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Phosphate-Solubilizing Bacterial Endophytes Isolated from Cherry Tomato (*Lycopersicon esculentum*) Leaves

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ABSTRACT

Excessive utilization of chemical fertilizers can disrupt soil fertility, with specific concern regarding the contamination of soil by phosphate fertilizers. This contamination poses risks to the environment, affecting soil, air, and water quality. Countries relying heavily on chemical fertilizers in their agricultural practices are particularly susceptible to this escalating environmental issue. However, the introduction of natural products derived from endophytic microbes offers a novel approach to sustainable agricultural applications. These endophytes have demonstrated the ability to enhance crop yield and production, presenting an opportunity to promote sustainable farming practices. Within this research, a total of 23 endophytic bacteria were extracted from Lycopersicon esculentum (cherry tomato) leaves. Evaluating their phosphate solubilization efficiency, it was observed that three bacterial isolates, namely Bacillus licheniformis, Bacillus inaquosorum, and Bacillus pumilus, exhibited the capacity to solubilize complex calcium phosphate, forming visible clear zones around their colonies. Furthermore, the phosphorus solubilization index for these three endophytic bacterial isolates ranged from 1.84 to 2.18 over a five-day incubation period. The results revealed that B. licheniformis displayed the highest potential for phosphate solubilization. Consequently, B. licheniformis represents a promising alternative for enhancing phosphorus nutrition in cherry tomato (L. esculentum) cultivation, thus contributing to agricultural sustainability.

Keywords: *Phosphate, Bacterial endophytes, Lycopersicon esculentum, Agricultural sustainability*

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INTRODUCTION

The agricultural system faces a significant challenge in meeting the increasing food demand. As the global human population has doubled, there is an urgent need to ensure sufficient food production (Tahira *et al*., 2024). Cherry tomato (*Lycopersicon esculentum*) is a crop of great economic importance, cultivated worldwide. Its high vitamin A content, potassium, and antioxidants such as ascorbic acid, lycopene, and tocopherols contribute to its beneficial effects on human health (Pushpa, 2021). However, the excessive use of chemical fertilizers, particularly phosphate fertilizers, on cherry tomato crops poses health hazards to humans and has negative impacts on soil ecosystems (Guo *et al*., 2021). Countries extensively employing chemical fertilizers in agriculture inevitably face the worsening of this environmental problem, resulting in health issues for humans and pollution of water, air, and land (Guo *et al*., 2021). Consequently, there is a pressing need to explore new, effective methods for enhancing tomato plant growth (Mącik *et al*., 2020). One viable alternative to chemical fertilizers is the utilization of biofertilizers, which provide nutrients to tomato plants through their root systems, control soil-borne diseases, and improve soil health (Kour *et al*., 2020). Therefore, it is crucial to discover efficient methods for enhancing nutrient uptake in cherry tomato plants (Dong *et al*., 2023). Endophytes play a significant role in promoting plant growth by enhancing microbial processes that increase nutrient availability and assimilation by plants (Mushtaq *et al*., 2023).

MATERIALS AND METHODS

L. esculentum leaf sampling
A total of 300 leaves from healthy and mature *L. esculentum* plants cultivated in greenhouses and small farms in Jeddah were carefully chosen for analysis. These leaves were placed in sterile plastic bags and subsequently processed in a Microbiology Laboratory. The identification and verification of the plant's nomenclature and family were conducted using the specified procedures (Su *et al*., 2021).

Isolation of endophytic bacteria
Endophytic bacteria were obtained from leaves of *L. esculentum* using an adapted technique outlined in a previous study (Nordin *et al*., 2021). Leaf tissues were disinfected and then divided into 5×5 mm segments, with four pieces placed on nutrient agar plates. Incubation was carried out at 28 °C for 24 hours. Daily observations were made to assess the growth of bacterial endophytes on the Petri dishes.

Purification and maintenance of endophytic bacteria
To achieve purification, the endophytic bacteria were subjected to multiple rounds of sub-culturing until a completely pure culture of the bacteria was obtained. All cultured samples were incubated for 24 hours at a temperature of 28 °C. The pure cultures were then preserved on slant agar cultures at a

temperature of 4 °C.

Morphological identification of isolated bacteria

Phenotypic identification of all bacterial isolates was conducted based on pure cultures cultivated on an NA (nutrient agar) medium. The bacterial colonies were assessed for various characteristics, including cell shape, size, colony morphology, pigmentation, color, elevation, margin, and texture. Furthermore, Gram staining was performed to examine the arrangement of the colonies (Ijoma *et al*., 2021).

