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Selection and Bioassay of Halotolerant Nitrogen-Fixing Endophytic Bacteria for Promoting Rice Seedlings (*Oryza sativa* L.) under Saline Conditions

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ABSTRACT

Nitrogen-fixing endophytic bacteria play an important role in improving nitrogen availability and supplying growth-promoting factors to plants. This study evaluated the potential of endophytic bacterial isolates obtained from various plants growing under saline conditions based on their nitrogenase activity, indole-3-acetic acid (IAA) production, and ability to promote rice seedling growth. A bioassay was conducted using rice seedlings grown in Fahraeus medium adjusted to a salinity level of 4 dS m^{-1}. The experiment was arranged in a randomized complete block design with eight treatments consisting of an uninoculated control and seven selected endophytic bacterial isolates, with three replications. The measured responses included shoot length, root length, shoot dry weight, root dry weight, and total seedling biomass. Based on the bioassay results, two superior isolates, A1 and A2, were identified. The isolates exhibited nitrogenase activities of 1.223 and 1.780 µmol mL^{-1} g^{-1} h^{-1}, respectively, and produced IAA at concentrations of 7.819 and 7.794 mg L^{-1}, respectively. They increased root length by 13.66% and 15.44%, respectively, and resulted in numerically higher total seedling dry biomass, with values 51.99% and 34.68% higher than the uninoculated control, respectively. These findings indicate that the selected halotolerant nitrogen-fixing endophytic bacterial isolates can promote rice seedling growth under saline conditions and have potential for further development as biological fertilizers for rice cultivation on saline soils.

KEYWORDS: Nitrogen-fixing endophytic bacteria, superior isolates, bioassay.

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1. INTRODUCTION

Rice is an important staple food for more than half of the world's population. It provides adequate calories as well as various vitamins, minerals, and other essential nutrients. Its nutritional content is relatively high compared with other staple foods (Mohidem et al., 2022). The demand for rice continues to increase from year to year. However, the conversion of agricultural land to non-agricultural uses has reduced the availability of land for rice cultivation, thereby constraining rice production. Marginal lands have therefore received increasing attention as potential areas for expanding rice cultivation, including saline soils. Saline soil is widely distributed along coastal areas of northern Java. According to Sukarman et al. (2018), Indonesia has approximately 12.020 million ha of saline soils, equivalent to about 6.20% of the country's total land area, with substantial areas distributed along coastal regions, including Cimrutu Village.

Cimrutu Village is located in Cilacap Regency, Central Java Province, Indonesia. Most agricultural land in the village is cultivated with rice. Because Cimrutu Village is located near the coast, its agricultural ecosystem is characterized by saline soils. High soil salinity can adversely affect crop growth and agricultural productivity. In rice-growing areas, high to very high salinity levels can reduce rice productivity by up to 90% (Rachman et al., 2018). Soil structure in saline environments is susceptible to degradation, while soil aeration and permeability may be reduced because of the high concentration of soluble salts, particularly NaCl. Salinity

also restricts plant water uptake because the osmotic potential of saline soil water becomes sufficiently low to reduce the water potential gradient required for water movement into plant tissues. Under severe conditions, this restriction can cause physiological dehydration and eventually plant death.

High soil salinity can also induce nutrient deficiencies. Saline soil ecosystems generally have relatively low nitrogen (N) availability. Nitrogen is an essential macronutrient required by plants for vegetative growth, chlorophyll synthesis and photosynthesis, as well as protein and other organic compound formation.

The use of biofertilizers is one approach to improving rice productivity while reducing the negative effects associated with excessive reliance on inorganic fertilizers. Biofertilizers are biologically active products containing microorganisms that facilitate nutrient supply directly or indirectly, decompose organic matter, improve fertilizer-use efficiency, and enhance soil fertility and health (Akmalia and Budi, 2026). Biofertilizers can improve soil fertility, increase nutrient availability, and support soil biodiversity. Endophytic bacteria are among the microorganisms with potential for use as biofertilizer components.

Endophytic bacteria inhabit plant tissues and establish associations with their hosts without causing detrimental effects. Depending on the bacterial species, these associations may involve the production of secondary metabolites that benefit the host plant (Sen et al., 2026). Endophytic bacteria perform several beneficial functions, including biological nitrogen fixation, production of phytohormones such as indole-3-acetic acid (IAA), and suppression of plant pathogens (Tarun et al., 2026). Nitrogen-fixing endophytic bacteria have considerable potential for improving plant growth, crop productivity, and soil health and consequently may contribute to sustainable agricultural production (Rana et al., 2023).

