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Original Research Article

Isolation and Characterization of Plant Growth Promoting Endophytic Bacteria From *Peganum Harmala L*

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Abstract: *Endophytes are increasingly recognised as a sustainable alternative to chemical fertilisers and pesticides in modern agriculture. Syrian rue (Peganum harmala L.) is a medicinal plant of importance, yet no prior studies have reported the isolation of bacterial endophytes from its stem.* ***Objective:*** *To isolate, identify, and evaluate the plant growth-promoting and biocontrol potential of bacterial endophytes from the stem of Peganum harmala.* ***Methods:*** *This experimental study involved the isolation of six bacterial endophytes from the stem of P. harmala. Strains were identified using 16S rRNA gene sequencing, which revealed the presence of Bacillus stercoris, B. tropicus, Staphylococcus simiae, Cladifontibacillus erzurumensis, B. subtilis, and B. mobilis. Biochemical characterisation was performed to assess phosphate solubilization, siderophore production, ammonia and HCN production, hydrolytic enzyme activities (cellulase, protease, and pectinase), and indole-3-acetic acid (IAA) production with and without tryptophan supplementation. Antifungal activity against Aspergillus niger and Rhizoctonia solani was tested. Furthermore, the impact of bacterial inoculation on tomato seed germination and seedling growth (Solanum lycopersicum) was evaluated under controlled conditions.* ***Results:*** *B. mobilis demonstrated the strongest phosphate solubilization ability (4.0 ± 0.2 mm), while both B. tropicus and B. mobilis showed superior siderophore, ammonia, and HCN production. All isolates exhibited diverse hydrolytic enzyme activities, with B. stercoris showing the highest cellulase activity (13.8 ± 0.4 mm), B. tropicus demonstrating the greatest protease activity (7.6 ± 0.5 mm), and both B. stercoris and C. erzurumensis producing prominent pectinase zones (24 ± 0.3 mm). IAA production ranged between 4.9 and 5.9 µg/mL without tryptophan and 10.6 and 12.8 µg/mL with supplementation. Except for S. simiae, all isolates exhibited strong antifungal activity. Among plant assays, B. tropicus notably enhanced tomato seed germination compared to the uninoculated control.* ***Conclusion:*** *The identified endophytic bacterial strains, particularly B. tropicus and B. mobilis, demonstrated significant plant growth-promoting and antifungal properties. These strains hold potential as eco-friendly biofertilizers and biocontrol agents, offering sustainable alternatives to chemical inputs in agriculture.*

Keywords: Endophytic Bacteria, Biofertilizer, Peganum harmala, Biocontrol, and Bacillus sp

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Introduction

The modern agricultural landscape increasingly relies on chemical fertilizers, pesticides, and growth regulators to meet the growing demand for increased crop yields, driven by the escalating need for food production and the expanding global population. Therefore, there is a renewed awareness in the adoption of eco-friendly, sustainable, and organic agronomic practices. A growing number of countries around the world are adopting environmentally friendly agricultural approaches, with a particular emphasis on the use of bio-fertilizers and bio-pesticides as a promising avenue (21).

Endophytic niches are considered to shield bacteria from various environmental stresses and can be found in a wide range of plants, including both herbaceous and woody species. They are well-known for forming this relationship with a wide range of plant taxa. Many bacterial endophytes have been found in practically every plant part or tissue. However, the bacteria are very small and cannot be identified based on morphology. Therefore, marking of other parts of the body is added, namely nutrition and growth (14). The consequence of a positive interaction between bacterial endophytes and the plants they inhabit is better adaptation to the environment. Bacteria play a crucial role in promoting plant growth through mutualism by enhancing nutrient absorption and mineral solubilization. Furthermore, this connection helps protect the host plant from several diseases (6). Endophytic bacterial strains have a major impact on crop plant health and maintenance through nitrogen fixation, phosphate solubilization, siderophore and phytohormone synthesis, and stress tolerance (9). There are two kinds of beneficial bacteria: Plant growth promoting bacteria (PGPB) create phytohormones such as auxins (Indole acetic acid) and siderophores for iron availability, which directly improves plant growth by increasing nutrient availability and producing phytohormones such as auxins (Indole acetic acid), biosynthesis as a response to biotic and abiotic stresses, biocontrol activity and play a role in ethylene inhibition (15).

