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Effects of AC Phosphatase Gene-Identified Rhizobacteria (PGPR) Strains on Flowering and Flower Quality in Geranium (*Pelargonium* sp.)

Sardunyada (*Pelargonium* sp.) Çiçeklenme ve Çiçek Kalitesi Üzerinde AC Fosfataz Gen Bölgesi Tanımlanan Rizobakteri (PGPR) Suşlarının Etkileri

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Abstract

In recent years, geraniums (*Pelargonium* sp.) have become one of the most popular and widely cultivated flowering potted plants worldwide, including in Turkey. One of the primary goals in geranium cultivation is to achieve high-quality and continuous flowering. Phosphorus plays a crucial role in flower quality. Although phosphorus is sufficiently present in soils, only a small fraction is available for plant uptake. Soil bacteria can solubilize this unavailable phosphorus, making it accessible to plants. This study was conducted to investigate the effects of *Bacillus megaterium* and *Pseudomonas putida* rhizobacteria, identified by their AC Phosphatase gene regions, on the flowering and flower quality of geraniums. The results showed that rhizobacteria applications positively impacted flowering and flower quality in geraniums. Flowering occurred two weeks earlier in the treated geranium seedlings, and significant increases were observed in plant height, branch number, leaf number and area, inflorescence number, and the number of flowers per inflorescence compared to the control group. Additionally, protein isolation from leaves indicated that, besides morphological traits, protein profiles were also positively affected. In conclusion, the findings demonstrate that rhizobacteria have significant positive effects on flowering, flower quality, and protein profiles in geraniums. The use of rhizobacteria and similar biostimulants in agriculture can contribute to environmentally friendly and sustainable agricultural production, potentially making horticultural production, including ornamental plants, more resilient to climate change

Keywords: Ornamental plants, *Pelargonium*, PGPR, protein profile

Özet

Son yıllarda sardunya (*Pelargonium* sp.), Türkiye de dâhil olmak üzere dünya genelinde en popüler ve yaygın olarak yetiştirilen çiçekli saksı bitkilerinden biri haline gelmiştir. Sardunya yetiştiriciliğinde temel hedeflerden biri, yüksek kaliteli ve sürekli çiçeklenmenin sağlanmasıdır. Fosfor, çiçek kalitesinde önemli bir rol oynar. Toprakta yeterli miktarda fosfor bulunmasına rağmen, bitkiler tarafından alınabilir kısmı oldukça sınırlıdır. Toprak bakterileri, bu kullanılmayan fosforu çözerek bitkilerin alabileceği hale getirebilir. Bu çalışma, AC Fosfataz gen bölgeleri ile tanımlanan *Bacillus megaterium* ve *Pseudomonas putida* rizobakteri suşlarının sardunyaların çiçeklenmesi ve çiçek kalitesi üzerindeki etkilerini belirlemeyi amaçlamıştır. Sonuçlar, rizobakteri uygulamalarının sardunyaların çiçeklenme ve çiçek kalitesi üzerinde olumlu etkiler sağladığını göstermiştir. PGPR uygulamaları ile sardunya fidelerinde çiçeklenme iki hafta erken gerçekleşmiş ve kontrol grubuna kıyasla bitki boyu, dal sayısı, yaprak sayısı ve alanı, çiçek salkımı sayısı ve salkım başına düşen çiçek sayısında önemli artışlar gözlemlenmiştir. Ayrıca, yapraklardan yapılan protein izolasyonları, morfolojik özelliklerin yanı sıra protein profillerinin de olumlu etkilendiğini göstermiştir. Sonuç olarak, bulgular rizobakteri uygulamasının sardunyaların çiçeklenme, çiçek kalitesi ve protein profilleri üzerinde önemli olumlu etkiler sağladığını ortaya koymuştur. Rizobakteri ve benzeri biyostimulantların tarımda kullanımı, çevre dostu ve sürdürülebilir tarımsal üretime katkı sağlayarak, süs bitkileri de dâhil olmak üzere bahçe bitkileri üretimini iklim değişikliğine daha dayanıklı hale getirebilir.