These characteristics provide a rationale for isolating and screening endophytic bacteria to identify superior isolates that can potentially be used as biofertilizers in saline soils. In this study, endophytic bacterial isolates were obtained from several plant species growing under saline conditions, including rice.

The objective of this study was to obtain superior nitrogen-fixing endophytic bacterial isolates based on their morphological characteristics, nitrogenase activity, and IAA production, and to evaluate the effects of selected nitrogen-fixing endophytic bacterial isolates on rice seedling growth through a bioassay under saline conditions.

2. MATERIALS AND METHODS

2.1 Materials

The bacterial isolates used in this study were obtained from roots and leaves of plants growing in five different rhizosphere ecosystems. Samples were collected from lowland rice (Oryza sativa L.) at coordinates 07°37′29.3″S, 108°49′00.3″E; purun tikus (Eleocharis dulcis) at 07°37′29.6″S, 108°49′00.1″E; water lily (Nymphaea sp.) at 07°37′29.1″S, 108°48′56.3″E; small water hyacinth (Monochoria vaginalis) at 07°37′29.1″S, 108°48′56.3″E; and wild grass (Cyperus iria) at 07°37′29.1″S, 108°48′56.3″E. All sampling sites were located in Cimrutu Village, Patimuan District, Cilacap Regency, Central Java, Indonesia.

Jensen's Nitrogen-Free Malate Bromothymol Blue (JNFB) medium was used for the isolation and presumptive screening of nitrogen-fixing bacteria. Fahraeus liquid medium adjusted to a salinity level of 4 dS m^{-1} was used for the rice seedling bioassay. The rice cultivar used was the salt-tolerant cultivar INPARI 34, obtained from the Indonesian Center for Rice Research, Sukamandi, West Java, Indonesia.

2.2. Isolation of Endophytic Bacteria

Endophytic bacteria were isolated following the method of James and Olivares (1997). Root and leaf samples were first washed thoroughly under running water and subsequently rinsed with distilled water. The plant tissues were cut into approximately 2–3 cm sections and dried with sterile tissue paper. Ten grams of plant tissue were washed thoroughly with sterile distilled water by shaking in a 250-mL Erlenmeyer flask for 30 min. The samples were then rinsed twice with sterile distilled water before surface sterilization with 0.2% $HgCl_2$ for 20–30 min. After sterilization, the samples were rinsed six times with sterile distilled water and homogenized by grinding or blending.

Serial dilutions ranging from 10^{-3} to 10^{-4} were prepared, and 0.5 mL from each dilution was inoculated onto solid Jensen's Nitrogen-Free Malate Bromothymol Blue (JNFB) medium. The medium contained malic acid, K_2HPO_4, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, NaCl, $CaCl_2$, KOH, 1.64% Fe-EDTA solution, micronutrients, bromothymol blue, yeast extract, agar, and distilled water. The inoculated plates were incubated at 34°C for 2–3 days.

2.3. Purification of Bacterial Isolates

Distinct bacterial colonies were selected using an inoculating needle and streaked onto JNFB medium using the streak-plate method, followed by incubation for 24–48 h. The purification process was repeated five times by transferring the isolates onto fresh JNFB medium to ensure that the bacterial cultures were free from contamination by other microorganisms. The purified bacterial isolates were subsequently transferred by streaking onto JNFB agar slants. Bacterial purity was further examined microscopically.

2.4. Characterization of Endophytic Bacterial Isolates

The bacterial isolates were characterized based on their colony morphology through visual observation. Microscopic observations were conducted to determine bacterial cell morphology. Gram staining was performed to classify the isolates as Gram-positive or Gram-negative bacteria. Differences in Gram-staining reactions are associated with differences in the structural components of the bacterial cell walls.

2.5. Nitrogenase Activity Assay

Nitrogenase activity was determined for the selected isolates using the Acetylene Reduction Assay (ARA) to evaluate their ability to fix atmospheric N_2. Bacterial isolates were grown in semi-solid JNFB medium. The cotton plugs used to seal the culture tubes were replaced with rubber stoppers. After sealing, approximately 10% of the remaining headspace gas, equivalent to approximately 1 mL, was withdrawn using a micro syringe and replaced with an equal volume of acetylene (C_2H_2). The cultures were then incubated for 1 h.