In this study, Peganum harmala L. was selected for the isolation of endophytic bacteria. It is a glabrous herb native to the eastern Mediterranean region that grows naturally in semi-arid temperatures, steppe areas, and sandy soils. It grows to be a 0.3-0.8 m tall shrub with short creeping roots, white blossoms, and spherical seed capsules containing over 50 seeds. It is an antibacterial, anti-inflammatory, and analgesic medicinal herb (5). This work aimed to isolate endophytic bacteria from Peganum harmala L. and evaluate their plant growth-promoting properties. To assess the potential of isolates for enhancing plant growth, isolated bacterial endophytes were inoculated into the seeds of tomato (Solanum lycopersicum), which was selected as the test crop.

Objectives: This research work was conducted to isolate endophytic bacteria from P. harmala L., investigate their plant growth-promoting activities, and evaluate their biocontrol potential against pathogenic fungi under in vitro conditions.

Methodology

Isolation of endophytic bacteria: Endophytic bacteria were isolated from the herbaceous plant Peganum harmala L., collected in Chakwal, and have been deposited in the Herbarium of Pakistan (ISL) at QAU

(Voucher number 133625). Plant (Peganum harmala L.) was carefully removed from the soil, washed to remove soil debris, and further divided into three parts, i.e., roots, stem, and leaves. Sterilization was performed by first washing the plant parts with 20% ethanol for 5 minutes, followed by a 2-minute treatment with 70% ethanol. Finally, the samples were rinsed with sterile distilled water (24). For further studies, pure and morphologically different colonies were chosen and preserved in glycerol stocks at -80 °C.

Molecular identification of isolated endophytic bacteria involved the use of 16S rRNA Gene sequence analysis. The extraction of DNA (total genomic) was achieved through the simple boiling method (13). Subsequently, the 16S rRNA gene amplification was performed using colony PCR, employing the 27F (GAGAGTTTGATCCTGGCTCAG) and 1492R (CTACGGCTACCTTGTTACGA) (universal bacterial) primers, resulting in a product of 1465 bp (7). For polymerase chain reaction, a total of 35 cycles (consisting of denaturation at 94 °C for 30 sec, annealing at 56 °C for 30 sec, and extension at 72 °C for 90 sec), preceded by an initial denaturation stage (94 °C for 5 min) and followed by a final extension stage (72˚C for 7 min). The Gene JET PCR purification kit (Thermo Scientific) protocol was used to purify the amplified PCR product, which was then subjected to 16S rRNA gene sequencing using Sanger sequencing technology on an ABI platform, conducted by Macrogen. Sequences were analyzed using NCBI, and strains were identified based on similarity rank. Accession numbers were obtained after submitting sequences to GenBank. For the tree construction sequence, we aligned using BioEdit. Aligned sequences were used to construct a tree using the Mega X software.

Biochemical characterization of endophytes: For the Methyl Red test, the endophytic bacteria were cultivated in a broth culture medium and incubated at 28 ± 2 °C for 24 hours. Subsequently, five drops of Methyl red indicator were added to the tubes (McDevitt, 2009). In Simmons citrate agar medium, endophytic bacteria were grown and incubated for 24 hours at 28°C ± 2 °C. Citrate-positive bacteria produce an alkaline reaction and change the color of the medium from green to bright blue, whereas citrate-negative bacteria do not (20). To assess a microorganism's ability to degrade urea using the enzyme urease. Christener's urea agar slant was made, and bacterial isolates were inoculated and incubated for 24-48 hrs. The colour shifting to pink signifies a positive result, while no colour change implies a negative result (8).

