomenex, USA). The mobile phases were ultrapure water with 1% (v/v)

formic acid (phase A) and methanol with 1% (v/v) formic acid (phase B). Chromatographic separations were performed using gradient: 0-5 min, 5% B; 5-10 min, 20% B; 10-17 min, 20% B; 17-30 min, 25% B; 30-35 min, 30% B; 35-45 min, 70% B; 45-50 min, 70% B; 50-55 min, 5% B; 55-60 min, 5% B. Rosmarinic acid, the dominant compound, was monitored at 330 nm. The flow rate was 0.25 ml/min and the injection volume was 5µL. Samples were filtered through a 0.22 µm PTFE filter before injection. For the quantification of RA, a stock standard solution of RA was prepared in methanol at seven concentrations (1-100 µg/mL) to generate a calibration curve by plotting peak area versus concentration ($R^2 > 0.99$). Data analysis was carried out using LabSolutions GPC software.

### 2.6 Molecular Characterization of Selected Bacterial Isolates

For molecular characterization, bacterial isolates were initially cultured on selective solid media: Starch Casein Agar (SCA; HiMedia) for *Actinomycetes* and Nutrient Agar (NA; HiMedia) for *Bacillus* strains. Following 24 hours of incubation at 28 ºC, a single well-isolated colony was aseptically transferred into a sterile microcentrifuge tube for subsequent genomic DNA extraction.

Genomic DNA was extracted using the GeneMatrix Gram Plus & Yeast Genomic DNA Purification Kit (EURx, Gdansk, Poland) according to the manufacturer's instructions. The efficiency of the total DNA extraction of bacterial strains was checked using QFX fluorimeter (DeNovix Inc, USA).

The 16S rRNA gene was amplified using universal bacterial pair of primers 27F (5'-AGAGTTTGATCNTGGCTCAG-3') and 1492R (5'-ACGGYTACCTTGTTACGACTT-3'), as described by Fredriksson et al. (2013). Briefly, the total volume of each PCR reaction was 50 µL, consisting of: 25 µL of HotStart Taq2x MasterMix (BioLabs, Heidelberg, Germany), 1 µL of each primer (10 µM), 21 µL of nuclease-free water, and 2 µL of extracted DNA. The conventional polymerase chain reaction (PCR) was carried out using a TC-412 Thermal Cycler (United Kingdom). The thermal cycling protocol consisted of an initial denaturation step at 95 ºC for 5 minutes, followed by 35 cycles of denaturation at 94 ºC for 1 minute, annealing at 55 ºC for 30 seconds, and extension at 72 ºC for 1 minute. A final extension was performed at 72 ºC for 7 minutes. PCR products were resolved by electrophoresis on a 1% agarose gel, stained with ethidium bromide (1µg/mL in $ddH_2O$ for 30 minutes), using a Thermo Scientific EC300XL2 electrophoresis unit (China), and visualized under UV illumination with a BlueCube 300 imaging system (SERVA Electrophoresis, Germany). Amplified products were purified and submitted for the Standard-Seq method of Sanger sequencing technology at Macrogen Inc. (Amsterdam, The Netherlands). The obtained sequences were analyzed using the BLASTn algorithm (Basic Local Alignment Search Tool) (National Center for Biotechnology Information [NCBI], n.d.a) against the NCBI GenBank database (NCBI, n.d.b) to determine their phylogenetic affiliation.

### 2.7 Statistical Analysis

The results underwent statistical analysis using the Statistics 13.3 application from TIBCO Software Inc. The significance of the differences between the treatments was assessed using one-way ANOVA followed by Fisher's LSD examination ($p < 0.05$). The correlation matrix is constructed by calculating Pearson's correlation coefficient ($r$) between tested biochemical parameters (TPC, RA, DPPH RSC, and PAL activity) using the same software. Correlations were considered significant at $p < 0.05$ level. All graphs were generated using Origin software (OriginLab Corporation, USA).