The ethylene (C_2H_4) produced in the headspace was detected and quantified using gas chromatography. The gas chromatograph was operated at an initial temperature of 100°C, injector temperature of 150°C, detector temperature of 200°C, and final temperature of 100°C. The carrier and supporting gases were nitrogen (40 psi), hydrogen (1.5 kgf cm^{-2}), and air (0.5 kgf cm^{-2}), respectively (Montes et al., 2023).

An ethylene standard curve was prepared at concentrations of 0, 50, 100, 150, 175, 200, and 225 µg mL^{-1}. The resulting chromatograms were analyzed, and the standard curve was constructed by plotting ethylene concentration against the corresponding peak area. The resulting regression relationship was then used to determine the ethylene concentration in each sample. A 1-mL headspace gas sample was subsequently collected for measurement of the C_2H_4 concentration by gas chromatography. Quantitative nitrogenase activity was calculated according to the equation described by Hawkes (2001).

2.6. IAA Production Assay

The ability of the bacterial isolates to produce indole-3-acetic acid (IAA) was evaluated by culturing the isolates in Nutrient Broth (NB) medium, with and without the addition of 0.5 g L^{-1} L-tryptophan. The cultures were incubated at 28 ± 2°C for 48 h with shaking at 120 rpm. Following incubation, the cultures were centrifuged at 13,000 rpm for 10 min. IAA production was analyzed using a colorimetric method with Salkowski reagent containing 12 g L^{-1} $FeCl_3$ in
7.9 M H_2SO_4. The supernatant was mixed with Salkowski reagent at a ratio of 1:2 (1 mL supernatant + 2 mL reagent) and incubated for 25 min. Absorbance was then measured using a spectrophotometer at a wavelength of 530 nm (Peng et al., 2014).

2.7. Rice Seedling Bioassay

A bioassay was conducted to evaluate the ability of the endophytic bacterial isolates to promote plant growth. Rice seedlings were grown in Fahraeus medium containing $CaCl_2$, $MgSO_4$, KH_2PO_4, $Na_2HPO_4 \cdot 2H_2O$, ferric citrate, yeast extract, micronutrients, agar, and distilled water.

The bioassay was conducted using 100-mL test tubes containing 90 mL of saline Fahraeus liquid medium adjusted to a salinity level of 4 dS m^{-1}. Rice seeds were surface-sterilized with 0.2% $HgCl_2$ for 2 min and subsequently treated with 70% ethanol for 1–2 min. The seeds were rinsed three times with distilled water and germinated in Petri dishes lined with sterile germination paper moistened with sterile distilled water. Germination was conducted for five days at 30°C.

The germinated seedlings were transferred to Fahraeus medium using sterile mesh and supporting tubes. The upper part of each tube was supported using plastic material and a pipe opening so that the shoots remained above the medium while only the roots were immersed in the Fahraeus solution.

2.8. Experimental Design and Statistical Analysis

The bioassay experiment was arranged in a randomized complete block design (RCBD). The treatment factor consisted of eight levels of halotolerant endophytic bacterial isolates: an uninoculated control, isolate 1, isolate 2, isolate 3, isolate 4, isolate 5, isolate 6, and isolate 7. Each treatment was replicated three times, resulting in 24 experimental units. All treatments were applied to Fahraeus medium adjusted to a salinity level of 4 dS m^{-1}.

The data were first tested for normality to determine their distribution. Normally distributed data were subsequently subjected to analysis of variance (ANOVA) using SPSS. When the treatment effect was statistically significant, Duncan's multiple range test was performed at the 5% significance level (Gomez and Gomez, 1995).

3. RESULTS AND DISCUSSION

3.1. Isolation of Nitrogen-Fixing Endophytic Bacteria

Nitrogen-fixing endophytic bacterial isolates were obtained from plant tissues, including roots and leaves. Isolation was conducted using five different plant samples with three replications. JNFB (Jensen's Nitrogen-Free Bromothymol Blue) solid medium was used as a selective medium for nitrogen-fixing endophytic bacteria. After approximately three days of incubation,

distinct bacterial colonies were selected and purified on agar slants.

A total of 13 pure isolates were obtained and subsequently grown in semi-solid JNFB medium. After 10 days of incubation, all 13 isolates caused a color change in the medium from green to blue and produced a white pellicle below the surface of the medium. This observation is consistent with Nuriyanah et al. (2023), who reported that the growth of endophytic bacteria in semi-solid medium can be indicated by the formation of a white pellicle below the medium surface accompanied by a change in medium color.

