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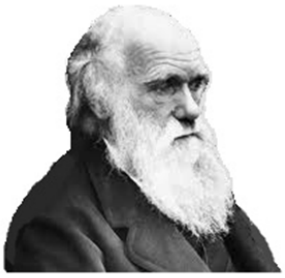

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Salt-tolerant endophytic *Pseudomonas putida* isolated from *Aronia prunifolia* root with plant growth-promoting potential

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ABSTRACT

Endophytes play a pivotal role in sustainable agriculture due to their capacity to generate numerous agriculturally significant metabolites. This study focuses on isolating a salt-tolerant, fluorescent green pigment-producing endophytic bacterium from the leaf samples of *Aronia prunifolia* cultivated on 2.5% Sodium chloride (NaCl) supplemented nutrient agar. After thoroughly examining the roots' isolated strain, EB-3, it has been identified as *Pseudomonas putida*. This identification was based on a detailed analysis of its morphological and biochemical characteristics, coupled with the scrutiny of its 16S rDNA sequence. Extensive biochemical and functional studies have revealed the diverse capabilities of *P. putida*. This bacterium excels in producing hydrogen cyanide (HCN), siderophore, indole acetic acid (IAA), and demonstrates proficiency in phosphate solubilization. These findings emphasize the potential of *P. putida* as a valuable bacterial inoculant for sustainable agriculture, especially in challenging environments. The versatility of this strain in producing beneficial metabolites underscores its crucial role in promoting agricultural resilience and productivity. Consequently, *P. putida* emerges as a promising ally in advancing sustainable agricultural practices, particularly in stressful conditions.

***Keywords*:** *Pseudomonas putida, Aronia prunifolia,* PGPR, Endophytic bacteria

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1. INTRODUCTION

Endophytic bacteria, which peacefully coexist within plant tissues, are gaining recognition for their crucial role in boosting plant growth and productivity without causing harm. With a growing focus on sustainable agriculture, there is increased interest in microbial alternatives to chemical inputs, with microbial endophytes being recognized as significant contributors. These microorganisms bring various benefits, such as phosphate solubilization, plant hormone production, and stress resistance, all promoting optimal plant development [1].

In the quest for sustainable solutions, isolating and characterizing endophytes from previously unexplored sources is vital. This study highlights *A. prunifolia*, commonly known as Purple Chokeberry, a native plant valued for its juice and jam production applications and admired for its ornamental qualities. Notably, the fruit of black chokeberry is renowned for its outstanding antioxidant content [2, 3].

Plants have evolved complex mechanisms to establish symbiotic relationships with microorganisms, particularly within their root systems. The Plant Growth-Promoting Rhizobacteria (PGPR) group, consisting of diverse bacterial species, plays a crucial role in influencing plant growth and health [4]. Pseudomonas, a versatile genus known for adaptability and metabolic capabilities, has emerged as a significant player in plant-microbe interactions [5]. While the positive effects of certain Pseudomonas strains on plant growth are well-documented, their specific contributions to Aronia, renowned for its antioxidant-rich berries, remain relatively unexplored.

The intricate interaction between plants and microorganisms within the root microbiome has long fascinated researchers trying to uncover the secrets behind enhanced plant growth. This study investigates the isolation and characterization of Pseudomonas bacteria in the roots of Aronia, aiming to reveal their potential role as PGPR. The relationship between plants and these microbial residents holds significant implications for agricultural sustainability and ecosystem resilience. By uncovering the mysteries of the plant-microbe partnership in Aronia, we hope to provide valuable insights that could contribute to sustainable agricultural practices and enhance overall ecosystem resilience.

2. MATERIALS AND METHODS

2. 1. Plant Sample Collection

In carefully collecting plant samples, we chose healthy *A. prunifolia* specimens, selecting a population entirely free from any signs of disease. This focus on disease-free plants is crucial to maintaining the integrity of our subsequent analyses.

To prepare the root samples for further investigation, we followed a detailed protocol. Each root underwent a thorough washing procedure to remove any external debris that could interfere with the subsequent isolation process. Afterward, we carried out a meticulous surface sterilization using a solution made of 75% ethanol and 0.2% $HgCl_2$. This strict sterilization protocol aimed to eliminate any external contaminants, ensuring that our analyses solely focused on the inherent characteristics of the plant and its associated microbiota.

The isolated and sterilized root samples were then carefully sectioned and underwent an incubation period on nutrient agar supplemented with 2.5% NaCl. This incubation occurred in a controlled environment, kept in darkness, at a temperature of 28 °C, spanning of 24 to 72

hours. We specifically chose these incubation conditions to promote the optimal growth and development of potential endophytic bacteria within the plant roots.

