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Isolation, characterization and screening of *Bradyrhizobium* from soybean plants for salinity tolerance

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Abstract

Salinity stress is a major limiting factor affecting the growth and productivity of soybean (*Glycine max*), particularly in arid and semi-arid regions. This study aimed to isolate and characterize *Bradyrhizobium* strains from soybean root nodules and screen them for salinity tolerance. A total of thirty-four *Bradyrhizobium* isolates were isolated from soybean samples from Adilabad and Hyderabad district saline soil regions and evaluated for their ability to withstand varying levels of salt stress. Results showed that ASB16 and HSB2 isolates exhibited high salinity tolerance and maintained their symbiotic efficiency with soybean even at high salt concentrations. These salt-tolerant *Bradyrhizobium* strains could potentially be used as bioinoculants to improve soybean productivity in saline soils, offering a sustainable solution for crop production in salt-affected regions.

Keywords: Soybean, *Bradyrhizobium*, salt tolerance

Introduction

Soybean, known as the "Wonder crop" of the 20th century, is the world's most important seed legume. It provides 25% of global edible oil and two-thirds of protein concentrate for livestock. Soybean is a key source of protein (40%) and vegetable oil (20%), offering numerous nutritional and health benefits. Its growth and yield are often limited by abiotic stresses like salinity, drought and extreme temperatures. Among these, salinity stress is particularly more harmful than in crops like wheat, rice and cotton (Prawan and Kumar, 2023) [8]. It negatively affects soybean germination, nodule formation, plant growth and overall yield.

Salinity is a major factor limiting plant growth by causing osmotic and ionic stress, leading to toxicity (Kamran *et al.*, 2019 [1]; Hernandez, 2019) [2]. High salinity disrupts cellular components like membranes, proteins and nucleic acids (Hashem *et al.*, 2019) [3]. This disruption causes cell membrane peroxidation, leading to leakage of vital components which negatively impacts plant growth and metabolism (Kamran *et al.*, 2019) [1]. Salinity stress reduces plant growth, physiological function and yield (Egamberdieva *et al.*, 2015) [4]. It is a major issue in irrigated areas, lowering crop yield and quality. The primary negative effects of salinity on plants include osmotic stress and ion toxicity (Zahra *et al.*, 2014) [5].

Bradyrhizobium is a soil bacterium that is important to soybean plants because it helps them fix nitrogen and access nutrients. *Bradyrhizobium japonicum* is a type of *Bradyrhizobium* that helps soybean plants use nitrogen from the air through a symbiotic relationship. This process is essential for economical soybean production. *Bradyrhizobium* colonizes the roots of soybean plants, helping them absorb more water and nutrients. *Bradyrhizobia* can vary in their tolerance to salt with some strains being more salt-tolerant than others.

In light of these challenges, the present study aims to isolate, characterize and screen various *Bradyrhizobium* isolates to identify those with effective salinity tolerance.

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2. Materials and Methods

2.1 Collection of root samples and Rhizosphere soil samples

The root nodules of Soybean plants and rhizosphere soils were collected from Adilabad and Ranga Reddy districts. Samples collected from the above-mentioned places were preserved safely and brought to the laboratory for isolation.

2.2 Isolation of *Bradyrhizobium* from soybean root nodules

Healthy, large pink nodules were carefully selected, washed, and surface sterilized using 95% alcohol followed by 0.1% acidified mercuric chloride. After multiple rinses with sterile water, the nodules were crushed in sterile distilled water. A loopful of the suspension was streaked on Yeast Extract Mannitol Agar (YEMA) with Congo red. The plates were incubated at 30 °C for 4 days (Vincent, 1970)^[6]. Single colonies were selected and re-streaked on YEM agar for purity. The pure cultures of the isolates were preserved on YEMA slants for future studies (Fig 1).

2.3 Cultural and morphological characterization

Morphological characteristics of the colony of each isolate were observed by growing on a YEMA medium incubated at 28 ± 2 °C for 3 to 4 days. Cultural characterization of the isolates examined by different characteristics of colonies such as shape, elevation and margin were recorded.

2.3.1 Growth on YEMA-congo red medium

Pure cultures were streaked on Yeast Extract Mannitol Agar (YEMA) with 0.25% Congo red and incubated at 28 ± 2 °C for 3-4 days.