The change in medium color from green to blue indicates an increase in pH. This may be associated with the ability of the endophytic bacteria to metabolize or utilize acidic compounds in the medium using available carbon sources (Alqahtani et al., 2024). Further characterization of the endophytic bacterial isolates was therefore conducted using Gram staining.

3.2. Characterization of Nitrogen-Fixing Endophytic Bacteria

Microscopic observations showed that the 13 isolates consisted of coccoid and rod-shaped bacterial cells. Two staining reactions were observed: violet, characteristic of Gram-positive bacteria, and red, characteristic of Gram-negative bacteria (Table 1).

Table 1. Characterization of Endophytic Bacterial Isolates Obtained from Plants Growing in Saline Soil

No.	Isolate code	Cell shape	Colony color	Gram reaction
1	Aa_1	Coccus	Red	-
2	Aa_2	Coccus	Red	-
3	Aa_3	Rod	Violet	+
4	Ba_1	Coccus	Red	-
5	Ba_2	Coccus	Violet	+
6	Bd_1	Rod	Red	-
7	Bd_2	Rod	Violet	+
8	Cd_1	Coccus	Red	-
9	Cd_2	Coccus	Violet	+
10	Dd_1	Rod	Red	-
11	Dd_2	Rod	Violet	+
12	Ea_1	Rod	Violet	+
13	Ea_2	Rod	Red	-

Based on Table 1, the Gram-negative isolates were Aa1, Aa2, Ba1, Bd1, Cd1, Dd1, and Ea2, whereas the Gram-positive isolates were Aa3, Ba2, Bd2, Cd2, Dd2, and Ea1. The isolates selected for the subsequent bioassay were Gram-negative bacteria with either coccoid or rod-shaped cells. The selection of isolates was partly based on their Gram reaction, considering that nitrogen-fixing endophytic bacteria are frequently dominated by Gram-negative bacterial groups (Iniguez et al., 2004). Similarly, Muangthong et al. (2015) reported that among endophytic nitrogen-fixing bacteria isolated from sugarcane roots, stems, and leaves, approximately 71% were Gram-negative bacteria.

The Gram-negative isolates appeared red following Gram staining (Figure 1). This reaction occurs because Gram-negative bacteria cannot retain the primary crystal violet stain during the decolorization process. Their cell envelope structure allows the primary stain to be removed during ethanol treatment, resulting in subsequent staining by the counterstain (Pelczar and Chan, 2012). Based on the Gram-staining results, seven endophytic bacterial isolates, namely Aa1 (A1), Aa2 (A2), Ba1 (A3), Bd1 (A4), Cd1 (A5), Dd1 (A6), and Ea2 (A7), were selected for the bioassay using rice seedlings grown under saline conditions.