Molecular identifications of isolated bacteria

The identification of the chosen bacterial isolates was accomplished through the utilization of 16S rDNA gene sequences. A modified version of the genotypic method outlined by (Kassa *et al*., 2024) was employed for bacterial identification.

Screening of the isolated bacteria, in vitro, for phosphate solubilization

To perform the phosphate solubilization assay, 10 µl of bacterial broth cultures that were shaken overnight were inoculated onto the central region of modified Pikovskaya's medium. This medium consisted of 5.0 g $Ca3(PO4)$, 0.2 g NaCl, 0.2 g KCl, 0.1 g $MgSO4·7H2O$, 0.00025 g $MnSO4·7H2O$, 0.00025 g $FeSO4·7H2O$, 0.5 g $(NH4)2SO4$, 0.5 g yeast extract, 10.0 g glucose, 20.0 g agar, and 1,000 ml distilled water. The plates were then incubated at a temperature of 28 ± 2 °C in darkness for 3-5 days (Pereira & Castro, 2014).

Determination of phosphate-solubilizing capacity

The phosphorus solubilization index (PSI), which represents the semi-quantitative determination of the bacterial isolates' phosphate-solubilizing ability, was assessed using the following formula established by (Aliyat *et al*., 2022). The measurement involved determining the total diameter of the halo zone, which was achieved by obtaining the length of the halo zone from one edge to the opposite edge. Similarly, the length of the colony diameter was measured using the same procedure. The PSI was calculated as the ratio of the sum of the colony diameter and halo zone to the colony diameter itself.

RESULTS AND DISCUSSION

Morphology of endophytes bacteria isolated from plant leaves

A total of 23 bacterial strains were examined for visible morphological distinctions, including variations in colony shape, color, elevation, margin, and texture **(Table 1)**. Furthermore, Gram staining was conducted to assess the arrangement of the colonies **(Table 2 and Figure 1)**.

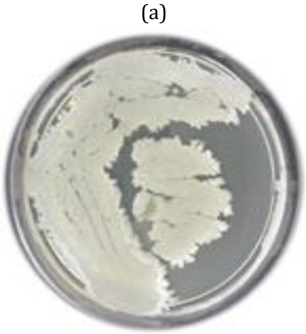
Table 1. Phenotypic characterization of bacterial endophytes isolated from *L. esculentum* leaves.

Serial No.	Bacterial isolates	Margin	Elevation	Texture	Appearance	Optical Property	Pigmentation
1	EB1	Convex	Entire	Smooth	Shiny	Opaque	Brown
2	EB2	Filiform	Irregular	Rough	Dull	Opaque	-
3	EB3	Entire	Convex	Smooth	Shiny	Opaque	-
4	EB4	Entire	Convex	Smooth	Shiny	Opaque	-
5	EB5	Irregular	Irregular	Smooth	Shiny	Translucent	-
6	EB6	Filiform	Irregular	Rough	Dull	Opaque	-
7	EB7	Filiform	Irregular	Rough	Dull	Opaque	-
8	EB8	Entire	Convex	Smooth	Shiny	Opaque	-
9	EB9	Entire	Convex	Smooth	Shiny	Opaque	-
10	EB10	Curled	Irregular	smooth	Shiny	Translucent	-
11	EB11	Filiform	Irregular	Rough	Dull	Opaque	-
12	EB12	Entire	Convex	Rough	Shiny	Opaque	-
13	EB13	Entire	Irregular	Smooth	Shiny	Transparent	-
14	EB14	Entire	Convex	Smooth	Shiny	Opaque	-
15	EB15	Entire	Convex	Smooth	Shinny	Transparent	-
16	EB16	Filiform	Irregular	Rough	Dull	Opaque	-
17	EB17	Filiform	Irregular	Rough	Dull	Opaque	-
18	EB18	Entire	Convex	Smooth	Shinny	Transparent	-
19	EB19	Entire	Convex	Smooth	Shinny	Opaque	-
20	EB20	Entire	Convex	Smooth	Shinny	Transparent	-
21	EB21	Entire	Convex	Smooth	Shiny	Opaque	-
22	EB22	Filiform	Irregular	Rough	Dull	Opaque	-
23	EB23	Entire	Convex	Smooth	Shinny	Opaque	-

Table 2. Presents the dimensions, color, and results of the Gram staining test conducted on the bacterial endophytes extracted from *L. esculentum* leaves.

