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Original Research Article

MORPHOLOGICAL CHARACTERIZATION OF ROOT INHABITING ENDOPHYTIC BACTERIA

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Abstract *Endophytic bacteria (EBs) are beneficial to stimulating plant growth. However, little information about the EBs associated with soybean plant roots is available. This study examined the diversity of ten EBs isolated from soybean root tissues. Morphological and biochemical characterization methods revealed significant variation among the isolates. Most isolates displayed smooth colony surfaces, regular shapes, and rod-shaped cells. However, Bacterial Strain-5 and 10 exhibited rough surfaces and irregular shapes, while Bacterial Strain-4 displayed round cell shapes instead of the typical rod morphology. Moreover, the isolates demonstrated diverse pigmentation, with strains showing various shades of white, creamy, light yellow, pinkish red, Creamish, yellow, and red. In KOH test, three strains (2, 6, and 9) showed positive reactions to KOH, while the remaining seven strains (1, 3, 4, 5, 7, 8, and 10) showed negative reactions. The catalase test confirmed that four strains (1, 2, 6, and 9) were gram-negative, and the six strains (3, 4, 5, 7, 8 and 10) were gram-positive. In the gram staining test, seven strains (1, 2, 3, 6, 8, 9, and 10) were gram-negative, while the remaining three strains (4, 5, and 7) were gram-positive. Finally, in the starch hydrolysis test, seven strains (1, 2, 3, 6, 8, 9, and 10) were gram-negative, whereas the three strains (4, 5 and 7) were gram-positive. This study will help us identify various EBs that could play a role in the nodule formation and adaptation of soybean plants in diverse soil conditions in Pakistan*.

Keywords: *Soybean; Bacterial Endophytes; Morphological; Biochemical; characterization*

Introduction

Soybean (*Glycine max* L.) is a leguminous crop belonging to the *Fabaceae* family found in East Asia, widely grown for its safe and frequent use (Campo *et al.*, 2009). It is one of the most cultivated legumes worldwide due to its high protein content and important industrial by-products (Stacey *et al.*, 2004; Masuda and Goldsmith, 2009). It is grown in tropical and subtropical regions and temperate climates where the daytime soybeans need an inch of rain per week during critical growing phases (Nyoki and Ndakidemi, 2014). It is the oldest and most important crop in the world. It originated in China and has been cultivated for more than 5000 years. Soybean has attained significant global significance as an agricultural crop. It is cultivated in over 100 countries, with the United States, Brazil, and Argentina leading the production charts. Soybeans serve multiple purposes, such as producing vegetable oil, animal feed, and biodiesel. Additionally, they play a vital role in producing various food products, including tofu, soy milk, and soy sauce (Liu *et al.*, 2021).

Soybeans are extensively cultivated globally, with a record-breaking production of over 352 million metric tons in 2020. The United States remains a significant player, producing over 96 million metric tons in the same year. Brazil, Argentina, China, and India are also notable soybean producers. It is a legume crop that contributes significantly to global economic growth and sustainable agriculture. Due to its symbiotic relationship with endophyte bacteria in root nodules, soybean has a considerable capacity for nitrogen fixation. Due to the positive effects of endophytes on plant growth promotion, biocontrol, and disease resistance, endophytic microorganisms are currently considered a significant bioresource for contemporary agriculture (Peixoto Neto *et al.*, 2002). Endophytes live in root nodules, which are a component of the root system. Endophytes inhabit the apoplast of plants, which are the cell walls' intercellular spaces and the xylem vessels of those

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plants' roots, stems, and leaves. They can be found in tissues, flowers, fruits, and seeds (Brader *et al.*, 2014).