2.3.2 Growth on Glucose Peptone Agar and Lactose Agar

Freshly prepared glucose peptone agar and lactose agar was streaked with cultures and incubated at 28 ± 2 °C for 3-4 days (Vincent, 1970)^[6].

2.3.3 Hofer's Alkaline Broth Test

YEM broth with thymol blue was prepared and the pH adjusted to 11.0 using sodium hydroxide. 10 ml of broth was distributed into test tubes, capped and autoclaved. Log-phase cultures were inoculated and incubated at 28 ± 2 °C for 7 days (Subbarao and Tilak, 1977)^[7].

2.3.4 Oxidase Test

Oxidase reagent (1% N, N, N-tetramethyl-p-phenylenediamine) was prepared and stored at 25-30 °C. Filter paper strips dipped in the reagent were used to test individual colonies. Oxidase-positive colonies turned lavender, dark purple, or black within 5 minutes.

2.3.5 Catalase Test

A strain smear was placed on a glass slide, and a few drops of H₂O₂ were added. The presence of gas bubbles indicated a positive catalase test, confirming the enzyme's ability to hydrolyse H₂O₂ into water and oxygen.

2.3.6 Methyl Red Test

Methyl red broth tubes were inoculated with isolates and incubated at 35 °C for 48 hours. After adding 5 drops of methyl red indicator, a red colour indicated a positive test, while a yellow colour indicated a negative test.

2.3.7 Voges-Proskauer Test

Alpha-naphthol and potassium hydroxide were added to the culture broth. A cherry red colour indicated a positive result, while a yellow-brown colour indicated a negative result (Bhagya

et al., 2019)^[15].

2.3.8 Starch Hydrolysis Test

Bradyrhizobium was inoculated on Starch Agar Medium. After 24 hours, iodine (0.1N) was added. A clear zone around the bacterial colonies indicated starch utilization (Nigam and Xaxlo, 2016)^[9].

2.3.9 Gelatin liquefaction test

The test was performed to determine the capability of *Bradyrhizobium* to produce gelatinase enzyme as use of gelatin as a media source. The degradation of gelatin indicates the presence of the gelatinase enzyme. The actively grown cultures were inoculated in nutrient gelatin medium and incubated for 48 hours.

2.3.10 Urea Hydrolysis Test

To test for urease production, *Bradyrhizobium* strains were grown in urea broth with phenol red. A deep pink colour indicated urease activity, showing ammonia production and an alkaline environment. Absence of the pink colour indicated a negative result.

2.4 Nodulation test

All *Bradyrhizobium* isolates significantly influenced nodules formation on the roots of soybean in LBJA. Inoculation of *Bradyrhizobium* isolates helped formation of nodules indicating their effectiveness along with control (Broughton and Dilworth, 1970)^[10].

2.5 Screening of isolates for salt tolerance

Bradyrhizobium isolates were screened for their tolerance to increased concentrations of NaCl on agar plates according to the method described by Wade *et al.* (2014)^[11]. Each isolate was grown on yeast extracts-mannitol (YM) liquid media (Vincent, 1970)^[6] to logarithmic phase and the optical density at 600 nm (Fig 4).

3. Results and Discussion

3.1 Isolation of *Bradyrhizobium*

A total of thirty-four isolates (ASB1 to ASB20, ARB1 to ARB6, HSB1 to HSB5, HRB1 to HRB3) were isolated from soybean root nodules and purified them by quadrant-streak method. Single colonies were selected and re-streaked on YEM agar for purity.

3.2 Colony and Morphological Characteristics

The colonies on YMA medium of all the isolates were isolates were Gram-negative, non-spore-forming rods, small, milky-white or white, translucent, and circular with continue smooth margin, reaching 2-4 mm in diameter after 7 days of incubation at 28 °C (Fig 2 and Fig 3).

3.2.1 Growth on YEMA-congo red medium

The confirmatory tests were done by the Congo red test. It is used to differentiate *Rhizobia* from *Agrobacterium* contamination. *Rhizobia*'s inability to absorb Congo red dye serves as a distinguishing characteristic. Out of thirty-four isolates thirty isolates did not absorb the congo red dye.