Plant growth-promoting activities

Phosphate solubilization: NBRIP medium was used to test the ability of bacterial isolates to solubilize inorganic phosphate (25). The bacterial endophytes were spot-inoculated on a phosphate medium, and the plates were incubated at 37°C for a week. Isolates that show positive outcomes produce a visible halo around the colonies.

Ammonia production: Selected endophytic bacterial isolates were cultured in 5mL peptone water and incubated for 48 hrs at 37°C. The isolates' ability to generate ammonia was evaluated by adding 0.5mL of Nessler's reagent. The development of the color from yellowish to brown indicates positive results (22).

HCN production: The method described by Ahmed et al. (30) was used for HCN production. TSA medium was supplemented with 4.4g (g/L) glycine. The bacterial isolates were spot-inoculated on this medium. The streaked plates were coated with filter paper that had been drenched in a solution of 2% Na2CO3 and 0.5% picric acid. For four days, the media plates were wrapped in parafilm and stored at 30˚C. The appearance of an orange to red color indicated the production of HCN.

Indole acetic acid: Fresh bacterial cultures were cultivated in a nutrient broth medium in the presence and absence of tryptophan at a temperature of 37˚C. Following a 24-hour incubation period, 1mL of the culture was transferred to an Eppendorf tube and subjected to centrifugation at 1000 rpm for 5 minutes. The resulting supernatant was collected in a test tube and then combined with 2mL of Salkowski's reagent. The appearance of pink color signifies the production of indole acetic acid. Subsequently, the absorbance of the solution was determined at a wavelength of 530nm using a UV Spectrophotometer after 25 min. For comparative purposes, the production of IAA was evaluated as a control (27).

Extracellular enzyme activity: Selected isolates were checked for protease activity on nutrient agar medium supplemented with 2% w/v skim milk powder (3). Fresh bacterial cultures were selected using a sterile cotton swab and spotted onto the respective medium. After 24 hrs, the plates were observed. The formation of clear zones around the test substance indicates positive protease activity.

The cellulase activity of the isolates was evaluated on medium supplemented with Congo red cellulose agar. Fresh bacterial isolates were spotted on the medium. Following incubation for 24 hours at 37°C, the halo zones indicate positive activity (17).

The bacterial strains were tested for pectinase activity on a specific medium supplemented with polygalacturonic acid as a substrate source. Bacterial isolates were spot-inoculated on the medium, and after the incubation period, the plates were flooded with a 1% iodine solution to check for clear zone formation surrounding the colonies (18).

Antifungal activity: Bacterial endophytes were tested for antifungal activity against Rhizoctonia solani and Aspergillus niger, a dual culturing technique was employed to screen the bacterial isolates on medium containing PDA and TSA in 1:1. The inoculation of bacteria on a media plate was done 2 cm away from the fungal disc, while the plate which was left uninoculated with the fungal disc was considered as a control (18). After incubating the plates at 30°C for up to 7 days, the antifungal activity was examined.

Plant growth promotion assay (In vitro): In the present study, tomato seeds are selected as experimental plants to assess the ability of bacterial isolates to enhance seed growth. This study aimed to provide insights into the plant growth-promoting (PGP) capacities of bacterial strains isolated from Peganum harmala L. Seeds inoculated with bacteria and non-inoculated seeds (serving as the control) were positioned onto filter paper plates within a plant growth chamber, where they were subjected to meticulously regulated conditions, encompassing a consistent temperature of 25˚C and a relative humidity of 60%. The resulting plantlets' root length was measured (1).

Statistical analysis: The data obtained from this experiment were analyzed using Microsoft Excel and Origin 2022.

Results

Isolation and identification of bacterial isolates through 16S rRNA gene analysis: Six morphologically diverse bacterial endophytes were selected for further analysis; the morphological characteristics of the endophytic bacteria are presented in Table 1.