## 3. Results and Discussion

In this study, six bacterial isolates were isolated from the rhizosphere of nettle (*Urtica dioica* L.), comprising three isolates belonging to the genus *Bacillus* (Bac1, Bac2, Bac3), and three isolates belonging to the *Actinomycetes* (Act1, Act2, Act3).

### 3.1 Physiological and PGP Characterization of the Isolates

The interaction between rhizospheric microbes and plants is influenced by various factors, including temperature, soil pH, salinity, the host plant, rhizobacterial strains, and the chemical and physical properties of the soil. In this study, at temperatures of 5 and 41 ºC, minimal growth was observed for isolates Bac2, Bac3, and Act1 (Table 1). Rhizobacteria that are tolerant to cold or can survive at high temperatures possess a significant advantage in enhancing plant growth and productivity under adverse environmental conditions. The *Bacillus* strains examined in this study contribute to the valuable biodiversity of cold- and heat-tolerant rhizobacteria.

**Table 1.** Physiological and PGP characterization of the isolates isolated from nettle rhizosphere

| | Isolates | Bac1 | Bac2 | Bac3 | Act1 | Act2 | Act3 | % of positives |
|---|---|---|---|---|---|---|---|---|
| Temp.* | 5 ºC | − | + | − | − | − | − | 16.6 |
| | 28 ºC | ++ | ++ | ++ | ++ | ++ | ++ | 100 |
| | 41 ºC | − | − | + | + | − | − | 33.3 |
| pH | 4.5 | ++ | ++ | ++ | + | + | + | 100 |
| | 7 | ++ | ++ | ++ | ++ | ++ | ++ | 100 |
| | 9 | ++ | ++ | ++ | − | ++ | + | 88.3 |
| NaCl | 0.9% | ++ | ++ | ++ | ++ | ++ | ++ | 100 |
| | 3% | ++ | ++ | ++ | + | ++ | + | 100 |
| | 5% | − | − | + | − | − | − | 16.6 |
| | 7% | − | − | + | − | − | − | 16.6 |
| IAA** | | − | − | + | − | − | − | 16.6 |
| Siderophore | | − | + | − | − | − | − | 16.6 |
| HCN | | + | + | + | + | + | + | 100 |
| Mineralization P | | + | − | + | − | − | − | 33.3 |
| Solubilization P | | − | − | − | + | − | + | 33.3 |
| Lipase | | + | + | + | + | + | + | 100 |
| Amilase | | − | + | − | + | + | + | 66.6 |
| Pectinase | | − | − | − | + | + | + | 50.0 |
| Protease | | − | − | − | − | + | − | 16.6 |
| Celulase | | + | + | + | + | + | − | 66.6 |
| Urease | | − | + | + | + | + | + | 66.6 |
| % of positives traits | | 47.6 | 61.9 | 71.4 | 61.9 | 61.9 | 57.1 | |

*temperature, pH, % NaCl: − complete absence of growth; + minimal growth; ++ optimal; +++ abundant growth; ** (+) positive reaction / produces / decomposes; (−) negative reaction / does not produce / does not decompose.

Optimal growth on substrates with pH values of 4.5, 7, and 9 was exclusively recorded in the genus *Bacillus*. These results are consistent with those of Getahun et al. (2020), who also found that *Bacillus* isolates have a high tolerance to unfavorable environments and that they grew successfully in media that ranged from 5 to 12 pH values.

Salinity emerges as a prominent abiotic stress factor significantly impeding the growth and productivity of plants. According to Sharma et al. (2016), the use of PGPR that tolerates high salinity stands out as a pivotal approach to effectively enhance plant growth in saline soils. In the present study, when substrates included 3% NaCl, all

isolates exhibited optimal or at least minimal growth. Conversely, on substrates with a higher percentage of NaCl, growth was absent in most isolates. Tolerance to higher doses of salt was shown by the *Bacillus* isolate Bac3. In the study conducted by Moncheva et al. (2002), optimal growth of actinomycetes was observed on substrates containing lower NaCl percentages (1.5, 3, and 5%), while substrates with 7, 15, and 20% NaCl demonstrated no growth of actinomycetes, which is in line with our results.