3.2.2 Hofer's Alkaline Broth

Out of thirty-four isolates six isolates exhibited growth (positive) in Hofer's Alkaline broth. Twenty-eight isolates showed no utilization (negative) of Hofer's alkaline broth even after incubating for seven continuous days. Lack of growth in the high-pH broth confirmed the presence of *Rhizobium*.

3.2.3 Growth on Glucose Peptone Agar

When tested for the utilization of Glucose Peptone Agar (GPA), it was observed that twenty-eight isolates were positive and six isolates showed poor to no growth.

3.2.4 Growth on Lactose Agar

All the isolates were grown on Lactose agar medium and thirty isolates showed no growth on the medium for all the isolates and four isolates showed growth on it.

3.3 Biochemical characterization

Out of thirty-four isolates thirty isolates shown positive and four isolates shown negative to oxidase test, twenty-eight isolates shown positive and six isolates shown negative to catalase activity. Twenty-six isolates tested negative for the methyl red test, indicating no red colour change and eight isolates shown positive. The thirty-two isolates shown negative for Voges-proskaur test and two isolates show positive. Out of all twenty-four isolates were not able to show starch hydrolysis, shown negative for gelatin hydrolysis and ten isolates were able to show starch hydrolysis and shown positive for gelatin hydrolysis. Twenty-nine isolates shown positive for both urea hydrolysis, nitrate reduction and five isolates shown negative. Similar results were obtained in studies conducted by Nguyen *et al.* (2022)^[12] and Nohwar *et al.* (2019)^[13].

3.4 Nodulation test

The nodulation capacity of the isolates was tested using the Leonard jar assembly. After 45 days, plants inoculated with the isolates showed significant nodulation, unlike the control, confirming the symbiotic efficiency of *Bradyrhizobial* isolates on soybean. Thirteen isolates (ASB2, ASB5, ASB7, ASB9, ASB11, ASB16, ASB19, ASB20, ARB2, ARB5, ARB6, HSB2, HSB5) demonstrated good symbiotic effectiveness. Similarly, Muindi *et al.* (2021)^[14] prepared the Leonard jar assemblies and observed the symbiotic efficiency of native rhizobia isolates inoculated on cowpea plants after 45 days of sowing. They found that out of twenty isolates, nine isolates were showed ability to form nodules in the host plant.

3.5 Screening of isolates for salinity tolerance

A total of thirteen isolates were tested spectrophotometrically for salt tolerance at 600 nm, its values ranging from 0.391 to 2.679 (Graph 1). Among them ASB16 has highest salt tolerance i.e., 2.679 followed by HSB2 (2.116), ASB2 (1.964), ASB19 (1.954), ARB5 (1.912), ARB2 (1.875), HSB5 (1.765), ASB20 (1.742), ASB11 (1.673), ARB6 (1.612), ASB7 (1.423), ASB9 (1.321) and least was shown by ASB5 (0.391) (Table 1).



