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Isolation of Zinc Solubilizing Bacteria and its Application in Improving Soil Fertility

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Abstract: Zinc (Zn) is an important micronutrient for the growth and development of plants and animals. It is essential for various physiological functions as it acts as a cofactor for multiple enzymes. The present study aimed to isolate Zinc Solubilizing Bacteria (ZSB) from diverse soil samples and organic substrates, and evaluate their potential as bio-inoculants for stimulating plant growth. A total of 25 bacterial isolates were obtained from vermicompost, paddy field soils, and rhizospheres of various fruit and vegetable trees. Among these, the most potential isolate was identified as *Acinetobacter baumannii* and *Acinetobacter calcoaceticus* based on 16S rRNA sequencing. The consortium of *A. baumannii* and *A. calcoaceticus* demonstrated significantly enhanced Zn solubilization, reaching 1058 mg/L within 24 h, which was notably higher than the solubilization potential observed for individual isolates (797 mg/L and 784 mg/L Zn solubilization, respectively in 7 days). Zn tolerance assays demonstrated tolerance of *A. baumannii* and *A. calcoaceticus* up to 1200 ppm and 800ppm of Zn. Apart from Zn solubilization, the isolates showed diverse plant growth promoting properties, including phosphate solubilization, Indole-3-acetic acid production, nitrogen fixation, ammonia synthesis and hydrolytic enzyme activity. The activity of bacterial consortium significantly enhanced stem height, root length, and leaf length and width of *Macrotyloma uniflorum* (horse gram). Overall, the study indicates good potential of the constructed consortium to be used as a bio-inoculant in agriculture.

IndexTerms **- Acinetobacter spp, bio-inoculants, consortium, plant growth promoting properties, zinc solubilizer, zinc tolerance**

I. INTRODUCTION

The rising global population, along with rapid urbanization, has significantly increased food demand and placed tremendous pressure on agriculture. Consequently, the agricultural systems commonly face challenges including low land availability and reduced soil fertility (Robinson, 2024). Among the nutrient deficiencies in soil, zinc (Zn) deficiency is a widespread constraint in agriculture (Sethi et al., 2025). It is commonly reported in studies from Australia, Africa, India, Pakistan, China and Brazil (Khokhar et al., 2024; Hacisalihoglu 2020; Khan et al., 2022). Zn deficiency is a serious agricultural challenge since it leads to reduced biomass, chlorosis, stunted growth, delayed maturation and poor yield (Nandal and Solanki, 2021; Singh et al., 2024).

Consuming foods deficient in Zn ultimately causes human health issues, and also poses a threat to food security for the growing population worldwide (Pal et al., 2025). In humans, Zn deficiency is described as a “hidden hunger”. It is estimated that nearly 17% of the population is Zn deficient (Yilmaz and Yilmaz, 2025). A lack of Zn in the diet can lead to hair loss, cognitive impairment, skin disorders, general weakness and infertility in men (Upadhayay et al., 2022). In pregnant women, Zn deficiency can negatively impact fetal brain development, leading to long-term neurological and cognitive impairments (Abregu et al., 2022). It also increases susceptibility to infections and delays wound healing (Chasapis et al., 2020).

The problems of nutritional deficiencies in soil along with growing population and food security threat has made sustainable nutrient management strategies imperative, and they require urgent attention (Bhardwaj et al., 2024). Zn plays a central role in plant biochemical and physiological processes, including enzyme activation, chlorophyll formation, nitrogen metabolism and protection against oxidative stress (Khan et al., 2022). Unfortunately, supplementation of Zn through fertilizers is not effective in the long term because they form stable and insoluble salts of ZnS, ZnO, $ZnCO_3$, and $Zn_3(PO_4)_2$ in soil, which are inaccessible to plants (Solanki et al., 2020). Besides, soil pH, organic matter, and mineral composition influence Zn solubility (Noulas et al., 2018). Hence, its bioavailability is limited even in Zn rich soils (Hussain et al., 2018). Excess amounts of phosphorus, iron and manganese can also immobilize Zn through precipitation or adsorption (Nandal and Solanki, 2021).