Serial No.	Bacterial isolates	Size	Color	Gram morphology	Gram staining
1	EB1	Medium	Creamy	Rods	Negative
2	EB2	Small	Creamy	Short rods	Positive
3	EB3	Small	Yellow	Rods	Negative
4	EB4	Small	Creamy	Short rods	Positive
5	EB5	Medium	Creamy/ White	Short rods	Positive
6	EB6	Medium	Creamy	Short rods	Negative
7	EB7	Small	Creamy	Rods	Negative
8	EB8	Small	Creamy/ Orange	Rods	Negative
9	EB9	Small	Creamy	Short rods	Negative
10	EB10	Medium	White	Short rods	Positive
11	EB11	Medium	Creamy	Short rods	Positive
12	EB12	Small	Light orange	Short rods	Negative
13	EB13	Small	-	Short rods	Negative
14	EB14	Small	Creamy/ White	Rods	Negative
15	EB15	Small	-	Short Rods	Negative
16	EB16	Medium	Creamy	Rods	Positive
17	EB17	Medium	Creamy	Rods	Negative
18	EB18	Small	Light green	Short Rods	Negative
19	EB19	Small	Orange	Rods	Positive
20	EB20	Small	-	Short Rods	Negative
21	EB21	Medium	Creamy	Short Rods	Negative
22	EB22	Medium	Creamy	Short Rods	Negative
23	EB23	Small	Creamy	Short Rods	Negative

(a)



(b)

(c)

Figure 1. Morphological appearance of endophytic bacteria strains. (a) *B. licheniformis*, (b) *B. inaquosorum*, and (c) *B. pumilus.*

Molecular identifications of isolated bacteria

DNA extracted from the bacterial isolates was subjected to PCR amplification using a universal bacterial primer pair. The resulting PCR products appeared as distinct and strong bands on the agarose gel within the expected size range of 523 to 761bp. For molecular identification of the strains at the species level, their sequences were compared to those of known species already published in the NCBI databases, with a similarity threshold of 95-100% as shown in **Table 3**.

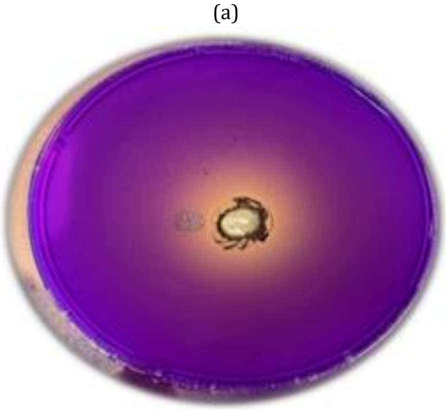
Table 3. Displays the molecular identification of the bacterial strains obtained in this study, along with their corresponding GenBank accession numbers and the percentage of identity.

Bacterial strains isolated in the current study			
Bacterial species	**Strain Code.**	**Accession No.**	**Identity (%)**
B. licheniformis	EB47	GQ280084.1	97.11%
B. inaquosorum	EB33	JN700079.1	98.46%
B. pumilus	EB17	MW301392.1	95.72%

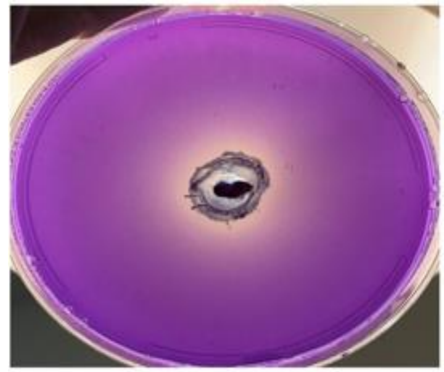
Phosphate solubilization assessment

Phosphate solubilization screening revealed that among the 23 endophytic bacterial isolates, three isolates demonstrated significant phosphate solubilization activity compared to the control strain. Consequently, these three isolates were selected based on their ability to effectively solubilize tri-calcium phosphate, resulting in the formation of distinct halo zones on Pikovskaya's medium supplemented with tricalcium phosphate. Generally, the size of the halo zones increased proportionally with the colony diameter **(Figure 2)**. The phosphate solubilization index of the endophytic bacteria on Pikovskaya's medium ranged from 1.84 to 2.18. The isolate *B. licheniformis* exhibited the highest phosphate solubilization index of 2.18, attributed to its proficient utilization of tricalcium phosphate on PVK medium. This was followed by *B. inaquosorum* and *B. pumilus* with solubilization indexes of 2 and 1.84, respectively.