Endophytic bacteria are Bacillus stercoris (ZP1), Bacillus tropicus (ZP2), Staphylococcus simiae (ZP6), Cladifontibacillus erzurumensis (ZP8), Bacillus subtilis (ZP9), and Bacillus mobilis (ZP10) identified through 16s rRNA gene analysis; their closest match, along with accession number and similarity index, is shown in Table 2.

Table 1: Colony morphology of the selected bacterial isolates from P. harmala L.

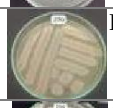
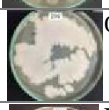
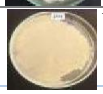
Isolates	Images of colonies	Color	Form	Size (mm)	Texture	Elevation	Margin	Gram staining
ZP1		**White**	**Irregular**	**2-3**	**Dry**	**Flat**	**Undulate**	**+**
ZP2		White	Circular	0.6-0.8	Creamy/ sticky	Raised	Entire	+
ZP6		White	Irregular	0.1-0.2	Slightly creamy	Flat	Entire	+
ZP8		Light yellow	Circular	0.8-0.9	Dry	Raised	Entire	+
ZP9		Off- white	Irregular	0.7-0.8	Dry, Sticky	Flat	Undulate	+
ZP10		Yellow	Irregular	0.8-0.9	Dry	Slightly raised	Entire	+

Table 2: Percent similarity of 16s rRNA partial gene sequence from our study and those from GenBank and their accession number.

Strains ID	Closest match	Accession no.	Similarity Index
ZP1	*Bacillus stercoris*	OR915514	99%
ZP2	*B. tropicus*	PP097213	98%
ZP6	*Staphylococcus simiae*	OR915517	98%
ZP8	*Cladifontibacillus erzurumensis*	OR826649	98%
ZP9	*B. subtilis*	OR915518	99%
ZP10	*B. mobilis*	OR921268	95%

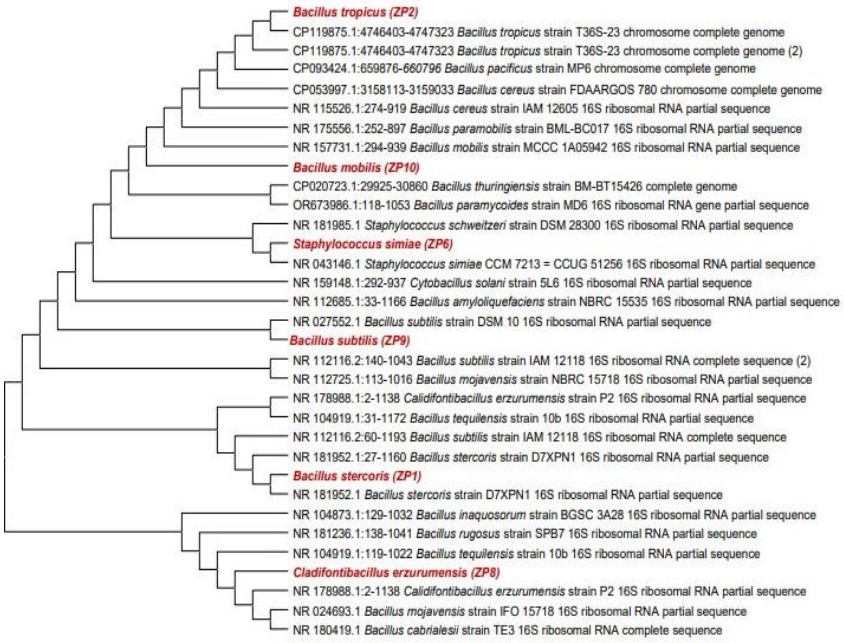


Figure 1: Phylogenetic tree of bacterial isolates from Peganum harmala L.