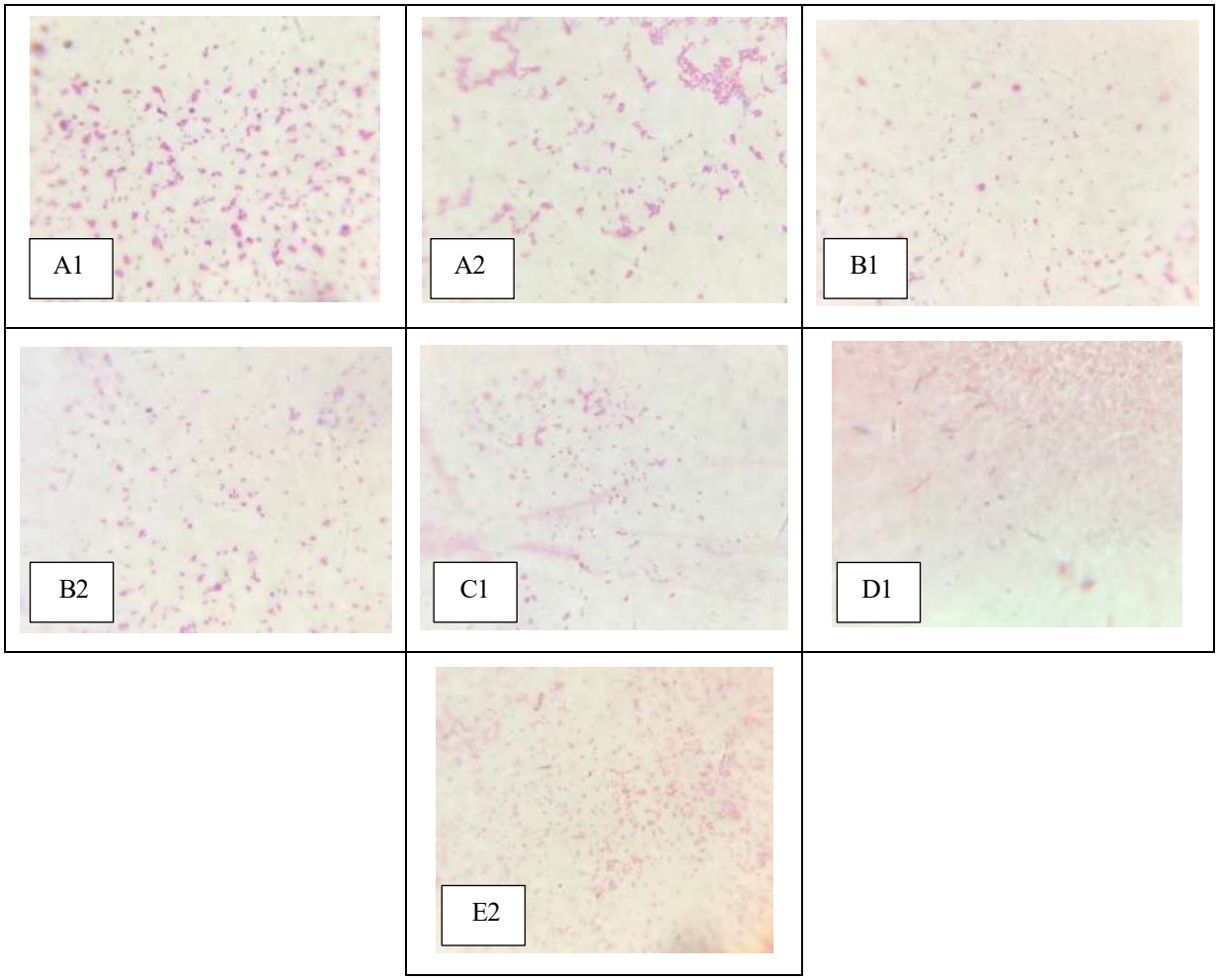

Figure 1. Cell morphology of Gram-negative nitrogen-fixing endophytic bacterial isolates

3.3. Bioassay of Endophytic Bacterial Isolates on Rice Seedlings

Inoculation with nitrogen-fixing endophytic bacterial isolates significantly affected root growth but did not significantly affect shoot growth. The parameters evaluated in the bioassay included shoot length, root length, shoot dry weight, and root dry weight of rice seedlings at 21 days after treatment. The results are presented in Table 2.

Table 2. Effects of Nitrogen-Fixing Endophytic Bacterial Isolates on Root and Shoot Growth of Rice Seedlings Grown in Saline Fahraeus Liquid Medium

Isolate treatment	Shoot length (mm)	Root length (mm)	Increase in root length (%)
Control	59,00	112,33 a	-
A1	78,33	127,67 bc	13,66
A2	69,33	129,67 c	15,44
A3	66,67	121,00 abc	7,72
A4	63,00	114,00 a	1,49
A5	63,33	122,33 abc	8,99
A6	66,67	117,00 a	4,16
A7	63,00	117,33 ab	4,45

Note: Values followed by the same letter are not significantly different according to Duncan's multiple range test at the 5% significance level.

As shown in Table 2, the uninoculated control had shorter roots than most of the treatments inoculated with endophytic bacteria. The observed increase in plant height and root length may be associated with the ability of beneficial bacteria to transform nutrients from unavailable forms into forms that can be taken up by plants (Shaukat et al., 2006).

No significant differences were observed among treatments in shoot length. This may indicate that the effects of the endophytic bacteria were more pronounced in the root system than in the shoot. Harni et al. (2023) reported that endophytic bacteria can stimulate lateral root formation and increase root development, thereby expanding the plant's capacity to acquire nutrients.