Anahtar Kelimeler: Süs bitkileri, *Pelargonium*, PGPR, protein profili

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1. Introduction

Plant growth promoting rhizobacteria (PGPR) have successfully established themselves in various environments within soil ecosystems due to their high adaptability and rapid growth rates. As a result, they are considered essential components in agricultural production, owing to their natural genetic potential (Sezen and Külekçi, 2020; Ünlü et al., 2023). PGPRs usually colonize plant root systems, facilitating growth and suppressing the proliferation of harmful microorganisms (Srivastava and Govil, 2007; Eid et al., 2009; Sharma and Kaur, 2010). Various studies have shown that PGPRs are utilized worldwide as plant growth regulators in the cultivation of ornamental plants, owing to their capacity to improve plant growth parameters (García Fraile et al., 2012; Flores Félix et al., 2013; Zulueta Rodriguez et al., 2014; Karagöz et al., 2016). PGPRs enhance plant development by synthesizing growth hormones, maintaining microbial balance in the rhizosphere, and modifying mineral availability to improve nutrient absorption (Sıddıqui, 2006; Şevik, 2010).

Additionally, inoculation of PGPR has been found to enhance plant survival and root colonization more effectively than free-living PGPR found in the soil. These bacteria can increase the plant's capacity to uptake and utilize nutrients due to their ability to solubilize minerals, reduce ethylene levels, fix nitrogen, and bind phosphorus (Tütüncü et al., 2024). By producing phytohormones and regulating the microbial balance in the rhizosphere, PGPR positively influence plant growth and facilitate the uptake of essential mineral nutrients (Kisvarga et al., 2022; Ünlü et al., 2023). Enhanced nutrient assimilation and absorption can improve the plant's nutritional status and resource use efficiency, resulting in overall improved growth and development (Paradikovic et al., 2019; Mohamed et al., 2021). PGPRs influence plant growth and health through both direct and indirect mechanisms. These bacteria can promote plant growth by fixing nitrogen, producing phytohormones, and enhancing the uptake of iron and trace elements through the production of bacterial siderophores (Tütüncü et al., 2024). They also solubilize mineral and organic phosphates and convert other nutrients into plant-absorbable forms. Research has demonstrated that PGPR can enhance plant growth and yield under abiotic stress conditions by facilitating the uptake of essential nutrients and enzymatically reducing ethylene levels (Samancıoğlu and Yıldırım, 2015).

Nitrogen, phosphorus, and potassium are the most critical nutrients for plant species, with phosphorus being second only to nitrogen in importance. Phosphorus is essential for energy transfer within plants, the transport and storage of compounds such as sugars and starches, and the formation of nucleic acids (DNA), playing a crucial role in gene transfer. Additionally, phosphorus is vital for flower and fruit formation, root development, cell division,

and new cell formation, making it indispensable for plant growth and reproductive success (Çakmakçı et al., 2009). Although phosphorus is abundant in soils, only a limited fraction is readily available for plant uptake. Certain root-associated bacteria can solubilize this unavailable phosphorus, making it accessible to plants. PGPR promote plant growth by enhancing the solubility and uptake of minerals like phosphorus, which acts as a direct mechanism of their beneficial effects (Gyaneshwar et al., 2002). The influence of PGPR on phosphorus solubilization primarily arises from their effect on soil pH. Organic acids, including gluconic acid and citric acid, as well as proton (H+) pumping by root bacteria, can modify soil pH, facilitating the transformation of phosphorus into a form that is readily absorbable by plants (Seshadri et al., 2000). Among the best phosphate-solubilizing rhizobacteria are species belonging to the genera *Pseudomonas, Bacillus*, and *Rhizobium* (Antoun and Prevost, 2006).

In this study, the effects of bacterial isolates belonging to the *Bacillus* species, identified through genomic methods and *in vitro* studies as having PGPR potential and high AC phosphatase production efficiency, on seedling quality and flowering of *Pelargonium zonale* seedlings were investigated.