During the incubation process, we singled out colonies showing a distinctive fluorescent-green pigment for further examination. These colonies were identified as potential endophytic bacteria and were meticulously isolated for subsequent analyses. The isolation process was carried out with precision to ensure the purity of the obtained strains, and each isolated bacterium was distinctly labeled for future reference and investigation.

To maintain the viability of the purified bacterial strains for future studies, we used a preservation method involving 30% glycerol. This meticulous preservation ensures the isolated strains' longevity and stability, safeguarding their integrity and allowing for sustained viability over time. The preserved strains are stored at an appropriate temperature, preserving their physiological characteristics for prospective investigations and analyses.

2. 2. Identification of Isolates

In our quest to identify the potential bacterial endophytes, we took a thorough approach by combining both morphological and biochemical assessments to understand their basic characteristics. This involved evaluating cell shape, Gram reaction, spore formation, motility, as well as the production of catalase, oxidase, and growth at different temperatures [6].

Expanding beyond traditional methods, we integrated molecular analyses into the identification process. We used the CTAB technique to extract DNA from bacterial isolates, ensuring high-quality genetic material for subsequent analysis [7]. Molecular tests employed universal primers designed to amplify the 16S rDNA region, providing insights into the genetic makeup of the bacterial endophytes [8].

The obtained DNA sequences underwent a thorough examination using the Basic Local Alignment Search Tool (BLAST), a robust tool for comparing genetic sequences and identifying similarities with known sequences in public databases. This comparative analysis helped identify closely related bacterial species and clarified the isolates' taxonomic placement. To visually represent the evolutionary relationships among the bacterial isolates, we constructed a phylogenetic tree using the neighbor-joining approach [9, 10].

2. 3. Characterization of *P. putida* for PGP Traits

A systematic exploration of specific attributes was undertaken in the comprehensive characterization of *P. putida* for Plant Growth-Promoting (PGP) traits. The focus was on discerning key characteristics that contribute to the positive influence of these bacteria on plant growth and development.

2. 4. Detection of Indole-3-acetic Acid (IAA) Production

We employed a specific method to examine *P. putida*'s ability to produce the essential IAA. The bacteria were grown in a nutrient broth supplemented with tryptophan, a precursor crucial for IAA biosynthesis. The inclusion of tryptophan in the nutrient medium acted as a trigger, encouraging the bacteria to produce IAA. After the incubation period, we carefully analyzed the supernatant of the bacterial culture using the Salkowski reagent, a reliable indicator for detecting the presence of IAA. When the Salkowski reagent reacts with IAA, it creates a distinctive pink color, serving as a visible marker to quantify the production of IAA [11].

2. 5. HCN Production

The technique of Bakker and Schippers (1987) was followed for the detection and quantification of hydrogen cyanide using King's B medium supplemented with 0.44% of L-glycine. Whatman No. 1 filter paper strips were placed into test tubes containing liquid media that had been infected with 100 µL of bacterial inoculum after being steeped in a 0.5% picric acid + 2% Na_2CO_3 solution. After sealing the tubes with parafilm and cotton plugs, they were incubated for 48 hours at 28±2 °C. The filter paper's color changed from yellow to either light brown or reddish brown, signifying the production of HCN.

2. 6. Siderophore Production

We assessed the production of siderophores by *P. putida*, a crucial trait for acquiring iron and an important characteristic for promoting plant growth. We spotted cultures on Chrom Azurol S agar plates to determine siderophore production. A color shift from blue to orange around the colony indicated the presence of siderophores. This trait holds importance as it signifies the bacterium's capability to aid in iron uptake, benefiting bacterial metabolism and potentially enhancing iron availability for the associated plant [12].

2. 7. Phosphate Solubilizing Activity

We evaluated *P. putida*'s ability to solubilize phosphate, a crucial trait for nutrient availability, using Pikovskaya's agar plates containing tricalcium phosphate. The presence of a solubilization zone around the colony signified the bacterium's capacity to enhance phosphorus availability, ultimately benefiting plant uptake [13].

2. 8. Detection of Ammonia (NH_3) Production

Qualitative detection of ammonia production was done by the method given by Bakker and Schippers [14]. Bacterial isolates were grown in peptone water for 2-3 days at the optimum growth temperature. After incubation, 1 ml of Nessler’s reagent was added to each tube. Tubes showing a faint yellow color indicated a small amount of ammonia, and deep yellow to brownish color indicated a maximum amount of ammonia**.**

3. RESULTS AND DISCUSSION

3. 1. Isolation and Identification of Endophyte Bacteria

In our investigation, we successfully isolated endophytic bacteria from the roots of *A. prunifolia* using a careful surface sterilization technique to eliminate epiphytic microorganisms. The absence of microbial growth on the control plate confirmed the effectiveness of the sterilization method as shown in the Figure 1. The identification of EB-3 as an endophytic bacterium of *A. prunifolia* was strongly supported by the aseptic conditions maintained during the surface sterilization process.