One of the most important PGP properties is the ability to produce Indole-3-Acetic Acid (IAA), an auxin hormone controlling various physiological processes in plants. In this research, IAA production was observed only in isolate Bac3, while other isolates did not exhibit the production of this hormone. In contrast to our research, several studies have demonstrated that isolates of *Bacillus* and actinomycetes possess the ability to produce IAA (Kang et al., 2019; Xie et al., 2024). However, although many bacterial species are known to produce IAA, an estimated 20% of bacteria isolated from the rhizosphere lack this ability (Ali et al., 2015).

Iron supply to plants and bacteria involves the production of low-molecular-weight molecules with a high affinity for $Fe^{3+}$, known as siderophores. Among the examined isolates, siderophore production was identified only in Bac2. In contrast to our results, Rajabi-Khamseh et al. (2020) found that all *Bacillus* isolates produced siderophores.

Phosphorus is an essential nutrient for plants, having a key role in nucleic acids, phospholipids, ATP, and various metabolic and biochemical pathways, including biological nitrogen fixation and photosynthesis. The ability to mineralize organic phosphorus compounds and thereby supply plants with this essential nutrient was observed in 33.3% of the examined isolates, including Bac1 and Bac3, while this characteristic was not confirmed in other isolates. The ability of isolates to solubilize inorganic phosphates was observed in 33.3% of the tested isolates in this study, specifically in two isolates, Act1 and Act3. Similar results were obtained by Zhang et al. (2021) and Babu et al. (2025), who also confirmed the ability of actinomycetes and *Bacillus* isolates to mineralize and solubilize phosphorus compounds.

Hydrolytic enzymes play a crucial role in maintaining soil fertility. These enzymes hydrolyze complex compounds, such as polysaccharides, proteins, and urea, into simpler forms, thereby enhancing soil fertility. Additionally, hydrolytic enzymes are known to induce paralysis and death in pathogenic microorganisms, especially fungi. Thus, microorganisms capable of producing one or more hydrolytic enzymes can be applied to combat a range of plant-pathogenic fungi and bacteria, as well as to enhance plant growth (Gomes et al., 2001). In this research, isolates Act1 and Act2 produced the highest number of examined enzymes, suggesting that these isolates can be further explored as potential bioagents for controlling phytopathogens.

### 3.2 Effect of Selected Isolates on Seed Germination and Plant Yield Parameters

Based on physiological, PGP, and biochemical characteristics, the most effective isolates, Bac3 and Act1, were selected for investigating their impact on seed germination, initial growth, and plant yield. Low seed vitality and seed dormancy are unfavorable traits for farmers as they lead to reduced productivity. Remarkable increases in the germination rate, vigor of seedlings, and sprout growth in essential crops have been documented following the application of PGP bacteria (Fiodor et al., 2023). Similar stimulatory effects of PGPR on germination and early seedling development have been reported in basil and sage, where improvements were attributed to enhanced nutrient availability and phytohormonal modulation (Çakmakçı et al., 2020; Pagliarini et al., 2023; Uçar et al., 2023). In this study, both applied isolates had a positive effect on the germination rate of basil and sage seeds, as well as on the length of the roots and shoots of the seedlings. Seven days after inoculation, the basil and sage seed germination rates were the highest in the variant with applied Act1 (Table 2). In this variant, basil seed germination increased by 76%, while sage seed germination improved by 117% compared to the control. Comparable improvements in germination induced by actinomycetes and *Bacillus* spp. have been previously

reported in aromatic plants and were associated with improved nutrient mobilization and hormonal stimulation (Moncada et al., 2021). The most favorable impact on root and shoot length, as well as the vigor index of basil and sage, was also noted in the variant with the actinomycetes isolate.