2. Material and Methods

2.1. Plant Material

Seedlings of *Pelargonium zonale* (geranium) were used as the plant material. The small, potted seedlings were commercially sourced and transplanted into 18 cm diameter pots containing a substrate mixture (1:1:1, v/v, peat, sand, perlite). The plants were grown in pots inside a non-heated polycarbonate greenhouse under natural daylight conditions. The plants were watered twice a week, applying 250 ml per pot. Additionally, during the summer, the greenhouse was covered with a shading net (providing 80% shade) to protect the plants from excessive sunlight.

2.2. Rhizobacteria Isolation from Soil

To isolate rhizobacteria, sixty soil samples were collected from various locations in Central Anatolia, Türkiye, at altitudes ranging from 1000 to 1680 meters. The samples were taken from soil around alfalfa, a leguminous forage crop. Each sample was transferred into bottles containing 50 mL of sterile saline water (0.9% NaCl) and subsequently into Luria-Bertani Broth (LB) medium. Samples in sterile saline water were shaken for 30-40 minutes, while those in LB medium were agitated in an orbital shaker for 4-6 hours to ensure homogenization. Afterward, aliquots from each sample were spread onto solid LB and Nutrient Broth (NB) media using the spread plate technique and incubated at 35°C for 12-24 hours.

Distinct colonies were then selected, purified, and transferred to NB medium for further culturing (Upadhyay et al., 2009).

This section of the study, conducted as part of a PhD thesis at the Faculty of Agriculture, Erciyes University, involved 55 bacterial strains from 6 species. The rhizobacteria were identified using SANGER sequencing and MALDI-TOF. Among these strains, the *B. megaterium* strain U2-1 (SANGER- OL673801 EU.U21) and *Pseudomonas putida* 9-4-2 strain (MALDI-TOF) were selected for further analysis. The AC Phosphatase gene regions of these strains were identified using PCR analysis, and their phosphate-solubilizing capabilities were assessed under *in vitro* conditions.

2.3. DNA Isolation from Rhizobacteria

Bacterial DNA was extracted using a modified version of Wilson's (2001) method. Bacterial isolates were grown in 10 mL of Nutrient Broth (NB) at 28°C (±2°C) with continuous shaking at 150 rpm for 24 hours. Post-incubation, 1.5 mL of the culture was centrifuged at 10,000 × *g* for 5 min to collect the cells. The cell pellet was then resuspended in Tris-EDTA buffer, with the subsequent addition of 10% SDS and proteinase K, and incubated at 37°C for 1 h. Following this, 5M NaCl and CTAB/NaCl solution were added, and the mixture was incubated at 65°C for 10 min until a white precipitate appeared. The samples were treated with chloroform/isoamyl alcohol (24:1, v/v) and centrifuged. DNA was then precipitated from the supernatant using isopropanol. The DNA pellets were air-dried and finally dissolved in TE buffer.

2.4. DNA Amplification and Identification of Bacterial Strains

Bacterial DNA was tested using AC Phosphatase-specific primers F (5'-AAGAGGGGCATTACCACTTTATTA-3') and R (5'-CGCCTTCCCAATCRCCATACAT-3') to identify strains possessing the AC Phosphatase gene (Raddadi, 2008). Each PCR reaction consisted of a reaction mixture prepared with 16.9 µl of sterile water, 2.5 µl of 10x PCR buffer, 0.5 µl of dNTP mix (10 nM), 0.2 µl of each primer (0.3 pM), 2 µl of MgCl2 (2.3 mM), 0.20 µl of DNA Taq polymerase (0.5U), and 2.5 µl of bacterial DNA (30 ng), resulting in a total volume of 25 µl. PCR amplification was conducted (denaturation at 95°C for 3 minutes, followed by 25 cycles of denaturation at 94°C for 1 minute, annealing at 57°C for 1 minute, and extension at 72°C for 1 minute, elongation step was performed at 72°C for 10 minutes). The resulting PCR products underwent analysis by electrophoresis on a 1% agarose gel. To identify bacterial isolates, 16S rDNA sequences were amplified using universal primers (16S forward 5'-AGAGTTTGATCCTGGCTCAG-3' and 16S reverse 5'-CCGTCAATTCCTTTGAGTTT-3')

following Edwards et al. (1989). The amplified products were sequenced and compared against microbial databases for species identification.