Endophytes are microscopic organisms associated with host plant tissues, such as fungi and bacteria, that secrete diverse bioactive compounds to stimulate plant growth without causing any harm (Strobel et al., 2004). Endophytes are involved in various biological processes, including the synthesis of siderophores, the production of plant hormones, the fixation of nitrogen, the solubilization of immobilized phosphorus, and the cycling of nutrients (Kusari *et al.*, 2012). Normally, plant roots absorb water and nutrients to support the growth of the plant tissues. In addition, they release a rich source of organic acids, amino acids, and sugars into the soil, which encourages the colonization of the soil with microorganisms at the surface of the plant roots. Any seed's germination also releases low molecular weight organic chemicals into the environment, luring rhizosphere and rhizoplane bacteria to the area (Thrall *et al.*, 2007).

EBs exhibit vast diversity and play critical roles in ecosystem function and plant physiology. These bacteria can benefit crops, including improved plant growth, nutrient acquisition, and disease resistance. They can colonies every plant part, even the intracellular and intercellular areas of the inner tissues. EBs can enhance medicinal plants' growth, promoting seed germination and increasing root and shoot biomass (Vendan *et al.*, 2010). Furthermore, endophytes can synthesize indole IAA, which is important in plant development (Fouda *et al.*, 2021). Additionally, EBs can reinfect nonhost plants and, thus, have been termed “true endophytes” (Rosenblueth and Martínez-Romero, 2006). Moreover, they serve as biocontrol and biofertilizer agents (Botta *et al.*, 2013). As such, EBs have the potential to be used for the production of sustainable agricultural systems.

EBs can improve P availability for plants via phosphate solubilization, using mechanisms such as chelation, acidification, ion exchange, and production of organic acids (Nautiyal *et al.*, 2000). Additionally, EBs can secrete acid phosphatase, which enhances the availability of phosphorus in the soil (Van Der Heijden *et al.*, 2008). Siderophores are necessary for the development and growth of plants, especially in iron-limited environments, as they provide iron to plants (Ma *et al.*, 2016). By chelating iron, siderophores ensure iron availability to plants and thus promote their survival and growth. Multiple studies have demonstrated the positive influence of bacterial siderophore production on plant growth. EBs have also been found to produce antimicrobial compounds that can prevent the growth of pathogens such as *Botrytis cinerea* and *Cylindrocarpon destructans* (Hong *et al.*, 2018)

EBs can also protect medicinal plants from diseases through "biocontrol." This process involves the displacement of plant pathogens from their niche within plant tissues by the EBs, which can lead to increased plant health and disease resistance. EBs can boost the resistance in host plants against abiotic stresses, such as high levels of metals and salinity (Sheng *et al.*, 2011). Endophytic Bacteria have been studied for their potential in plant disease management. Endophytes are known for their various beneficial mechanisms responsible for plant defense mechanisms and stress management of the host plant (Singh *et al.*, 2020). Studies have shown that endophytic bacteria can control various plant diseases such as wilt, damping off, and rot (Latha *et al.*, 2019). Endophytic bacteria play an important role in maintaining the health of their host plants by conferring tolerance/resistance to the host plants from diseases (Oukala *et al.*, 2021).

Materials and Methods

Survey of Soybean Field

A total of 3 soybean fields in MNSUAM were surveyed to assess the bacterial endophytes. In each field, ten healthy soybean plants were carefully selected for observation. Approximately a total of 200g of root samples were collected at a depth 5-6 cm in the winter after one month at the time of blossoming from the surveyed fields packed in polythene bags and brought to the laboratory for bacterial isolation, and stored in the refrigerator at 4°C until further processing.

Isolation and purification of Bacteria

Within 24 hours of sample collection, the plant samples were carefully cleaned with running water, sterilized the surface with 2% sodium hypochlorite, and washed with sterile distilled water. The surface-sterilized plant root samples were combined in a sterile 0.85% saline solution (NaCl) and crushed using a double sterilized pestle mortal. After that 500 ml nutrient agar (NA) media was prepared and autoclaved at 121°C and 15 psi for 20 minutes. Then NA media were carefully poured on sterilized petri plates in a laminar flow chamber. The saline solution containing the released endophytic bacteria is evenly spread onto NA media plates and wrapped in the plates. After proper tagging, the plates were kept in the incubator for 24 hours at 28 °C. After 24 hours, bacterial colonies of different colors were observed and streaked into new plates containing NA media using the streaking method for purification.