Fig 1: Preservation of pure cultures of the isolates were on YEMA slants



Fig 2: Colonies of bacteria on isolated plates

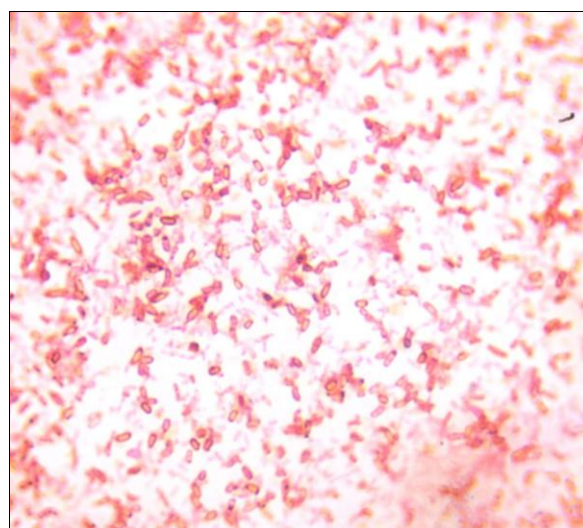


Fig 3: Cell morphology of *Bradyrhizobial* isolates by microscopic observation

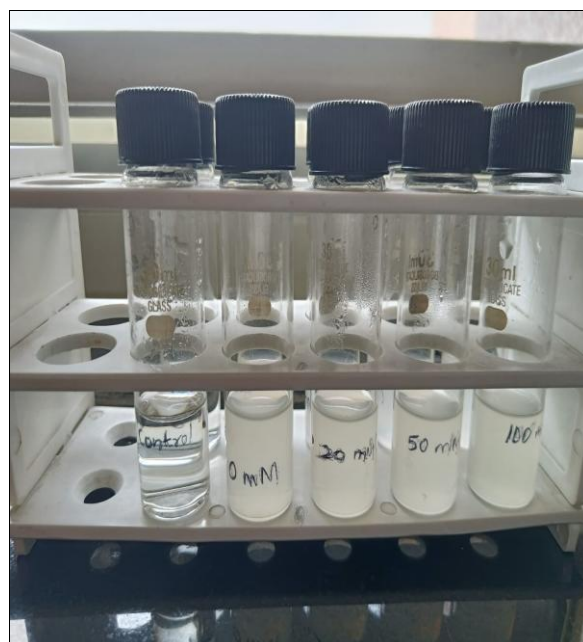
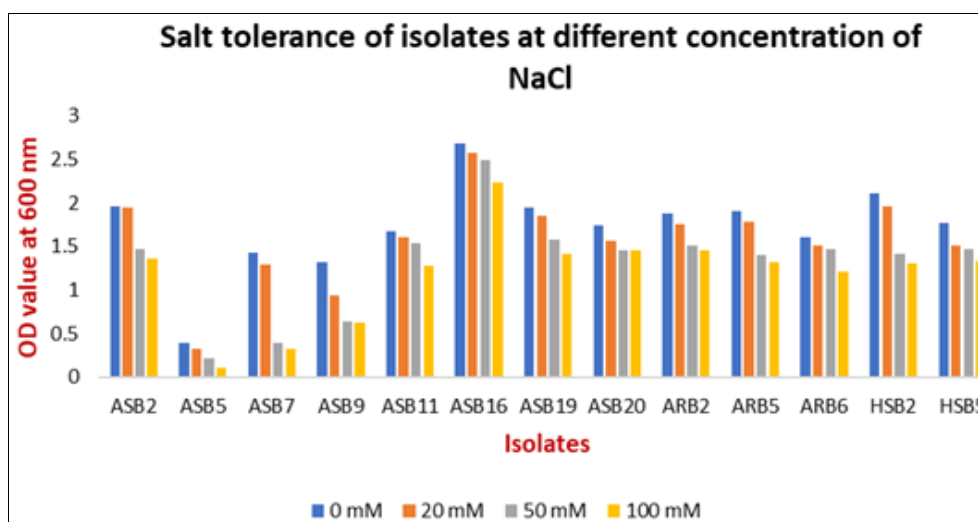


Fig 4: Growth of isolate in YEM broth at different concentrations of NaCl

Table 1: Screening of bacteria for salt tolerance

S. No	Isolate	Salt Concentration (mM NaCl)			
		0 mM	20 mM	50 mM	100 mM
		Growth (OD value)	Growth (OD value)	Growth (OD value)	Growth (OD value)
1	ASB2	1.964	1.942	1.471	1.362
2	ASB5	0.391	0.322	0.212	0.102
3	ASB7	1.423	1.297	0.396	0.321
4	ASB9	1.321	0.934	0.643	0.626
5	ASB11	1.673	1.612	1.537	1.280
6	ASB16	2.679	2.571	2.491	2.239
7	ASB19	1.954	1.853	1.586	1.414
8	ASB20	1.742	1.561	1.462	1.451
9	ARB2	1.875	1.763	1.509	1.460
10	ARB5	1.912	1.786	1.406	1.319
11	ARB6	1.612	1.507	1.464	1.213
12	HSB2	2.116	1.964	1.416	1.305
13	HSB5	1.765	1.514	1.472	1.337

**Graph 1:** Screening of the isolates for salt tolerance

4. Conclusion

The study successfully isolated, purified and characterized thirty-four *Bradyrhizobium* strains. The biochemical and growth tests confirmed the identity and physiological behavior of the isolates. Thirteen isolates showed high symbiotic effectiveness, promoting soybean nodulation. Additionally, ASB16 and HSB2 displayed the highest salt tolerance making them potential candidates for bio-inoculants to improve soybean productivity in saline soils. This research offers promising solutions for enhancing soybean growth under salinity stress.

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