Unlike other nutrient deficiencies which can be complimented with fertilizers, Zn deficiency can be best managed with Zn solubilizing bacteria (ZSB), which mobilizes insoluble Zn through the secretion of organic acids, chelators, siderophores and redox-based mechanisms (Upadhayay et al., 2021; Singh et al., 2024). ZSB are a type of Plant Growth Promoting Bacteria (PGPB) that has emerged as an effective alternative to chemical fertilizers. More often, the ZSB also demonstrate other traits of PGPB such as ACC deaminase activity, exopolysaccharide production, and phosphorus or potassium solubilization (Solanki et al., 2020). Various ZSB strains, including Bacillus, Pseudomonas, Burkholderia and Acinetobacter have been previously reported to improve seed germination, increase root length, stem height, leaves width, grain yield and the micronutrient content of edible parts (Upadhayay et al., 2021; 2022). A study conducted by Ramesh et al. (2014) reported that Zn solubilizing *Bacillus aryabhattai* significantly enhanced the root and shoot dry weight of soybean and wheat. Also, seed yield improvements of up to 30.7% in soybean and 48.0% in wheat were observed. The *B. aryabhattai* strain also contributed to an increase in Zn content within the edible portions of the plants. The study on Zn bio-fortification of fodder oat investigated by Chaudhary et al. (2021)

indicated that combining chelated Zn with foliar application and ZSB significantly enhances plant growth, providing taller plants and wider leaves.

Considering the relevance of ZSB in agriculture, the present study was carried out with an aim to isolate and characterize potential ZSB from diverse environments, construct a suitable consortium and study its effect as a bio-inoculant in improving soil fertility and growth of *Macrotyloma uniflorum* (horsegram) plant.

II. RESEARCH METHODOLOGY

2.1 Sample Collection

Rhizospheric soil samples were collected from sites surrounding banana, mango, pomegranate, papaya, and rice plants. These samples were obtained from a depth of approximately 10 cm to ensure the presence of an active microbial population. Additionally, samples were collected from vermicompost and rice fields. Each sample was carefully transferred into alcohol sterilized, zip-locked polyethylene bags and transported to the laboratory for microbiological analysis.

2.2 Enrichment and Isolation of ZSB

1 g of soil sample was suspended in 10 mL of phosphate-buffered saline (PBS, pH 7.0), mixed and allowed to stand for 10 min. 1 ml of this suspension was inoculated into Modified Pikovskaya's Medium (MPM) supplemented with 0.1% insoluble Zn salts, and incubated under static conditions at room temperature (RT, 28 ± 2°C) for seven days or until visible dissolution of insoluble Zn salts was observed. MPM was selected for this study since it provides optimal conditions for the selective growth of ZSB by providing a balanced nutrient composition, stable pH and reproducible results (Jagana et al., 2019).

For qualitative detection of ZSB, a loopful of the enriched culture was streaked onto sterile MPM agar plates supplemented with 0.1% insoluble Zn salts, and incubated at RT for 72h. Colonies exhibiting clear zones around their growth were considered potential Zn solubilizers. These colonies were further purified by repeated streaking on fresh MPM agar plates and maintained for subsequent characterization (Tamboli, 2019; Nivaas, 2019; Shahab and Ahmed, 2008). The diameter of both the colony and the halo zone was recorded up to the seventh day of incubation. The solubilization index (SI) and solubilization efficiency (SE) were calculated using the following formulas (Choudhary et al., 2024; Bhakat et al., 2021):

$$Solubilization\ Index\ (SI) = \frac{diameter\ of\ halo\ zone - diameter\ of\ colony}{diameter\ of\ colony}$$

$$Solubilization\ Efficiency\ (SE) = \frac{diameter\ of\ halo\ zone}{diameter\ of\ colony}\ x\ 100$$