Morphological characterization

The bacterium that grew on the culture plate was observed visually and identified morphologically by observing its colony shape, colony color, cell shape, and colony surface and conducting biochemical tests like Gram staining, 3% KOH test (Holt et al., 2000;

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Mubeen et al., 2015) Catalase (Reiner, 2010) and Starch hydrolysis test.

Statistical Analysis

All the bacterial isolates were subjected to statistical software for data analysis. Analysis of variance was performed on the recorded data with 5% significance level. CRD was used to compare statistics among the treatments in Statistics 8.1 Software.

Results

Samples of healthy soybean plant roots were collected at the flowering stage (Figure 1). Isolation was performed through the roots crushed method on NA media plate, and adequate colonies were observed (Figure. 2). Pure and single colonies of ten bacteria were successfully grown by subculturing them on NA media plates (Figure.3).

Figure 1: Survey and sampling of Soybean plants in MNSUAM field

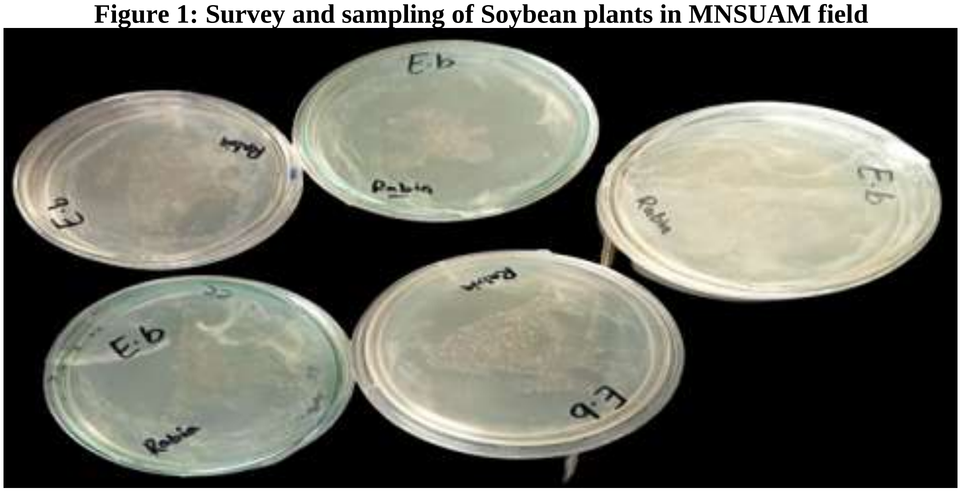

Figure 2: Isolation through root crushed method

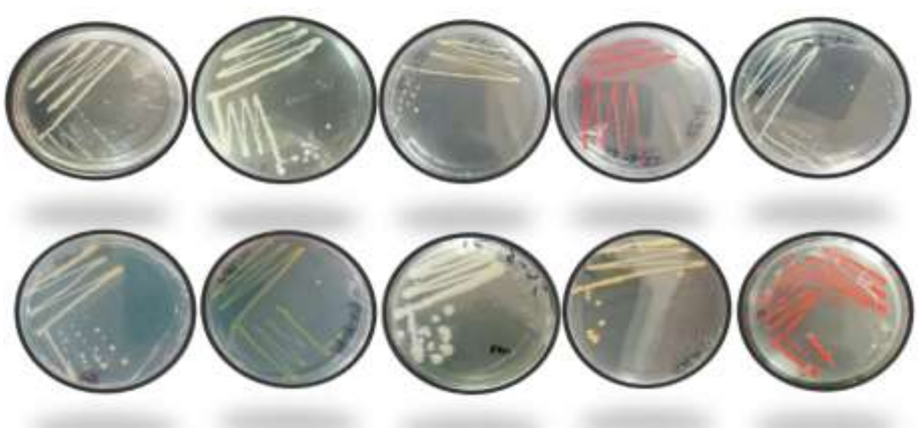

Figure 3: Purified 10 bacterial isolates

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All the bacterial isolates had smooth colony surfaces, except Bacterial Strain-5 and Bacterial Strain-10, which had rough surfaces. All bacterial isolates were regular in colony shape except Bacterial Strain-5 and Bacterial Strain-10, which were irregular. In cell shape, all the bacterial isolates were rod-shaped, except Bacterial Strain-4, which was round. In colony color, all bacterial isolates were tested in which three isolates viz., Bacterial Strain-2, 5, and 8 exhibited White pigmentation, Bacterial Strain-6 exhibited creamy White pigmentation, Bacterial Strain-3 exhibited light Yellow pigmentation, Bacterial