2.5. In Vitro Phosphate Solubilization of Bacterial Strains

The phosphate-solubilizing ability of *B. megaterium* strain U2-1 and *P. putida* strain 9-4-2 were assessed using NBRIP medium (Nautiyal et al., 2000). Bacteria were inoculated and incubated at 26°C. After 48 hours, cultures were transferred to NBRIP-BPB liquid medium. Tubes were incubated at 26°C for two weeks. Positive results were indicated by a color change in the medium from blue to light blue or transparent (Nautiyal, 1999; Coşkun, 2022). Phosphate solubilization was measured at 450 nm using a spectrophotometer, and results were compared to a control (NBRIP medium).

According to Pikovskaya (1948), solid PVK medium (obtained from HI-Medium Laboratories Pvt. Ltd.) was used for inoculation. Four bacterial strains were inoculated onto each plate in triplicate using sterile cotton swabs. After incubation at 30°C for one week, the diameter of the clear zone around the bacterial colonies and the colony diameters were measured. The clear zone diameter was calculated by subtracting the colony diameter from the total diameter (Nautiyal, 1999).

2.6. Preparation, Activation and Treatment of Rhizobacteria

Bacterial solutions were prepared from two rhizobacterial strains confirmed to possess the AC Phosphatase gene region through PCR analysis and exhibiting phosphate solubilization properties under *in vitro* conditions. Nutrient Agar (NA) and Nutrient Broth (NB) media were employed for bacterial activation and culture (Yılmaz, 2010; Ünlü et al., 2023). The rhizobacteria were inoculated onto NA plates and incubated at 35°C for 24 hours. A single colony from the activated bacteria was then transferred to NB medium. The inoculated broth was incubated overnight at 35°C while shaking at 180 rpm (Yılmaz, 2010). Bacterial solutions were prepared at a concentration of 3×10^8 cells/ml.

The experiment was conducted in triplicate, with two pots for each replicate. Bacterial culture prepared from the rhizobacterial strains were applied to potted geranium plants through irrigation water, administered a total of three times at 15-day intervals.

2.7. Morphological Measurements

Measurements and observations were recorded at the end of the 12th week to evaluate the effects of different rhizobacterial formulations on the quality and flowering of geranium seedlings. To assess the impact of PGPR applications on the seedlings, the following parameters were measured: plant height, number of leaves, leaf diameter, number of stem branches, number of flower inflorescences, length of the pedicel, and number of flowers per inflorescence.

2.8. Protein Isolation

Protein extraction from geranium leaf samples were processed using the TCA (Trichloroacetic acid)/acetone method (Walker, 2002). Eighty grams of tissue were ground in liquid nitrogen, and 1 g of the resulting powder was mixed with 4 mL of 10% TCA/acetone. The supernatant was discarded, and the pellet was suspended in 10% TCA/acetone with 5 mM DTT. This step was repeated, followed by a final suspension in 80% acetone with 5 mM DTT (1,4-Dithiothreitol) incubated at -20°C for 10 minutes. The pellet was then air-dried at 25°C.

2.9. Protein Concentration Measurement

The protein concentration was quantified using the "Bio-Rad Protein Assay" technique. Bovine serum albumin (BSA) served as the reference standard for constructing a calibration curve. Absorbance readings were taken at 660 nm for various concentrations of BSA (0.125, 0.250, 0.500, 0.750, 1, 1.5, and 2 mg/mL) as described by Bradford (1976).

2.10. SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis)

To assess variations in the protein expression profiles of the plants, the SDS-PAGE technique was utilized. Samples were analyzed through polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS). After electrophoresis, silver staining was applied to the gel. Subsequent visualization and analysis of the stained gel were carried out as outlined by Walker (2002).

2.11. Statical Analysis

The data collected were subjected to analysis of variance (ANOVA) using JMP software (version 13.2.0). Mean comparisons were made at significance levels of 0.05 and 0.001, applying the Duncan test.