2.3 Quantitative Analysis of Zinc solubilization

Bacterial isolates exhibiting the highest SI and SE were subjected to quantitative Zn solubilization broth assay. Each selected isolate was inoculated into sterile MPM broth containing 0.1% insoluble Zn salts. A 24h old culture adjusted to an optical density (OD_{540}) of 0.5 was used as the inoculum. The cultures were incubated at RT and samples were withdrawn every 48 h for the determination of insoluble Zn concentration in the medium (Yasmin et al., 2021). Zinc ion (Zn^{2+}) concentration in the supernatant was quantified using complexometric titration with EDTA as the titrant and Eriochrome Black T (EBT) as the indicator (Gangolli et al., 2014). The titration was conducted at pH 10.0–10.5 to ensure complex stability. During titration, Zn^{2+} ions form a stable 1:1 Zn–EDTA complex, displacing the EBT indicator and resulting in a color change from pink to blue at the endpoint (Simões et al., 2020).

The concentration of Zn^{2+} ions was calculated using the following formula:

$$Zn\ (g/100\ mL) = \frac{65.38 \times 0.02 \times (\mathrm{T} - \mathrm{B})}{1000}$$

Where, 65.38 is the atomic weight of Zn, 0.02 is the molarity of EDTA, T is the burette reading of test solution (mL) and B is the burette reading of blank (abiotic control).

2.4 Development of Microbial Consortium

ZSB isolates exhibiting high SI and SE were selected for the development of microbial consortia. The consortia were formulated based on the biocompatibility of individual isolates, which was determined through a cross-streak assay described by Hossain (2024). Each isolate was grown overnight in nutrient broth and adjusted to an optical density (OD_{540}) of 0.5. The isolates were streaked as a single straight line on sterile Nutrient Agar (NA) plates. A second isolate was then streaked perpendicularly to intersect the first streak at the midpoint. The plates were incubated at RT for 24h. The presence of an inhibition zone at the intersection indicated incompatibility between the isolates, whereas its absence confirmed compatibility. Based on observed patterns, biocompatible isolates were combined to form microbial consortia. The efficiency of Zn solubilization by the developed consortia was evaluated through the in vitro Zn solubilization broth assay.

2.5 In Vitro Screening for Plant Growth-Promoting Properties of potential ZSB strains

2.5.1 Indole acetic acid

Indole-3-acetic acid (IAA) production by potential ZSB isolates was estimated following the method of Upadhayay (2021). Bacterial cultures (OD_{540} = 0.5) were inoculated into Luria broth supplemented with 50 µg/mL of L-tryptophan and incubated at RT for 48 h. After incubation, cultures were centrifuged at 5500 rpm for 10 mins. Two ml of the resulting supernatant were mixed with an equal volume of Salkowski's reagent and incubated in the dark for 30 mins. The appearance of a pink colour indicated IAA production, and absorbance was measured spectrophotometrically at 540 nm.

2.5.2 Nitrogen Fixation Activity

The nitrogen-fixing potential of ZSB isolates was determined using Norris Glucose Nitrogen-Free (NGNF) medium, following the method described by Othman et al. (2022). Cultures adjusted to 0.5 OD_{540} were inoculated in the medium and incubated at RT for 24 h. As the medium lacked a nitrogen source, only nitrogen-fixing bacteria were able to grow. The formation of a halo zone around the colonies, resulting from the dissolution of calcium carbonate, indicated organic acid production and confirmed nitrogen-fixing ability.

2.5.3 Phosphate, Potassium, and Silica Solubilization Activity

Phosphate solubilization was assessed using Pikovskaya's agar medium, where isolates (OD_{540} = 0.5) were spot-inoculated and incubated at RT for seven days. Potassium solubilization was evaluated using Aleksandrov's medium supplemented with insoluble potassium salts and bromothymol blue as a pH indicator to visualize solubilization. Silica solubilization was tested on basal medium containing insoluble silica salts. The development of clear halo zones around bacterial colonies was recorded as evidence of solubilization activity (Yasmin et al., 2021).