**Table 2.** Seed germination, root and shoot length (mm), and vigor index (VI) of basil and sage

| Isolates | Germination rate | | | | Root length | | Shoot length | | VI | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Basil | | Sage | | Basil | Sage | Basil | Sage | Basil | Sage |
| | No.* | % | No. | % | | | | | | |
| Bac3 | 11.6±1.1$^{b}$** | 58 | 18.0±0.7$^{b}$ | 90 | 10.4±0.4$^{b}$ | 24.6±0,5$^{b}$ | 17.9±0.1$^{b}$ | 20.0±0.2$^{a}$ | 1641 | 4095 |
| Act1 | 15.4±1.1$^{a}$ | 77 | 20.0±1.2$^{a}$ | 100 | 12.7±0.2$^{a}$ | 42.2±1.5$^{a}$ | 26.2±0.2$^{a}$ | 20.9±0.2$^{a}$ | 2976 | 6220 |
| control | 8.8±0.7$^{c}$ | 44 | 9.2±0.8$^{c}$ | 46 | 6.0±0.7$^{c}$ | 13.7±0.2$^{c}$ | 9.5±0.4$^{c}$ | 7.5±0.3$^{b}$ | 674 | 986 |
| CV (%) | 8.1 | | 5.7 | | 4.4 | 2.7 | 1.3 | 1.4 | | |
| LSD (0.05) | 1.8 | | 2.1 | | 0.6 | 1.3 | 0.6 | 0.3 | | |

*Average number of germinated seeds per Petri dish; % percentage of germinated seeds; **Values are means ± standard deviation (SD), n = 5. CV (%) = SD/mean × 100. Means within each column followed by different letters differ significantly according to Fishers LSD test ($p < 0.05$). LSD values are reported for each variable.

The plant hormone auxin (indoleacetic acid (IAA)) has a highly beneficial effect on seed germination. In this study, only isolate Bac3 was able to produce IAA, a compound known to stimulate seed germination, whereas isolate Act1 did not. However, the observed increase in germination percentage suggests that isolate Act1 contributed to promoting germination through alternative mechanisms. Notably, the application of Act1 resulted in the highest increase in germination percentage, indicating that its ability to solubilize phosphorus may have contributed to improved seed germination. This assumption is further supported by the findings of Fiodor et al. (2023), who reported similar results. Their study concluded that phosphate solubilization could influence seed germination, as evidenced by the positive effect of isolate AF8II13 on carrot seed germination in the absence of IAA synthesis.

Six weeks into the experiment, a positive impact of all isolates on the root and stem length of basil and sage was observed, although the increase was not consistently statistically significant (Table 3). Bac3 showed a stronger effect on root elongation, while Act1 predominantly enhanced shoot growth and biomass accumulation. Such strain-specific responses are commonly reported and are linked to differences in rhizosphere competence, nutrient mobilization efficiency, and metabolic interaction with host plants (Çakmakçı et al., 2020).