3. Results and Discussion

3.1. Identification of Bacterial Strains with Primers and in Vitro Analysis

In the PCR analysis conducted on the DNA of *B. megaterium strain* U2-1 and *P. putida* strain 9-4-2, specific bands of approximately 750 bp were observed (Figure 3.1). Additionally, the phosphate solubilization potential of these isolates was confirmed in liquid NBRIP medium. The phosphate solubilization index was determined to be 3.60 for *B. megaterium* and 3.45 for *P. putida* (Table 3.1). These results suggest that both isolates have significant potential for phosphate solubilization, which could be beneficial in promoting plant growth.

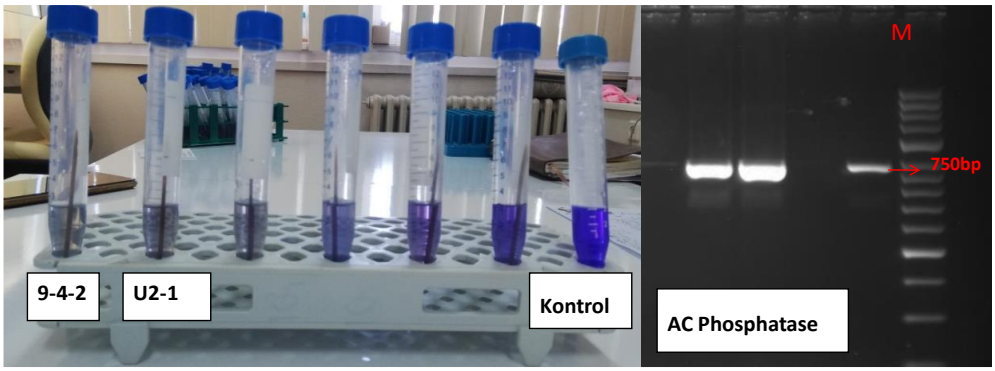

Figure 1. Evaluation of phosphate solubilization ability of *B. megaterium* (U2-1) and *P. putida* (9-4-2) in NBRIP-BPB liquid medium (left) and gel electrophoresis image showing AC Phosphatase gene presence determined by PCR analysis (right)

Table 1. Phosphate Solubilization Findings of Rhizobacteria Strains

Bacterial Strain	Zone Diameter (mm)	Colony Diameter (mm)	Phosphate Solubilization Index	Microplate Reading (nmol)
Control (water)	0.00	0.00	0.00	2.30
Bacillus megaterium (U2-1)	1.40	0.50	3.60	0.61
Pseudomonas putida (9-4-2)	1.20	0.40	3.45	0.66

3.2. Morphological Parameters Results

Overall, the application of PGPRs resulted in significantly higher seedling quality compared to the control group (Table 3.2, Figure 3.2). Specifically, in geranium plants, the flowering time was notably affected by the bacterial treatments. Plants treated with *B. megaterium* (U2-1) initiated flowering 16 days earlier than the control group, while those treated with *P. putida* (9-4-2) began flowering 19 days earlier. The first flower bud formation was observed 10 days after the PGPR application, followed by the appearance of flower clusters 14 days later, and full flowering occurred 3 days after that.

Plant height was found to nearly double with the rhizobacterial treatments. Additionally, the number of stems, leaf count, and leaf area increased significantly compared to the control plants. The pedicel length showed a statistically significant increase of approximately 1.4-fold over the control group. While only a single inflorescence formed in the control group, an average of 3.66 and 3.10 inflorescences were observed in plants treated with *B. megaterium* and *P. putida*, respectively. Moreover, the number of flowers per inflorescence increased by approximately 1.8-fold with the rhizobacterial treatments (Table 3.2, Figure 3.2).