Biochemical and plant growth-promoting properties of bacteria: The ability to use citrate as an energy source and other biochemical activities (Urea base agar, methyl red test) and plant growth-promoting traits (phosphate solubilization, siderophore production, IAA, Ammonia, and HCN production) were studied. A citrate test is used to determine a microorganism's ability to utilize citrate as a carbon source. Bacillus stercoris, Cladifontibacillus erzurumensis, Bacillus subtilis, and Bacillus mobilis exhibited positive results on Simmons citrate agar. Bacillus tropicus, Staphylococcus simiae, Cladifontibacillus erzurumensis, and Bacillus

subtilis exhibited urease enzyme activity. Bacillus tropicus and Staphylococcus simiae oxidized glucose with the release and stabilization of high quantities of acid end products in the methyl red test.

Among the bacterial isolates, zones were formed for Bacillus tropicus, Staphylococcus simiae, Cladifontibacillus erzurumensis, Bacillus subtilis, and Bacillus mobilis, confirming that these bacterial endophytes possess phosphate-solubilizing properties. All the bacterial isolates exhibited positive outcomes to produce ammonia and hydrogen cyanide (HCN).

The synthesis of IAA in endophytic bacterial strains in the presence and absence of tryptophan produced a range of optical densities, as shown in Table 3. Endophytic bacterial isolates were found to possess the ability to assimilate free nitrogen from the air.

Antifungal activity: All the Bacillus species isolated from P. harmala showed antifungal activity against

Aspergillus niger and Rhizoctonia solani are shown in Figure 1 (D).

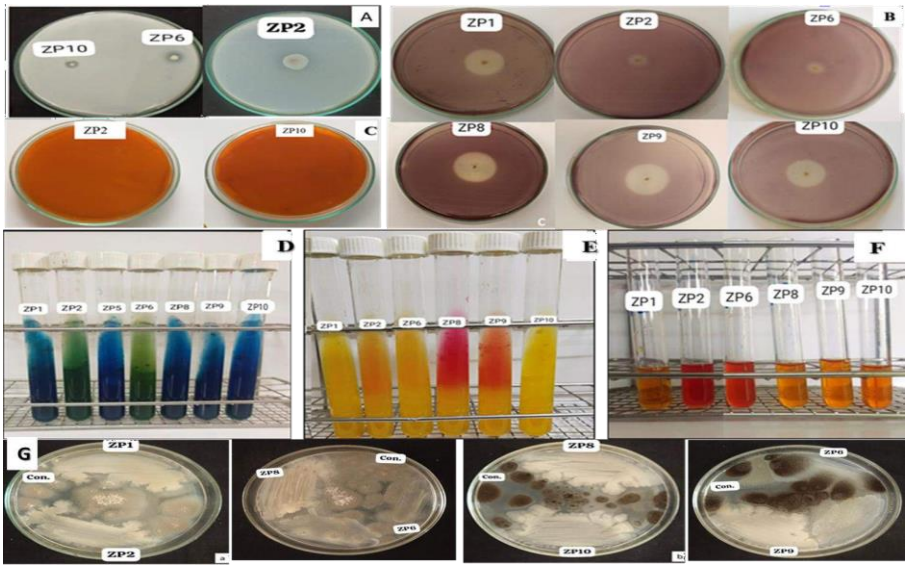


Figure 2: PGP and Biochemical properties of the bacterial isolates. A- Phosphate solubilization. B- Pectinase activity. C- Detection of HCN production. D- Detection of Citrate utilization. E- Detection of the urease test. F- Detection of methyl red test. G- (a) antifungal activity of Isolates against R. solani (b) Antifungal activity of Isolates against A. niger.

Extracellular enzyme activity: Every strain displayed affirmative cellulase and pectinase activity except ZP6. Isolates having cellulase and pectinase activity formed an approximate zone of 10-20mm.

Some of the isolated endophytes exhibited protease activity. For this activity, the largest zone size was exhibited by Bacillus tropicus, i.e.,> 7mm.