2.5.4 Amylase enzyme activity

Amylase production was determined by spot inoculating isolates (OD_{540} = 0.5) onto sterile starch agar plates containing 0.2% soluble starch and incubating them at RT for 72 h. Following incubation, plates were flooded with iodine solution. The rapid formation of a clear halo zone surrounding the bacterial colony indicated starch hydrolysis and amylase activity (Vithoba, 2023).

2.5.5 Cellulase Enzyme Activity

Cellulase activity was evaluated using Carboxymethyl Cellulose (CMC) agar medium with cellulose as the sole carbon source. A 24 h old culture (OD_{540} = 0.5) was spot-inoculated onto CMC agar plates and incubated at RT for 72 h. Post-incubation, plates were flooded with 0.1% Congo Red solution, followed by destaining with 1 M NaCl. The formation of a clear halo zone around the colonies indicated cellulase production (Vithoba, 2023).

2.5.6 NH_3 Production

Ammonia production was tested in peptone water (5 mL per tube), following the method described by Chaudhary et al. (2023). The medium was autoclaved, cooled and inoculated with bacterial isolates (OD_{540} = 0.5). It was then incubated at RT for 48–72 h. After incubation, 0.5 mL of Nessler's reagent was added to each tube and left for 5 mins at RT. The development of a brownish-orange coloration confirmed ammonia production.

2.5.7 Stress Tolerance

The tolerance of bacterial isolates to Zn stress was evaluated by growing them on NA plates supplemented with varying concentrations of Zn^{2+} ions (10–1200 ppm). Growth patterns were observed after incubation at RT for 48 h. The highest concentration permitting visible bacterial growth was recorded as the maximum tolerance level (Sultan et al., 2023).

2.6 Phenotypic and biochemical characterization of potential ZSB strains and their identification

Identification of the potential isolate was done based on cultural, morphological, biochemical and molecular tests. Gram characteristics of the isolates were determined using Gram staining and the 3% potassium hydroxide (KOH) tests to confirm cell wall type and Gram reaction.

2.6.1 Capsule staining

The presence of a capsule was confirmed using Maneval's staining technique (Hughes and Ann, 2007). A thin bacterial smear was prepared on a clean glass slide and air-dried without heat fixation. The smear was first stained with Congo red (negative stain) to create a dark background, followed by the addition of Maneval's stain as a counterstain. The appearance of a clear, unstained halo surrounding the bacterial cells indicated the presence of a capsule, while the absence of a halo denoted a negative result.

2.6.2 Haemolytic activity

Along with standard biochemical tests such as catalase and oxidase, hemolytic activity was assessed on freshly prepared blood agar plates (Karnwal, 2021). The bacterial isolates were streaked onto the plates and incubated at 37°C for 24–48 h. After incubation, the type of hemolysis was recorded as follows:

- α-hemolysis (Alpha): Partial lysis of red blood cells producing a greenish zone around colonies.
- β-hemolysis (Beta): Complete lysis of red blood cells producing a clear, transparent zone around colonies.
- γ-hemolysis (Gamma): No visible hemolysis, indicated by the absence of any change in the medium.

2.6.3 Molecular identification

For molecular identification, the most efficient ZSB was submitted to Hi-Gx360® Solutions, HiMedia Laboratories Pvt. Ltd. DNA extraction and amplification were followed by sequencing using BDT v3.1 chemistry on the 3500XL Genetic Analyzer. The obtained sequence data were subjected to similarity analysis using the NCBI BLAST database for species identification.