Strain-10 exhibited Pinkish Red pigmentation, Bacterial Strain-1 exhibited Creamish pigmentation, Bacterial Strain-7 exhibited Yellow pigmentation and rest one isolated Bacterial Strain-4 exhibited red pigmentation. All bacterial isolates in the colony surface were observed in which eight isolates viz., Bacterial Strain-1, 2, 3, 4, 6, 7, 8, 9 exhibited smooth surfaces, and the other two isolates Bacterial Strain-5 and 10 exhibited rough surfaces. In colony shape, all bacterial isolates were observed in which eight isolates viz., Bacterial Strain-1, 2, 3, 4, 6, 7, 8, and 9 exhibited regular shape, and rest two isolates Bacterial Strain-5 and 1o exhibited irregulars. In cell shape, all bacterial isolates were observed to be rod-shaped except Bacterial strain-4, which was round (Table. 1).

Table 1: Morphological characteristics of collected isolates

Sr. No	Isolates I	Colony Surface	Colony Shape	Cell Shape	Colony Color
1.	Bacterial Strain-1	Smooth	Regular	Rod shape	Creamish
2.	Bacterial Strain-2	Smooth	Regular	Rod shape	White
3.	Bacterial Strain-3	Smooth	Regular	Rod shape	Light Yellow
4.	Bacterial Strain-4	Smooth	Regular	Round shape	Red
5.	Bacterial Strain-5	Rough	Irregular	Rod shape	White
6.	Bacterial Strain-6	Smooth	Regular	Rod shape	Creamy White
7.	Bacterial Strain-7	Smooth	Regular	Rod shape	Yellow
8.	Bacterial Strain-8	Smooth	Regular	Rod shape	White
9.	Bacterial Strain-9	Smooth	Regular	Rod shape	Light Yellow
10.	Bacterial Strain-10	Rough	Irregular	Rod shape	Pinkish Red

After performing conventional biochemical tests, the three tests bacterial strain-2, 6 and 9, showed positive reactions to KOH and rest seven *viz.*, bacterial strain-1, 3, 4, 5, 7, 8 and 10 showed negative reaction to KOH. Hence was confirmed that in the KOH test *viz.*, Bacterial Strain-2, 6 and 9 during the experiment were gram-negative and rest seven isolates *viz.*, Bacterial Strain-1, 3, 4, 5, 7, 8 and 10 were gram-positive.