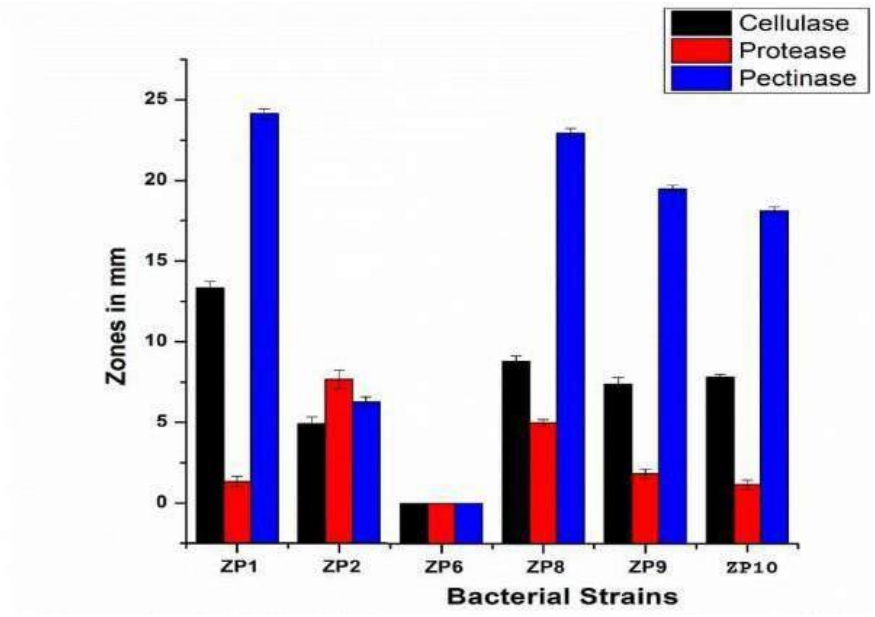


Figure 3: Graphical presentation of protease, pectinase, and cellulase-producing bacteria along with their zone diameter.

Indole acetic acid activity: Bacterial isolates were checked both in the presence and absence of L-tryptophan. Results revealed that the presence of tryptophan significantly enhanced IAA production, indicating its involvement in the IAA biosynthetic pathway. All the isolates generated more indole-3-acetic acid (IAA) in the presence of tryptophan, but significantly less in its absence. Only a few bacterial strains produced a substantially higher quantity of IAA compared to others. Isolate ZP9 produced the highest level of IAA, i.e., 12.8µg/mL, in the presence of tryptophan, while 5.8µg/mL was produced in the absence of tryptophan.

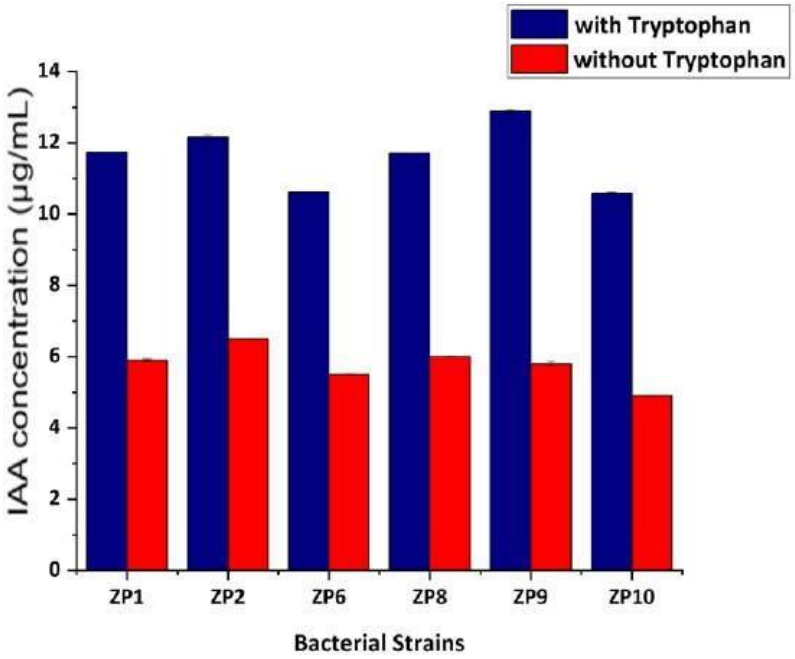


Figure 4: Indole acetic acid concentration with and without tryptophan

Endophytic bacteria affect the growth of Solanum lycopersicum (tomato): S. lycopersicum seeds inoculated with endophytic bacteria showed that B. tropicus, Cladifontibacillus erzurumensis, B. subtilis, and B. mobilis demonstrated a significant enhancement in the length of roots of the plantlets in comparison to the control.