**Table 3.** Effect of selected isolates on the root and stem length (cm) and fresh and dry yield (g) of basil and sage

| Isolates | Root length | | Stem length | | Fresh yield | | Dry yield | |
|---|---|---|---|---|---|---|---|---|
| | Basil | Sage | Basil | Sage | Basil | Sage | Basil | Sage |
| Bac3 | 4.6±0.07$^{a}$* | 5.1±0.07$^{a}$ | 13.3±0.22$^{b}$ | 9.0±0.14$^{a}$ | 13.8±0.18$^{a}$ | 10.2±0.11$^{a}$ | 1.6±0.7$^{a}$ | 0.7±0.07$^{b}$ |
| Act1 | 4.1±0.07$^{b}$ | 4.4±0.07$^{b}$ | 14.9±0.18$^{a}$ | 10.4±0.11$^{a}$ | 14.0±0.18$^{a}$ | 11.1±0.11$^{a}$ | 1.2±0.07$^{b}$ | 0.9±0.07$^{a}$ |
| Control | 3.1±0.07$^{c}$ | 5.0±0.07$^{a}$ | 12.5±0.18$^{b}$ | 9.6±0.11$^{a}$ | 8.3±0.11$^{b}$ | 6.25±0.08$^{b}$ | 1.0±0.07$^{c}$ | 0.6±0.07$^{b}$ |
| CV (%) | 1.8 | 1.45 | 1.4 | 1.24 | 1.3 | 1.1 | 22.0 | 9.6 |
| LSD (0.05) | 0.1 | 0.1 | 0.3 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 |

* Values are means ± standard deviation (SD), n = 5. CV (%) = SD/mean × 100. Means within each column followed by different letters differ significantly according to Fishers LSD test ($p < 0.05$). LSD values are reported for each variable.

Both inoculation treatments significantly increased fresh and dry biomass of basil and sage relative to the control. In basil, Act1 had a stronger effect on fresh biomass, increasing it by 68.6%, whereas Bac3 most effectively enhanced dry biomass, with a 60% gain. In sage, Act1 consistently produced the largest increases in both fresh

(77.6%) and dry (50%) biomass, highlighting its superior growth-promoting effect. These findings align with the outcomes reported by Çakmakçı et al. (2020), who also observed positive effects, such as increased biomass in saplings and roots of various medicinal and aromatic plants following inoculation with PGP bacteria. Many studies evaluate the beneficial impact of PGP microorganisms on the yield parameters of basil (Pagliarini et al., 2023) and sage (Uçar et al., 2023). The results obtained in this study are consistent with previous research, emphasizing that the positive effects of PGP bacteria depend on multiple factors, including the isolate's adaptability and its specific PGP properties. This is in line with findings that such synergistic effects on both germination and growth parameters are commonly associated with highly efficient rhizosphere colonizers capable of producing multiple plant-beneficial metabolites (Backer et al., 2018). However, contrasting results have also been reported, where no significant yield improvement was observed, likely due to environmental conditions, plant genotype, or strain specificity. The studies by Moncada et al. (2021) reported that inoculation with a commercial *Bacillus* spp. product had no significant effect on the basil plant growth. This variability highlights that PGPR efficiency is strongly context-dependent and influenced by plant-microbe-environment interactions.

In this study, the observed growth promotion can be attributed to a combination of direct and indirect PGPR effects, including phytohormone production, improved nutrient solubilization (phosphorus), and secretion of hydrolytic enzymes. These mechanisms collectively enhance root system development, nutrient uptake efficiency, and metabolic activity in plants. Moreover, improved plant nutrition may lead to increased carbon allocation toward growth and secondary metabolism, which is particularly relevant for medicinal species.