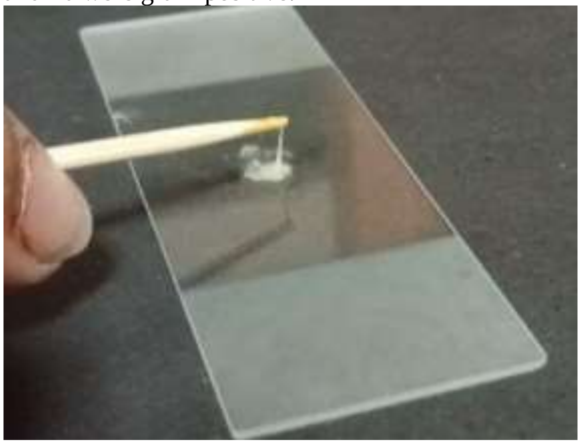

Figure 4: Bacterial Strain-5 KOH test

In the catalase test, four bacterial strain-1, 2, 6, and 9, showed a negative catalase reaction, and the rest, six *viz.*, bacterial strain-3, 4, 5, 7, 8, and 10 showed a positive catalase reaction. Hence, in the catalase test *viz.*, Bacterial Strain-1, 2, 6, and 9 during the experiment were gram-negative, and the rest of the nine isolates *viz.*, Bacterial Strain-3, 4, 5, 7, 8, and 10 were gram-positive.

Figure 5: Hydrogen peroxide formed bubbles in Bacterial Strain-4

In the gram staining test, seven bacterial strains *viz.*, 1, 2, 3, 6, 8, 9, and 10 exhibited pinkish under the microscope, and the rest three viz., 4, 5, and 7 were purple. Hence, the gram staining test confirmed that bacterial strains viz., 1, 2, 3, 6, 8, 9, and 10 were gram-negative, and the remaining four isolates *viz.*, *viz.*, 4, 5, and 7 were gram-positive.

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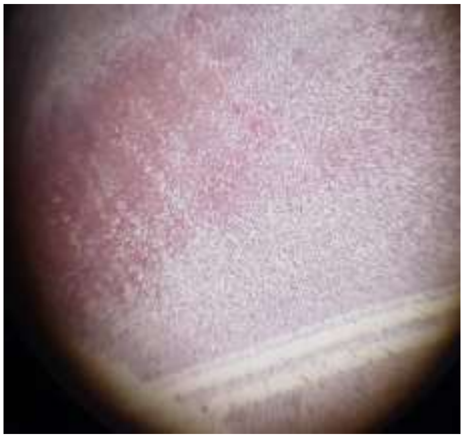

Figure 6: Bacterial Strain-6 Gram staining test slide under microscope

In the starch hydrolysis test, ten bacterial strains *viz.*, 1, 2, 3, 6, 8, 9, and 10, showed no reaction with gram iodine, and the remaining four *viz.*, 4, 5, and 7, showed a reaction with gram iodine. Hence, it was confirmed in the starch hydrolysis test that bacterial strains *viz.*, 1, 2, 3, 6, 8, 9, and 10 were gram-negative, and the rest of the four isolates *viz.*, *viz.*, 4, 5, and 7 were gram-positive (Table. 2).