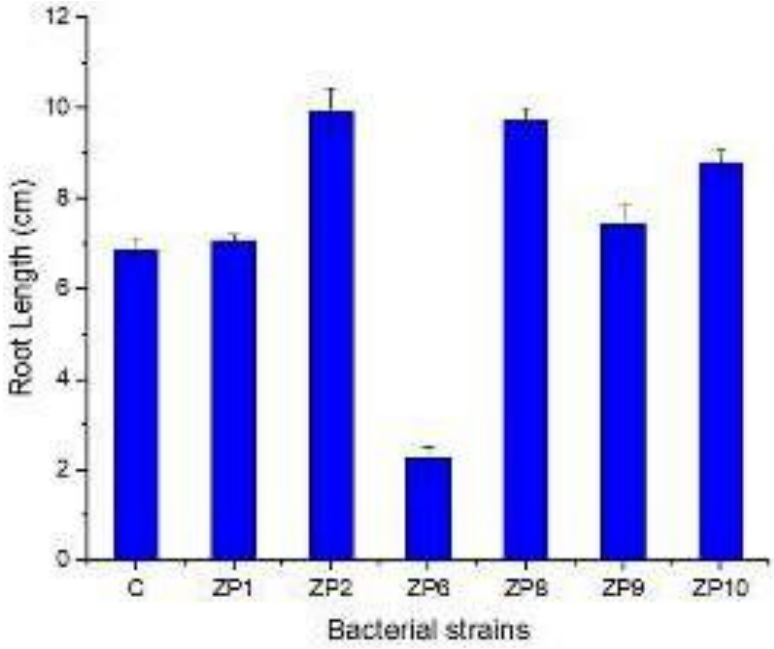


Figure 5: Effect of bacterial isolates on the root of S. lycopersicum

Table 3: Screening of Biochemical and Plant Growth Promoting Properties of Isolates

Strains ID	**ZP1**	**ZP2**	**ZP6**	**ZP8**	**ZP9**	**ZP10**
Strain name	***Bacillus stercoris***	***Bacillus tropicus***	***Staphylococcus simiae***	***Cladifontibacillus erzurumensis***	***Bacillus subtilis***	***Bacillus mobilis***
Biochemical Tests						
Simmons Citrate agar	+B	-	-	+B	+B	+B
Urea Base agar	-	+P	+P	+P	+P	-
Methyl red test	-	+R	+R	-	-	-
PGP properties						
Phosphate Solubilization	-	+	+	+	+	+
Ammonia production	+	+	+	+	+	+
HCN production	+	+	+	+	+	+
IAA production Without Tryptophan	5.9±0.04	6.5±0.01	5.5± 0.01	6.0± 0.01	5.8±0.05	4.9±0.01
With Tryptophan	11.7±0.01	12.2±0.07	10.6±0.01	11.7±0.01	12.8±0.05	10.6±0.02
Extracellular enzyme activity						
Protease activity	+	+	-	+	+	+
Pectinase activity	+	+	-	+	+	+
Cellulase activity	+	+	-	+	+	+

+ means growth, - means negative growth, P=pink, B=blue, R= red color

Discussion

In the current study, six bacterial strains were isolated from the plant P. harmala. These isolates belong to the genera Bacillus and Staphylococcus. Several species of the genus Bacillus are the second most abundant genus of all the isolated organisms. Endophytes from the Bacillus genus are known to aid in plant development in various plants (16). The current investigation found that most of the isolates solubilised phosphate, but the isolate Bacillus mobilis (isolate ZP10) showed significant activity (4.0±0.2mm), which may aid in boosting plant phosphorus availability. Sundara et al. (29) demonstrated that a phosphate-solubilising Bacillus megaterium enhances both the quantity of plant-available phosphorus and sugarcane production.