The molecular analysis of the isolated strain EB-3 revealed an impressive 100% similarity to the *P. putida* KT792731 sample available in the National Center for Biotechnology Information (NCBI) database. This high degree of sequence identity strongly supports the classification of our isolate as *P. putida*. Consistent with the molecular findings, the morphological characteristics of the isolate closely match the established features of *P. putida*.

This agreement in both molecular and morphological aspects substantiates the accurate identification of the bacterial isolate as *P. putida*.

Figure 1. Initiation of endophytic bacterial growth from roots.

Based on the 16S rDNA sequence, the phylogenetic analysis placed our isolate within the cluster of *P. putida* with robust bootstrap support (Figure 2). This clustering further underscores the close evolutionary affinity of our isolate with known *P. putida* strains. The comprehensive morphological and biochemically characterized tests confirmed its identification as *P. putida*. The isolates exhibited negative reactions to Gram staining under a light microscope, generally appeared as short rods, were motile, did not produce endospores, tested positive for catalase and oxidase, and could not grow at 41 ºC but thrived at 4 ºC. Collectively, these findings support the accurate classification of EB-3.

The morphological, physiological, and biochemical characterization results indicated that the isolates belonged to the genus *P. putida*, following the methods outlined in Bergey's Manual of Determinative Bacteriology. The isolation of *P. putida* from *A. prunifolia*, especially strain EB-3, carries significant implications within the scientific literature. While the presence of *P. putida* in the internal tissues of various plants has been previously documented [15-17], this study stands out by confirming its existence in *A. prunifolia*.

Endophytic bacteria play a crucial role in sustainable agriculture due to their ability to produce a diverse array of essential metabolites, including plant hormones, enzymes, organic acids, siderophores, HCN, antibiotics, and antifungal metabolites for agricultural applications [18, 19]. This research contributes to our understanding of the microbial diversity associated with *A. prunifolia* and underscores the potential agricultural benefits associated with the endophytic bacterium *P. putida*, specifically strain EB-3.