Significant differences in root length were observed following Duncan's multiple range test. The A1 and A2 treatments produced root lengths of 127.67 and 129.67 mm, respectively, and differed significantly from the control. In contrast, isolates A3, A4, A5, and A7 produced root lengths that were not significantly different from the uninoculated control. Isolates A1 and A2 increased rice seedling root length by 13.66% and 15.44%, respectively, compared with the control. A visual comparison of rice seedling root and shoot growth is presented in Figure 2.

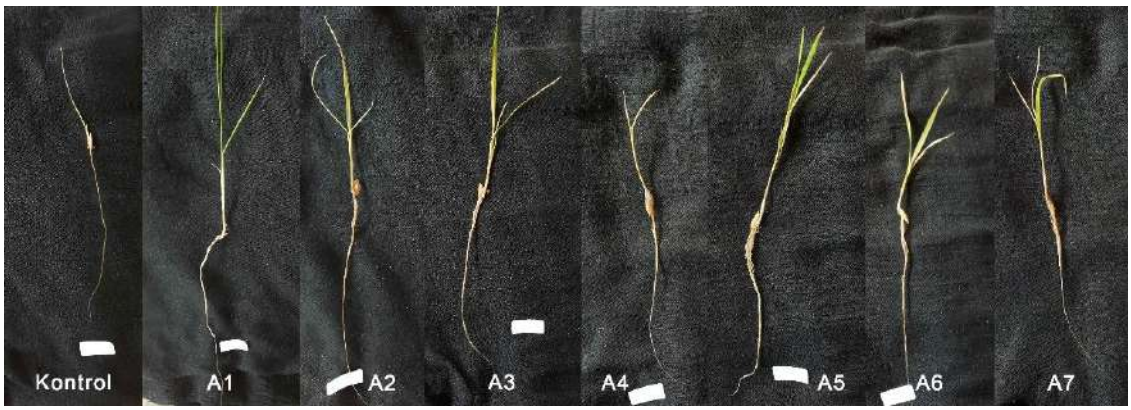

Figure 2. Root and shoot growth of rice seedlings compared with the uninoculated control at 21 days after planting

Figure 2 shows that uninoculated control produced the shortest roots compared with treatments inoculated with endophytic bacterial isolates. This response may be associated with the ability of endophytic bacteria to produce phytohormones, particularly indole-3-acetic acid (IAA). Roots are highly responsive to IAA, which can contribute to root elongation as well as the formation of lateral and adventitious roots (Leveau and Lindow, 2005).

In addition to shoot and root length, shoot dry weight and root dry weight were evaluated in the bioassay. The results are presented in Table 3.

Table 3. Effects of Endophytic Bacterial Isolates on Shoot and Root Dry Weight and Total Biomass of Rice Seedlings at 21 Days after Planting

Treatment	Shoot dry weight (mg)	Root dry weight (mg)	Total biomass (mg)	Increase in total biomass (%)
Control	6,33	11,00	17,33	-
A1	10,67	15,67	26,34	51,99
A2	7,67	15,67	23,34	34,68
A3	6,33	14,00	20,33	17,31
A4	7,00	13,67	20,67	19,27
A5	6,33	13,33	19,66	13,44
A6	7,67	13,00	20,67	19,27
A7	6,67	13,00	19,67	13,50

Note: The treatment factor did not significantly affect the measured responses based on analysis of variance at the 5% significance level.

Based on Table 3, the endophytic bacterial treatments did not significantly affect shoot dry weight or root dry weight. Nevertheless, inoculation with endophytic bacteria resulted in numerically higher total seedling dry biomass than the uninoculated control, with values 51.99% and 34.68% higher for isolates A1 and A2, respectively. Gofar et al. (2015) similarly reported that

endophytic bacterial isolates increased fresh and dry root biomass in rice. This response may be related to the ability of some endophytic bacteria to improve nitrogen availability and stimulate the production of plant growth-promoting phytohormones.

3.4. Ranking of Superior Endophytic Bacterial Isolates

Superior endophytic bacterial isolates were identified by ranking their performance based on shoot length, root length, shoot dry weight, root dry weight, and total biomass. The isolates were ranked according to their scores, with the lowest total score representing the best overall performance. The ranking results are presented in Table 4.