Results of this study showed that isolate B. tropicus (isolate ZP2) and B. mobilis (isolate ZP10) produced siderophores, which are consistent with the results presented by Wilson et al. (2006), in which different Bacillus species produced siderophores. Bacterial endophytes are capable of producing hydrogen cyanide (HCN). In our study, all species of two different genera produced HCN. B. subtilis (G5S) and B. velezensis (G6S), both isolated from the shoot system of Pelargonium graveolens, demonstrated HCN generation activity (2).

IAA is a key plant hormone that promotes plant growth in various ways. All the Bacillus sp. showed greater IAA production in the presence of tryptophan with the concentration of 10.6-12.8 µg/mL and comparatively less without the presence of tryptophan (4.9-5.9 µg/mL) which follow the results by Wagi and Ahmed (29) which showed that both the bacterial strains B. cereus (So3II) and B. subtilis (Mt3b) showed variable potential to produce bacterial IAA under different sets of growth and environmental conditions. Shim et al. (28) demonstrated that 90% of Bacillus species with high IAA production in the presence of tryptophan promote plant growth.

Most of the isolates suppressed fungal growth against Rhizoctonia solani and Aspergillus niger. According to Cho et al. (10), most Bacillus species have antifungal activity against phytopathogenic bacteria, such as R. solani demonstrated cellulolytic activity, but other species with antifungal activity showed only protease activity and no cellulolytic activity, which is in accordance with our current results, which showed that Staphylococcus samini (isolate ZP6) does not show antifungal activity, and their cellulase activity results are also not too significant. Furthermore, earlier findings involving bacterial isolates are consistent with our findings, such as those of B. subtilis (11), B. cereus, B. licheniformis, and B. megaterium (26), which also produce protease.

Bacterial endophytes play a crucial role in seed germination and seedling development. In this study, most of the strains tested were found to be positive for seed germination. All Bacillus strains produced promising results, but B. tropicus (isolate ZP2) had the best seed germination outcomes and increased root and shoot length. Hence, the PGP potential of the bacterial isolates can be enhanced by optimizing growth conditions for these isolates, which can be utilized for the optimal production of nutrients and their application in improving plant growth, leading to better yields in an eco-friendly manner.

Conclusion

The current rates of population growth demand the development of novel approaches for sustainable agriculture, with a crucial role being played by the utilization of beneficial microorganisms. Endophytes, which serve as an important resource, can be employed in various aspects of everyday life. Our current investigation reveals that endophytic bacteria derived from medicinal plants exhibit a wide range of plant growth-promoting traits. Notably, certain species belonging to the genus Bacillus are well recognised in this regard. Bacillus tropicus, Cladifontibacillus erzurumensis, and Bacillus mobilis have been proven to function as efficient facilitators of plant growth, with their modes of action mediated through the remarkable production of traits that promote plant growth. Future research should also encompass an examination of the stability and efficacy of the plant growth promotion traits following the inoculation of bacteria into natural (field) conditions to show their beneficial properties.

Declarations

Data Availability statement

All data generated or analyzed during the study are included in the manuscript.

Ethics approval and consent to participate

Approved by the department concerned. (24)

Consent for publication

Approved

Funding

Not applicable

Conflict of interest

The authors declared the absence of a conflict of interest.

Author Contribution

ZZB
Manuscript drafting, Study Design,
TY
Review of Literature, Data entry, Data analysis, and drafting articles.
ZKS
Conception of Study, Development of Research Methodology Design,

All authors reviewed the results and approved the final version of the manuscript. They are also accountable for the integrity of the study.

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