### 3.3 Biochemical Effects of Selected Isolates on Plants

Basil and sage belong to a group of medicinal and aromatic plants, best known for their high concentration of phenolic compounds, which exhibit strong antioxidant activity. The results presented in Table 4 reveal that the investigated plants are a rich source of phenolic compounds. The phenolic content in sage ranged from 22.4 mg/g (control) to 25.7 mg/g (Act1 treatment), while in basil it ranged from 25.5 mg/g (control) to 32.4 mg/g (Bac3 treatment). In the literature, the phenolic content in sage has been reported as 19.1 mg/g (Pop et al., 2015) and for basil as 41.12 mg/g (Gajula et al., 2009). This variation can be attributed to the ecological conditions of the different locations. The content of rosmarinic acid in sage ranged from 8 mg/g (control) to 14.0 mg/g (Bac3 treatment). As reported by Jurić et al. (2024), the rosmarinic acid content in *S. officinalis* was 5.73 mg/g. The rosmarinic acid content in *O. basilicum* ranged from 0.52 to 3.05 mg/g according to HPLC results of Zare et al. (2021). In this study, the values of rosmarinic acid content in basil ranged from 9.8 mg/g in control plants to 14.2 mg/g (Act1 treatment). Under PGPRs treatment, the contents of rosmarinic acid were significantly higher than in control plants. The PAL enzyme plays a crucial role in the biosynthesis of phenolic compounds. The PAL activity was significantly higher in plants treated with PGPR compared to the control plants. The PAL activity in sage plants ranged from 505.7 U/mg protein (control) to 704.6 U/mg protein (Bac3 treatment). Lower PAL activities were obtained for basil plants, where the control PAL activity was 86.7 U/mg protein, while significantly higher values were obtained for treated plants; basil treated with Bac3 had the highest PAL activity at 309.9 U/mg protein. This result aligns with the findings of Zare et al. (2021), which indicate increased PAL activity in inoculated basil plants.

The results presented in Table 4 reveal that the investigated plants are rich in phenolic compounds and rosmarinic acid and exhibit measurable PAL activity, indicating active phenolic metabolism. In this study, PGPR treatment appeared to activate the phenylpropanoid pathway, induce the PAL enzyme activity, and enhance the biosynthesis of phenolic compounds.

**Table 4.** Total phenolic content, rosmarinic acid content, and PAL activity in basil and sage

| Isolates | TPC | | RA | | PAL | |
|---|---|---|---|---|---|---|
| | Basil | Sage | Basil | Sage | Basil | Sage |
| Bac3 | 32.4 ± 0.7[a] | 22.5 ± 1.1[b] | 13.7 ± 0.7[a] | 14.0 ± 0.7[a] | 309.9 ± 15.0[a] | 704.6 ± 35.2[a] |
| Act1 | 31.5 ± 1.6[a] | 25.7 ± 1.1[a] | 14.2 ± 0.7[a] | 13.5 ± 0.7[a] | 132.1 ± 6.6[b] | 603.4 ± 30.2[b] |
| control | 25.5 ± 0.9[b] | 22.4 ± 0.8[b] | 9.8 ± 0.5[b] | 8.0± 0.4[b] | 86.7 ± 4.3[c] | 505.7 ± 25.3[c] |
| CV (%) | 3.6 | 4.3 | 5.0 | 5,1 | 4,9 | 5.1 |
| LSD 0.05 | 2.3 | 2.0 | 1.2 | 1.2 | 33.1 | 62.3 |

Values are means ± standard deviation (SD), n = 3. CV (%) = SD/mean × 100. Means within each column followed by different letters differ significantly according to Fishers LSD test ($p < 0.05$). LSD values are reported for each variable.

The antioxidant activity of the extracts was measured using the DPPH-RSC assay. All investigated plant extracts showed significant DPPH scavenging capacities in a concentration-dependent manner (Figure 1). The results showed an increase in the DPPH-RSC of plants treated with PGPR compared to the control. The $IC_{50}$ value, obtained from the graphs, was used as a standard measure for comparing scavenging capacities of the extracts. A lower $IC_{50}$ value indicates stronger scavenging capacity. Basil plant extracts treated with Act1 and Bac3 displayed lower $IC_{50}$ values than the control, at 1.61 and 1.63 mg/ml, respectively (Figure 1A). For sage plant extracts, the strongest DPPH radical scavenging capacity was observed in extracts obtained from plants treated with Act1, with an $IC_{50}$ value 1.82 mg/mL, followed by Bac3 (2.07 mg/mL) (Figure 1B). It should be noted that both treated plant extracts exhibited lower $IC_{50}$ values than the control sage plant (2.49 mg/mL), indicating higher antioxidant activity. Similarly, Zaman et al. (2022) reported that yeast extract increased DPPH antioxidant activity in suspension cultures of purple basil.