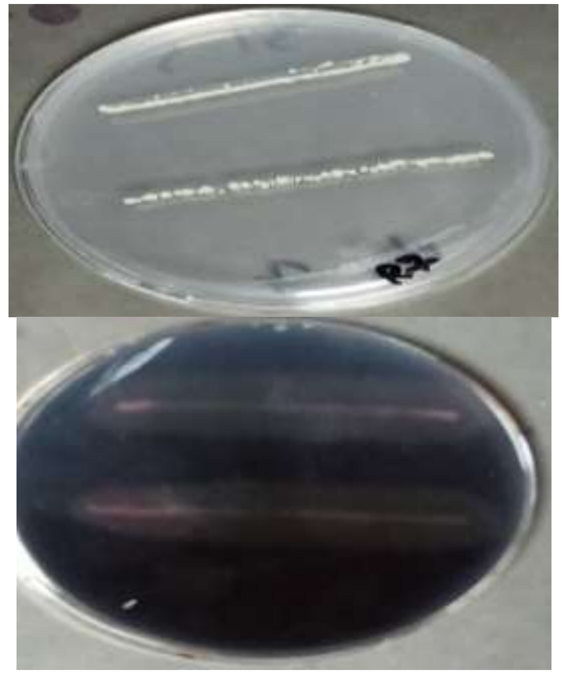

Figure 7: Bacterial Strain-7 Reaction with gram iodine

Table 2: Biochemical characteristics of collected isolates

Sr. No	Isolates	KOH	Catalase	Gram reaction	Starch Hydrolysis
1.	Bacterial Strain-1	-ve	-ve	-ve	-ve
2.	Bacterial Strain-2	+ve	-ve	-ve	-ve
3.	Bacterial Strain-3	-ve	+ve	-ve	-ve
4.	Bacterial Strain-4	-ve	+ve	+ve	+ve
5.	Bacterial Strain-5	-ve	+ve	+ve	+ve
6.	Bacterial Strain-6	+ve	-ve	-ve	-ve
7.	Bacterial Strain-7	-ve	+ve	+ve	+ve
8.	Bacterial Strain-8	-ve	+ve	-ve	-ve
9.	Bacterial Strain-9	+ve	-ve	-ve	-ve
11.	Bacterial Strain-10	-ve	+ve	-ve	-ve

All ten bacterial isolates, based on frequency in several isolations, were subjected to statistical software for data analysis. Analysis of variance was performed on the recorded data with 5% significance level.

Table 3: Mean of bacterial isolates frequency

Sr. No	Isolates	Mean
1.	Bacterial Strain-1	1.25
2.	Bacterial Strain-2	1.75
3.	Bacterial Strain-3	1.0
4.	Bacterial Strain-4	1.0
5.	Bacterial Strain-5	0.5
6.	Bacterial Strain-6	1.25
7.	Bacterial Strain-7	2.0
8.	Bacterial Strain-8	2.0
9.	Bacterial Strain-9	1.5
10.	Bacterial Strain-10	0.5

Discussion

Soybean hold global significance as a crop due to its abundant protein and oil content (Dukariya *et al.*, 2020). The protein in soybean is particularly valuable due to its rich amino acid profile, notably lysine, which is deficient in most cereal crops (Rana *et al.*, 2013). Over the past few years, there has been a growing interest in studying endophytic micro-organisms due to their significant role in the agricultural environment. These micro-organisms have captured attention due to their potential application in sustainable agriculture (Surjit and Rupa, 2014). Endophytes have been discovered in nearly all plants examined thus far (Ryan *et al.*, 2008). They reside within plant tissues such as flowers, fruits, leaves, stems, roots, and seeds, benefiting from the host plant's protection against environmental stresses and microbial competition (Kobayashi and Palumbo, 2000). The association between endophytes and plants has been shown to enhance plant health and assist the host plant in overcoming various biotic and abiotic stresses (Hasegawa *et al.*, 2006; Sapak *et al.*, 2008).

In this study, bacterial endophytes were isolated from the different soybean fields of MNSUAM.

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There was significant variation both morphologically and biochemically in the types of bacteria. The research involved the isolation of bacteria from healthy soybean plant roots at the flowering stage. The isolation was performed through roots crushed method on NA media plate, and an adequate number of colonies was observed. The diversity of ten endophytic bacteria obtained from various root tissues of soybean plants was evaluated in morphological and biochemical characterization techniques. The analysis of colony morphology provided valuable insights into the variations observed among the endophytic bacteria. The isolates were chosen for their dominance and uniqueness or differences from others in colony shape, colony color, cell shape, and colony surface. Most isolates exhibited smooth colony surfaces, regular colony shapes, and rod-shaped cells. However, Bacterial Strain-5 and Bacterial Strain-10 differed by displaying rough colony surfaces and irregular colony shapes. Additionally, Bacterial Strain-4 exhibited round cell shapes instead of the typical rod-shaped morphology observed in the other isolates. Notably, the bacterial isolates demonstrated diverse pigmentation, with different strains exhibiting White, creamy White, light Yellow, pinkish red, Creamish, Yellow, and red pigmentation.