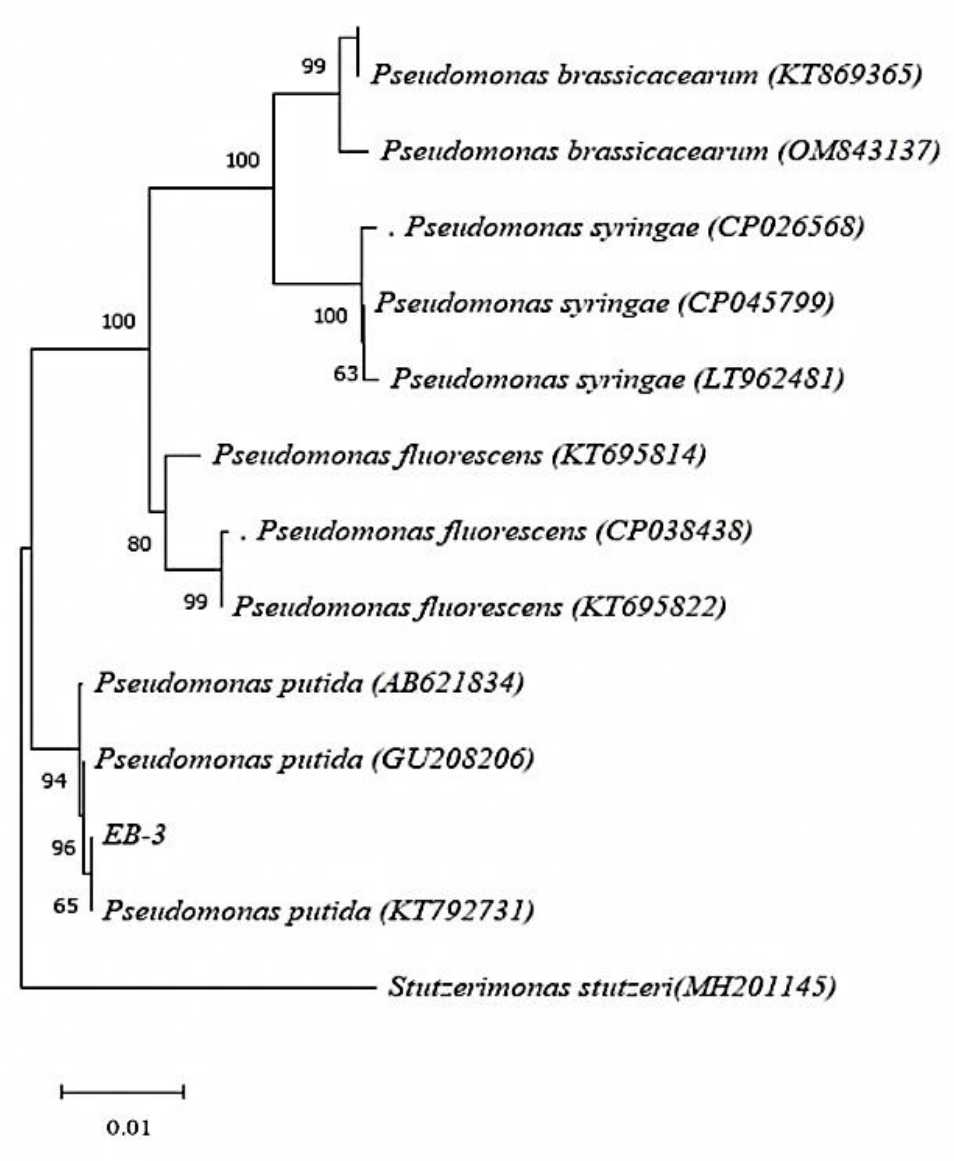

Figure 2. Phylogenetic tree based on the 16S rRNA gene sequences of strains of the *P. putida*

3. 2. Characterization of *P. putida* for Plant Growth Promoting Traits

The confirmation of IAA production by endophytic EB-3 was conducted through Salkowski's reagent test. The results revealed the development of a pink color upon the addition of Salkowski's reagent to the culture filtrate, indicating the successful production of IAA by the endophytic bacteria (Figure 3a).

The synthesis of IAA by endophytic microorganisms has been recognized as a potential factor contributing to the enhanced growth promotion observed in plants colonized by endophytes [20, 21]. This phenomenon underscores the significance of IAA as a plant growth regulator and suggests a potential role of endophytic bacteria, such as EB-3, in promoting plant development.

Several studies have highlighted the ability of various Pseudomonas species, including *P. putida*, to produce IAA [22, 23]. Moreover, documented instances show that inoculating seeds with *P. putida* substantially increased seedling root length, ranging from 2 to 3 times [24, 25]. These findings align with our study, supporting that endophytic *P. putida*, as demonstrated by strain EB-3, may positively influence plant growth through IAA production.