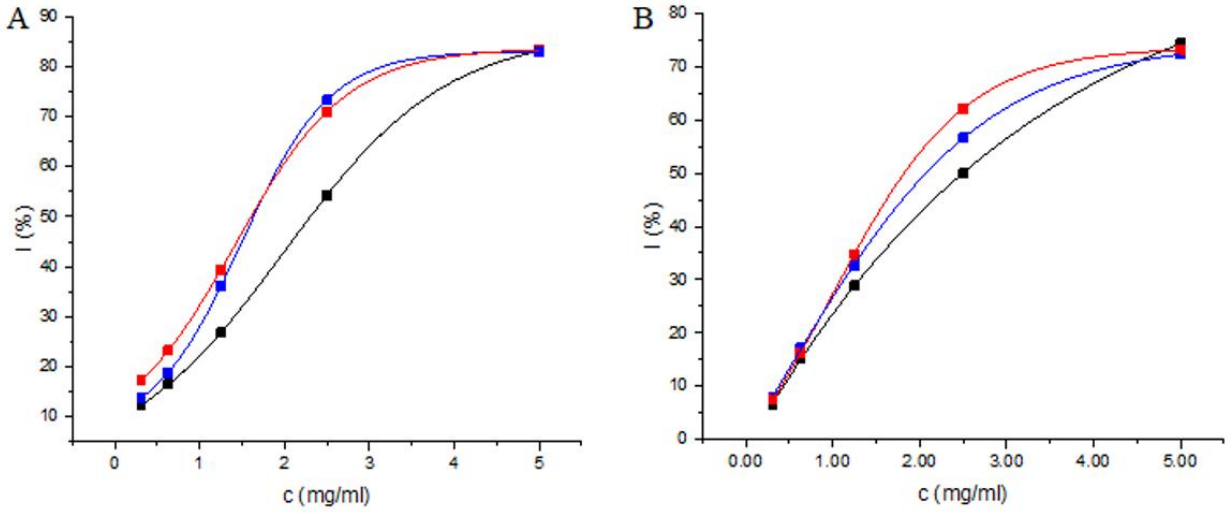


**Figure 1.** Percent of inhibition of DPPH radical in the presence of different concentrations of extracts of basil (A) and sage (B) plants

Blue line: extracts treated with Bac3; red line: extracts treated with Act1, and black line: control extracts (no PGPR treatment).

Table 5 shows the correlation coefficients (*r*) between total phenolic content (TPC), rosmarinic acid content (RA), PAL activity, and $IC_{50}$ value of DPPH scavenging activity in extracts from individual plants.

**Table 5.** Correlation coefficients (*r*) between DPPH-RSC, rosmarinic acid (RA) content, PAL activity, and total phenolic content (TPC)

| | Correlation coefficients for basil | | |
|---|---|---|---|
| Variable | RA | PAL | TPC |
| DPPH-RSC | -0.8772* | -0.9851* | -0.8894* |
| RA | | 0.9059* | 0.5804 |
| PAL | | | 0.8640* |
| | Correlation coefficients for sage | | |
| Variable | RA | PAL | TPC |
| DPPH-RSC | -0.6733* | -0.5146 | -0.6325 |
| RA | | 0.9689* | 0.0359 |
| PAL | | | -0.1978 |

* Marked correlations are significant at $p < 0.05$.

Correlation analysis revealed species-specific relationships between antioxidant capacity and phenolic metabolism. In basil, strong and statistically significant negative correlations were observed between DPPH-RSC ($IC_{50}$) and all measured biochemical parameters, indicating that higher levels of total phenolics, RA, and PAL activity were associated with enhanced antioxidant potential. In contrast, sage showed a significant association only between DPPH-RSC and RA content, while correlations with total phenolics and PAL activity were weaker and not statistically significant. A strong positive correlation exists between RA content and PAL activity in both species, suggesting a coordinated activation of the phenylpropanoid pathway.