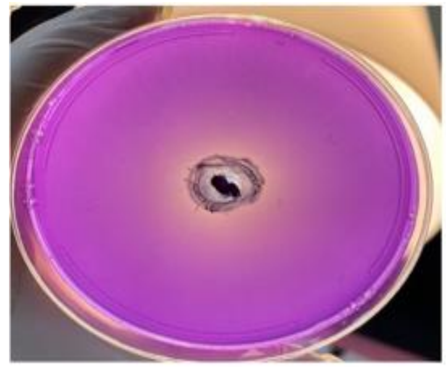
(a)



(b)



(c)



(d)

Figure 2. The formation of phosphate solubilizing zones by the bacterial endophytes. (a) Control, (b) *B. licheniformis*, (c) *B. inaquosorum*, and (d) *B. pumilus* on Pikovskaya's media was observed after incubation at 28 ± 2 °C in the dark for 3-5 days. Consequently, these isolates can be suggested as a cost-effective and environmentally friendly solution to mitigate the contamination caused by chemical fertilizers, thereby protecting the environment.

Endophytic microorganisms have gained significant attention due to their ability to protect their host plants against insect pests, pathogens, and herbivores (Chaudhary *et al*., 2023). Cherry tomato (*L. esculentum*) is a widely consumed crop known for its antioxidant and anticancer properties. The use of chemical fertilizers has a profound impact on the nutrient content of tomatoes. To address this concern, many countries have been exploring the substitution of inorganic fertilizers with cost-effective and environmentally friendly bio-fertilizers (Chen *et al*., 2022). Bacterial endophytes play a crucial role as plant growth promoters (Negi *et al*., 2023) and provide essential nutrients such as phosphate and nitrogen (Grabka *et al*., 2022). Therefore, this study aims to investigate the potential of these endophytic bacteria in promoting cherry tomato growth. A total of 23 bacterial isolates were identified from 300 samples of *L. esculentum* leaves. Most of these isolated bacteria have previously been reported as endophytes in various plant species (Nagah *et al*., 2024). Previous studies have highlighted the ability of Bacillus strains to solubilize zinc and enhance the growth of different plants, including tomatoes (*Solanum lycopersicum*) (Yadav *et al*., 2023). These endophytic bacteria produce natural bioactive compounds that play important roles in various processes. Numerous reports have discussed the diverse functions of endophytic bacteria (Abdelshafy Mohamad *et al*., 2020; Saqib *et al*., 2020; Mei *et al*., 2021). Several studies have demonstrated the ability of different endophytic bacterial species to release insoluble inorganic phosphate compounds, such as tricalcium phosphate (Wu *et al*., 2021; Hasan *et al*., 2024). Among the isolates, *B. licheniformis* exhibited high phosphate solubilization, making it a promising alternative for improving cherry tomato (*L. esculentum*) crops. Consistent with our findings, previous research has shown that Bacillus species are capable of solubilizing inorganic phosphate (Emami *et al*., 2019). Additionally, *Bacillus* spp. isolated from healthy tomato leaves (*L. esculentum*) have shown antagonistic activity against phytopathogenic fungi, as well as the ability to produce plant growth-promoting hormones and solubilize phosphate (Shah *et al*., 2020).

CONCLUSION

The use of chemical fertilizers has detrimental effects on the Earth's atmosphere, leading to air and groundwater pollution. Hence, it is crucial to explore endophytic bacterial isolates that can play a vital role in implementing safe agricultural practices. Numerous plants may harbor valuable bacterial isolates that warrant evaluation for diverse agricultural and environmental applications. In this particular study, the cherry tomato (L. *esculentum*) stands out as a host to abundant endophytic bacteria, particularly Bacillus species. These bacteria are widely recognized as biofertilizers due to their capacity to supply essential nutrients to crops, facilitate phosphate solubilization, and produce phytohormones. The positive interactions between the Bacillus species and the cherry tomato (*L. esculentum*) result in a range of beneficial effects, including phosphate solubilization, indole-3-acetic acid (IAA) production, and ammonia release.

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