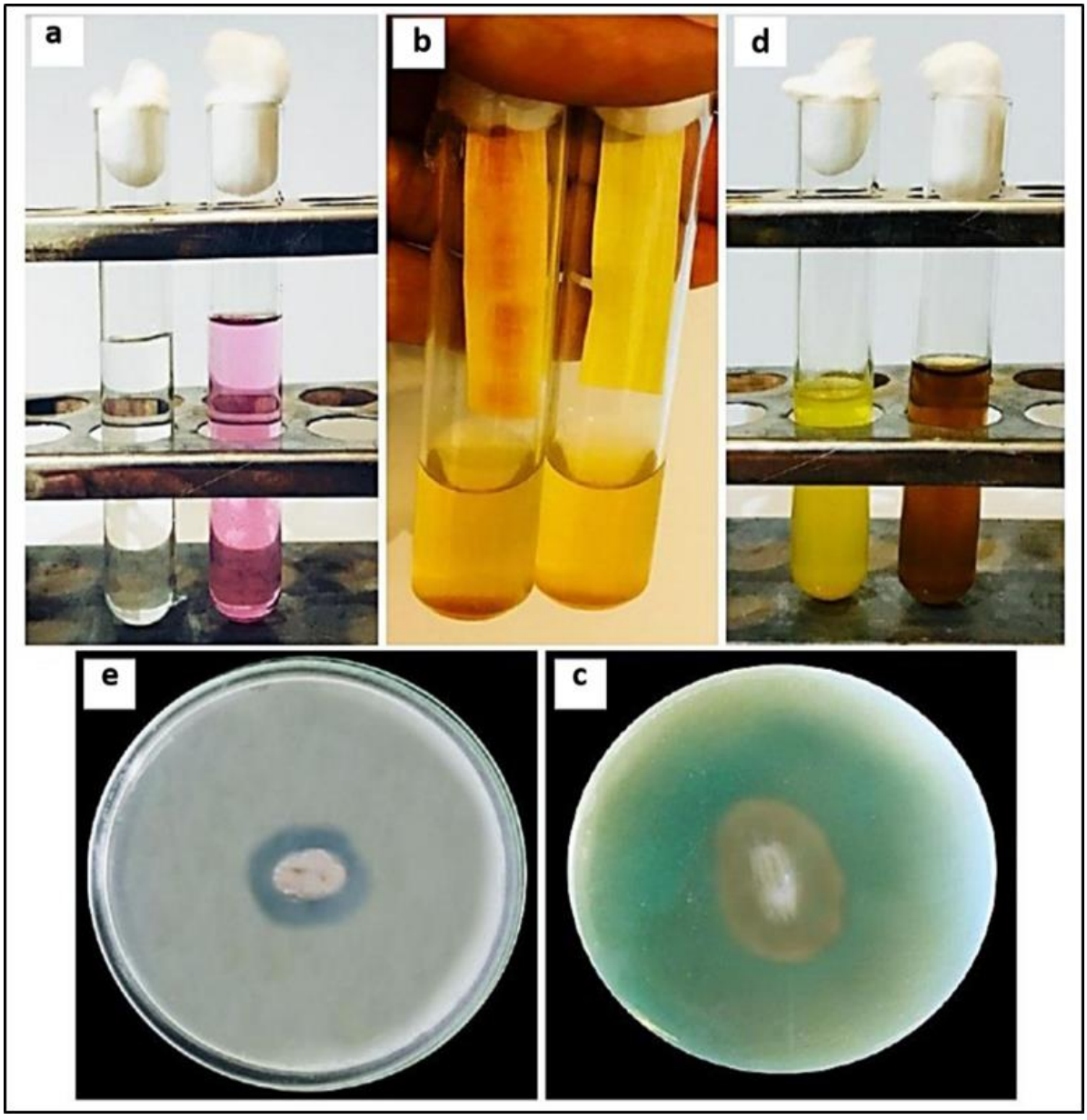

Figure 3. Screening of Plant growth promoting traits of *P. putida*: **a)** IAA production, **b)** HCN production, **c)** Siderophore production, **d)** NH_3 production, **e)** phosphate solubilizing.

Plant-symbiotic microorganisms have the ability to counteract plant-pathogenic microorganisms, known as "general suppression" or "general antagonism." This protective

mechanism is attributed to the production of HCN. Michelsen and Stougaard (2012) documented that HCN serves as a secondary metabolite produced by various antagonistic Pseudomonas species, effectively inhibiting the growth of hyphae belonging to *Rhizoctonia solani* and *Pythium aphanidermatum*.

The genus Pseudomonas, renowned for its PGP activities, has been reported to produce HCN and siderophores [27, 28]. Our results confirm these reports, which indicate that the isolated strain, EB-3, produces HCN and siderophores, as depicted in Figures (3b, c). This suggests that EB-3 could serve as an effective agent against plant-pathogenic microorganisms. In line with prior findings, *P. putida* is known to produce antimicrobial compounds such as siderophores, HCN, and hydrolytic enzymes, contributing to fungal cell-wall degradation, thereby reducing plant disease incidence and promoting plant growth [29, 30].

The agricultural value of *P. putida* lies in its robustness, competitive ability against pathogens, and capacity to produce secondary metabolites inhibiting pathogen growth. Analysis of various plant growth-promoting traits of isolate EB-3 revealed positive results for NH_3 and phosphate solubilization. The observed clear halo zones on peptone water agar and Pikovskaya media indicate ammonia production and inorganic phosphate solubilization activity (Figures 3d, e). These findings suggest that EB-3 can enhance lateral root development, mineral absorption, and the host plant's assimilation of nitrate and ammonium. Our results align with previous studies highlighting the significance of *P. putida* in stimulating plant growth, controlling pathogens, and its ability to produce ammonia and facilitate phosphate solubilization [31].

4. CONCLUSIONS

This research focused on exploring the root microbiome of Aronia and isolating Pseudomonas bacteria, specifically strain EB-3, sheds light on their role as PGPR. The confirmed production of IAA, HCN, and siderophores indicates their potential to boost plant growth and combat pathogenic microorganisms. The noted antimicrobial properties, characteristic of Pseudomonas, highlight their significance in agriculture.

These findings offer valuable insights for sustainable agriculture, emphasizing the potential of these bacteria to reduce dependence on chemical inputs and enhance ecosystem resilience. The study underscores the diverse benefits of the plant-microbe partnership in Aronia, suggesting broader applications for optimizing crop yields across various ecosystems. This knowledge sets the stage for future research, paving the way for innovative and sustainable agricultural practices contributing to overall ecosystem health.

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