Table 2. The effect of rhizobacteria treatments on flowering and seedling quality in geranium

Treatment	Flowering time	Plant height (cm)	Stem branching number	Leaf number	Leaf diameter (cm)	Flower: length of pedicel (cm)	Flower: inflorescence number	Inflorescence: flower number
***B. megaterium* (U2-1)**	29 June	21.5 [a]	3.5 [a]	27.66 [a]	8.8 [a]	22.5 [a]	3.66 [a]	32.33 [a]
***P. putida* (9-4-2)**	26 June	21[a]	2.5 [ab]	25.67 [a]	8.26 [b]	20 [b]	3.10 [a]	28.33 [a]
Control	15 July	12.67[b]	2 [b]	18.33 [b]	7.05 [c]	16 [c]	1.33 [b]	18.25 [b]
	*** p<0.05**	*	*	*	*	*	*	*

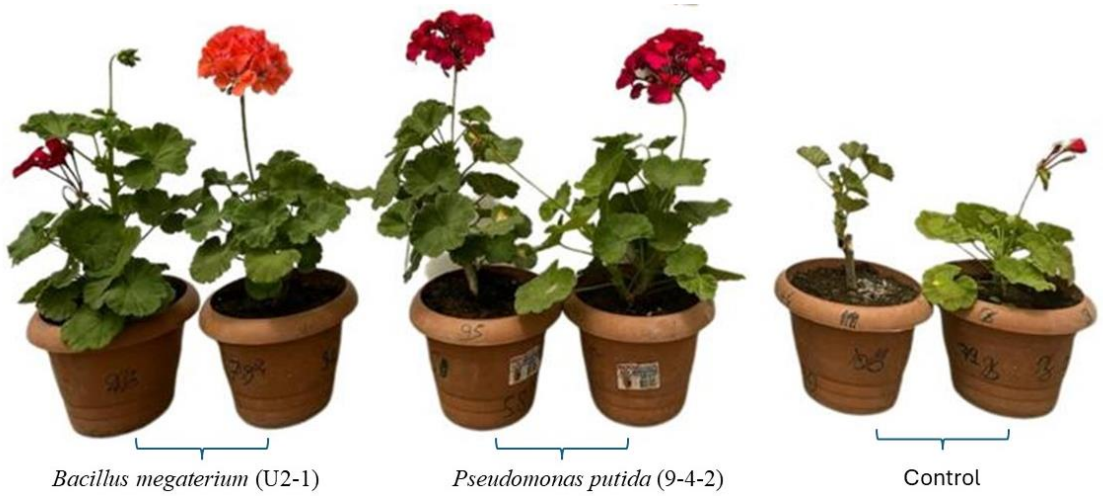

Figure 2. Visual of the effect of *B. megaterium* strain, *P. putida* strain and Control on flower quality

3.3. Protein Profiling Results

This study also aimed to determine the effects of rhizobacterial applications on the protein profile of geranium plants. The SDS-PAGE analysis revealed that the control group displayed a limited number of protein bands. In contrast, the most intense protein bands, ranging from 15 to 100 kDa, were observed in plants treated with *P. putida* (9-4-2). Similarly, *B. megaterium* (U2-1) treatments also produced dense and distinct protein bands compared to the control plants. As a result, the application of growth-promoting rhizobacteria, identified with the AC phosphatase gene region, led to notable increases and variations in the protein profile of geranium plants (Figure 3.3). Additionally, it was noted that *P. putida* produced more protein bands than *B. megaterium*.

(U2-1) (9-4-2) Control Marker

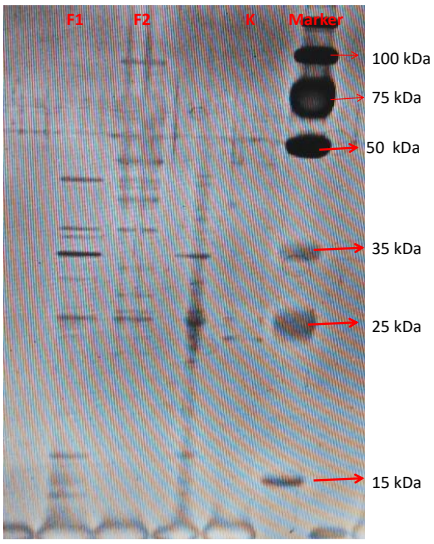

Figure 3. Gel image illustrating the effects of PCPR treatments on the protein profile in geranium