These differences may reflect species-specific variations in phenolic metabolism, compound composition, or extraction efficiency. While antioxidant activity in basil appears closely linked to phenolic content and biosynthetic enzyme activity, the response in sage may involve other contributing phytochemicals. This highlights the complexity of phytochemical interactions and the importance of species-specific analyses.

### 3.4 Molecular Characterization of Selected Bacterial Isolates

In light of the promising effects observed with the Bac3 and Act1 isolates, molecular characterization was conducted to accurately identify the strains.

Molecular analysis of the two selected bacterial strains Bac3 and Act1 began with the extraction of genomic DNA. The concentration of the extracted DNA was 0.435 ng/µL for *Bacillus* isolate Bac3 and 0.443 ng/µL for *Actinomycetes* isolate Act1. PCR amplification of the 16S rRNA gene produced amplicons of the expected size (~1460 bp), as confirmed by agarose gel electrophoresis.

The resulting PCR products were purified and submitted for Sanger sequencing. The obtained nucleotide DNA sequences were analyzed using the BLASTn alignments algorithm against the sequences deposited in NCBI GenBank database for taxonomic identification (accessed on 09 June 2025). The BLAST search yielded alignments of very high quality, but none of the alignments disclosed a score of 100% identity. Isolate Bac3 demonstrated 97.95% sequence identity with *Bacillus pretiosus* strain SAICEU11T BU-ISCIII 59 (GenBank accession number: NZ_JAOXJG010000059.1), while isolate Act1 showed 97.73% identity with *Streptomyces ardesiacus* NBRC 15402 (GenBank accession number: NZ_BEWC01000032.1).

## 4. Conclusions

This study demonstrates that selected plant growth-promoting rhizobacteria (PGPR) belonging to the genus *Bacillus* and actinomycetes group can effectively enhance growth, yield, and phytochemical quality of basil (*Ocimum basilicum* L.) and sage (*Salvia officinalis* L.). Both isolates improved seed germination and early seedling vigor, with the actinomycete isolate Act1 showing the most pronounced effects.

Under controlled pot conditions, PGPR inoculation significantly enhanced yield-related traits, with Act1 increasing fresh biomass by up to 77.6% in sage, while Bac3 improved basil dry biomass by up to 60%. In addition, both isolates promoted the accumulation of bioactive compounds, including up to 27% higher total phenolic content and more than 50% increased rosmarinic acid levels, indicating enhanced antioxidant potential and secondary metabolic activity. Therefore, the observed increase in RA content following PGPR treatment not only strengthens the plants' physiological resilience but also enhances their commercial and therapeutic value, making this a promising approach for sustainable cultivation of high-quality medicinal herbs. These responses are likely driven by multiple PGPR-mediated mechanisms, including improved nutrient availability and stimulation of plant secondary metabolism. Overall, PGPR application represents a sustainable strategy for improving both agronomic performance and medicinal quality of basil and sage.

Molecular identification revealed that isolate Bac3 is closely related to *Bacillus pretiosus* strain SAICEU11^T BU-ISCIII 59, while Act1 is affiliated with *Streptomyces ardesiacus* NBRC 15402.

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### Transparency of Data

Available data: The entire data set that supports the results of this study was published in the article itself.

### Author Contribution Statement

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|---|---|---|---|---|---|---|---|
| Conceptualization | ■ | | ■ | ■ | | | |
| Formal analysis | ■ | ■ | ■ | | | | |
| Investigation | ■ | ■ | | | | ■ | |
| Methodology | ■ | ■ | | | ■ | | |
| Resources | | | | | | | ■ |
| Supervision | ■ | | | | | | |
| Validation | | | | ■ | ■ | | |
| Visualization | ■ | ■ | | ■ | | | ■ |
| Writing – original draft | ■ | ■ | | | | | |
| Writing – review and editing | | | ■ | | | ■ | |

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