Table 4. Ranking of Superior Endophytic Bacterial Isolates

Isolate	Shoot length	Root length	Shoot dry weight	Root dry weight	Total biomass	Total score	Rank
A1	2	1	1	1	1	6	1
A2	1	2	2	1	2	8	2
A3	4	4	6	3	5	22	4
A4	7	7	4	4	3	25	5
A5	3	6	6	5	7	27	6
A6	6	3	2	6	3	20	3
A7	5	5	5	6	6	27	7

As shown in Table 4, isolate A1 had the lowest total score (6) and was therefore ranked first, followed by isolate A2 with a total score of 8 and a rank of second. The other isolates had total scores ranging from 20 to 27 and were ranked third to seventh. Based on the bioassay ranking, isolates A1 and A2 were identified as the superior isolates for promoting rice seedling growth under saline conditions compared with the other isolates. These superior isolates were subsequently analysed quantitatively for IAA production and nitrogenase activity using the Acetylene Reduction Assay.

3.5. IAA Production by Superior Endophytic Bacterial Isolates

As shown in Table 5, the IAA concentrations produced by isolates A1 and A2 were 7.819 and 7.794 mg kg^{-1}, respectively.

Table 5. IAA Production by Endophytic Bacterial Isolates

Isolate code	Replicate 1 (mg L^{-1})	Isolate code (mg L^{-1})	Replicate 1 (mg L^{-1})	Isolate code (mg L^{-1})
Control (standard solution)	0,000	-	-	0,000
A1	7,857	7,819	7,781	7,819
A2	7,630	7,743	8,008	7,794

IAA production by the two superior isolates was quantified using a colorimetric method with Salkowski reagent. The analysis was conducted to determine whether the nitrogen-fixing endophytic bacterial isolates were capable of producing IAA. Microbial IAA biosynthesis is generally associated with tryptophan-dependent pathways, in which tryptophan serves as a precursor. Endophytic microorganisms may synthesize IAA and thereby stimulate or enhance plant growth following colonization of plant tissues (Shi et al., 2009).

3.6. Nitrogenase Activity Determined by the Acetylene Reduction Assay

The nitrogenase activity analysis showed that isolate A2 had the highest nitrogenase activity, at 1.780 mg kg^{-1}, followed by isolate A1 at 1.223 mg kg^{-1} (Table 6).

Table 6. Nitrogenase Activity of Endophytic Bacterial Isolates

Isolate code	Replicate 1 (µmol mL^{-1} $g^{-1}h^{-1}$)	Replicate 2 (µmol mL^{-1} $g^{-1}h^{-1}$)	Replicate 3 (µmol mL^{-1} $g^{-1}h^{-1}$)	Mean ARA activity (µmol mL^{-1} $g^{-1}h^{-1}$)
A1	1,288	1,218	1,162	1,223
A2	1,790	1,694	1,856	1,780

The nitrogenase assay was conducted to evaluate the ability of the endophytic bacteria to fix atmospheric N_2. The nitrogen-fixing endophytic bacteria were grown in nitrogen-free medium (Baldani et al., 1992), after which nitrogenase activity was estimated based on ethylene (C_2H_4) production (Lindström and Mousavi, 2019). The ARA is an indirect method for evaluating nitrogenase activity based on the reduction of acetylene to ethylene, which can subsequently be quantified using gas chromatography (Doty et al., 2009). Nitrogenase activity can therefore be estimated from the concentration of ethylene detected by gas chromatography. Higher ethylene production reflects greater acetylene reduction activity and is therefore indicative of higher nitrogenase activity and a greater potential for biological nitrogen fixation (Montes-Luz et al., 2023).

4. CONCLUSION

Thirteen endophytic bacterial isolates were obtained from various plants growing in saline environments, of which two superior halotolerant endophytic bacterial isolates, A1 and A2, were identified based on their performance in the rice seedling bioassay. Both isolates were coccoid, Gram-negative bacteria and exhibited nitrogenase activities of 1.223 and 1.780 µmol mL^{-1} g^{-1} h^{-1}, respectively, as determined by the acetylene reduction assay and IAA at concentrations of 7.819 and 7.794 mg L^{-1}, respectively. Isolates A1 and A2 increased rice seedling root length by 13.66% and 15.44%, respectively, and resulted in numerically higher total seedling dry biomass by 51.99% and 34.68%, respectively, compared with the uninoculated control. These findings indicate the potential of isolates A1 and A2 as halotolerant nitrogen-fixing endophytic bacteria for promoting rice seedling growth under saline conditions and support their further evaluation as candidates for biological fertilizer development.

5. ACKNOWLEDGMENTS

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6. CONFLICTS OF INTEREST

The authors declare no competing financial interests or conflicts of interest relevant to this work.

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