Based on the results of this study, it was determined that PGPR treatments, specifically those associated with the AC phosphatase gene region, significantly enhanced both flowering time and flower quality in geranium plants. Several studies have reported similar findings, demonstrating that species such as *Azotobacter, Agrobacterium, Bacillus, Rhizobium, Salmonella, Chromobacterium, Arthrobacter, Pseudomonas, and Streptomyces* are important phosphate-solubilizing bacteria that improve phosphorus uptake in plants (Rodriguez et al., 1999; Whitelaw, 2000). In fact, phosphorus is known to initiate cell division, which is critical for determining plant growth and productivity (Singh and Reddy, 2011).

Our results align with the findings of Pasley et al. (2019), who noted that increased bioavailability of nitrogen (N) and phosphorus (P) resulted in enhanced plant height, collar diameter, chlorophyll content, and root morphology. Similarly, Asewar et al. (2003) demonstrated that the application of 20 kg P_2O_5 in conjunction with *B. megaterium* significantly boosted plant biomass and nitrogen accumulation in plant tissues. Additionally, Sharma (2013) reported that PGPR applications not only enhanced yield, plant biomass, and soil phosphorus levels, but also exhibited a significant residual effect in subsequent growing seasons. In ornamental plants, Padmadevi et al. (2004) demonstrated that *Azospirillum* sp. improved the floral characteristics of *Anthurium andreanum* Lind. Similarly, in roses, *Agrobacterium rubi* applications enhanced root development (Orhan et al., 2006). Furthermore, Mishra et al. (2010) observed a significant increase in flower yield of *Pelargonium graveolens* following treatments with *Bacillus subtilis* and *Pseudomonas fluorescens* compared to the control. Lastly, Parlakova Karagöz (2018) highlighted the beneficial effects of PGPR on plant growth and quality parameters in *Euphorbia pulcherrima* Willd. ex Klotzsch (poinsettia).

Furthermore, previous research has indicated that treatments with PGPR bacteria result in significant increases in proteins and enzymes directly linked to plant growth and defense mechanisms, including superoxide dismutase, peroxidase, catalase, polyphenol oxidase, phenylalanine ammonia-lyase, lipoxygenase, and phenolic compounds (Liang et al., 2011; Chakraborty et al., 2013). Another study conducted on maize plants demonstrated that *Bacillus* spp. PGPR treatments resulted in positive alterations in plant protein profiles (Gökçek et al., 2021).

4. Conclusion

In recent years, most studies on phosphate solubilization have focused on isolating soil microorganisms and evaluating them in vitro phosphate-solubilizing activities. Beyond these traditional approaches, this study investigated the effects of PGPR strains containing the AC phosphatase gene region on flowering and flower quality under greenhouse conditions. The results demonstrated that *Bacillus* and *Pseudomonas* species significantly contribute to the growth and development of ornamental plants, primarily due to their phosphate-solubilizing capabilities. These findings reveal that *Bacillus megaterium* (U2-1) and *Pseudomonas putida* (9-4-2) have the potential to serve as environmentally friendly bio fertilizers, reducing dependency on synthetic fertilizers. The data demonstrate that these bacterial strains not only enhance plant growth and flower quality but also contribute significantly to sustainable agricultural practices. Their role in maintaining and improving soil fertility highlights their importance in addressing environmental concerns associated with chemical fertilizers.

Moreover, this study underscores the advantages of replacing conventional fertilizers with natural alternatives, emphasizing their benefits for both agricultural productivity and ecosystem management. These results suggest that Bacillus and Pseudomonas species warrant further exploration in biotechnological and agricultural applications. Investigating their effects on various plant species and their performance under diverse environmental conditions will be essential for optimizing their use and enhancing their efficacy as bio fertilizers. Promoting the strategic use of bio fertilizers for sustainable and eco-friendly agricultural production could play a pivotal role in overcoming current ecological challenges and developing more resilient farming systems in the face of climate change.

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