The biochemical test results indicate that three bacterial strains (2, 6, and 9) showed positive reactions to KOH, while the remaining seven strains (1, 3, 4, 5, 7, 8, and 10) showed negative reactions. This confirmed that the three strains with positive reactions were gram-negative, while the seven strains with negative reactions were gram-positive. Regarding the catalase test, four bacterial strains (1, 2, 6, and 9) displayed negative reactions, while the remaining six strains (3, 4, 5, 7, 8, and 10) showed positive reactions. This confirmed that the four strains with negative reactions were gram-negative, whereas the six with positive ones were gram-positive.

In the gram staining test, seven bacterial strains (1, 2, 3, 6, 8, 9, and 10) appeared pink under the microscope, while the remaining three (4, 5 and 7) appeared purple. This confirmed that the seven strains with pinkish color were gram-negative, while the three strains with purple color were gram-positive. Lastly, in the starch hydrolysis test, seven bacterial strains (1, 2, 3, 6, 8, 9 and 10) did not react with gram iodine, whereas the remaining three strains (4, 5 and 7) showed a reaction. This confirmed that the seven strains with no reaction were gram-negative, while the three strains with a reaction were gram-positive. This result was in line with previous research by Li et al. (2019), which isolated many Gram-negative endophytes from soybean nodules. In contrast, other studies reported a low number of endophytic bacteria with the predominance of Gram positive bacteria (Bai *et al.*, 2002; Hung and Annapurna, 2004).

The biochemical test results confirmed that three bacterial strains (2, 6 and 9) were gram-negative, while the remaining seven strains (1, 3, 4, 5, 7, 8 and 10) were gram-positive. The KOH, catalase, gram staining, and starch hydrolysis tests consistently distinguished between the two groups of strains, providing valuable information about their gram status. Previous studies have documented a higher prevalence of Gram-negative bacteria within the plant tissues across multiple plant species (Stoltzfus *et al.*, 1997; Elbeltagy *et al.*, 2000). However, a subsequent study by Zinniel et al. (2002) observed an equitable distribution of Gram-negative and Gram-positive bacteria.

Conclusion

The study examined the diversity of ten endophytic bacteria isolated from soybean root tissues. Morphological and biochemical characterization methods revealed significant variation among the isolates. Most isolates displayed smooth colony surfaces, regular shapes, and rod-shaped cells. The biochemical tests performed on the bacterial strains yielded the following results: three strains (2, 6 and 9) showed positive reactions to KOH, indicating they were gram-negative, while the remaining seven strains (1, 3, 4, 5, 7, 8 and 10) showed negative reactions, indicating they were gram-positive. The catalase test confirmed that four strains (1, 2, 6 and 9) were gram-negative, and the six strains (3, 4, 5, 7, 8 and 10) were gram-positive. In the gram staining test, seven strains (1, 2, 3, 6, 8, 9 and 10) appeared pinkish, indicating they were gram-negative, while the remaining three strains (4, 5, 7, and 10) appeared purple, indicating they were gram-positive. Finally, in the starch hydrolysis test, seven strains (1, 2, 3, 6, 8, 9 and 10) did not show any reaction with gram iodine, indicating they were gram-negative, whereas the three strains (4, 5 and 7) showed a reaction, indicating they were gram-positive.

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Declarations

Data Availability statement

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

Ethics approval and consent to participate

Ethical approval was given from Ethical Review committee of department.

Consent for publication

The consent form was approved from Ethical Review committee of department.

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Conflict of Interest

Regarding conflicts of interest, the authors state that their research was carried out independently without any affiliations or financial ties that could raise concerns about biases.

Author Contribution

RR conducted research and wrote up initial draft of manuscript. MAM, AH, SA and MAS provided resources. SA, MAF, MWA an SA made final editing in the manuscript. All authors approved final version of manuscript.

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