2.7 Optimization of Zinc solubilization activity

The effect of different physicochemical parameters on the Zn solubilizing activity of the bacterial consortium was evaluated using the one factor at a time optimization approach. Each optimized parameter was applied in subsequent experiments to determine their cumulative effects on zinc solubilization efficiency (Khanghahi et al., 2018; Shaikh and Saraf, 2017). As a standard approach, 1ml culture suspension (0.2 OD_{540}) isolates were inoculated into 50 mL of modified St. Pikovskaya's broth supplemented with 0.15% ZnO. After incubation at RT for 24 h, the cultures were centrifuged, and the concentration of soluble Zn ions in the supernatant was determined using complexometric titration with Eriochrome Black T as the indicator and EDTA as the titrant. The optimised parameters included O.D (0.1–0.7), inoculum sizes (0.5–3.0 mL), pH (3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0), temperature (RT, 35°C, 45°C, and 55°C) oxygen (static and 120rpm), carbon sources (1% sucrose, xylose, mannitol and starch) and nitrogen sources (0.04% peptone, tryptone, meat extract, and beef extract).

2.8 Application of consortia as bio-inoculant

2.8.1 Preparation of the inoculant

A fresh culture of the ZSB isolates and their consortium was cultivated in 50 mL of nutrient broth medium and incubated for 24 h at RT under shaking conditions. After incubation, the culture broth was centrifuged to harvest the bacterial cells. The resulting cell pellet was washed twice and re-suspended in freshly autoclaved distilled water to obtain a standardized bacterial suspension with a final concentration of approximately 10^8 CFU/mL, which was subsequently used for seed priming.

2.8.2 Seed treatment and Pot assay

Horse gram (*Macrotyloma uniflorum*) seeds were selected for the pot assay experiment. The seeds were first surface-sterilized with 70% ethanol and rinsed thoroughly with sterile distilled water, followed by soaking in autoclaved distilled water for 12 h. The sterilized seeds were then subjected to seed priming by exposing them to different treatments indicated in Table 1, under shaking conditions for 4 h.

The soil used in the study was air-dried, sieved to remove debris, and sterilized by autoclaving to eliminate indigenous microbial populations. This ensured that the observed plant growth responses were due to the introduced bio-inoculants. Each pot was filled with 200g of sterilized soil and sown with five treated seeds. The pots were watered daily with sterilized distilled water to maintain moisture. After 15 days of sowing, a second application of the respective inoculant treatments was performed to reinforce bacterial colonization and enhance Zn solubilization in the rhizosphere. The plants were allowed to grow for 30 days before harvesting. Growth parameters such as shoot length, root length, and biomass were measured, and the results were analyzed using bar graphs and one-way ANOVA to determine statistical significance among treatments.

Table 1: Treatment of pots with ZSB

Sr. No.	Treatments
1	Control (Only seeds)
2	$ZnSO_4$ as zinc supplement
3	ZnO as zinc supplement
4	ZSB inoculant 1
5	ZSB inoculant 1+ ZnO supplement
6	ZSB Inoculant 2
7	ZSB Inoculant 2 + ZnO supplement
8	Consortium: ZSB Inoculant 1+ ZSB Inoculant 2
9	Consortium: ZSB Inoculant 1+ ZSB Inoculant 2 + ZnO supplement
Each treatment was performed in triplicates; Amount of ZnO added per pot (0.02g); Amount $ZnSO_4$ solution added per pot (0.0011g/mL)	

III. RESULTS AND DISCUSSION

3.1 Isolation of ZSB

A total of 25 bacterial isolates were obtained from diverse soil samples. Among these, 14 isolates showed Zn solubilization potential. The solubilization efficiency of the isolates obtained in this study is indicated in Table 2. The observed zones on Pikovaskya's medium supplemented with 0.1% Zn ranged from 1.78 cm to 3.07 cm. Based on the observations of qualitative test on MPM plates, isolate BR2B exhibited the highest solubilization activity, with SI of 3.71 ± 0.526 and SE of 241 ± 15.56, indicating its strong potential for Zn mobilization. In contrast, isolates BR2A and PA2 demonstrated the smallest halo zones of 1.78 cm and 1.85 cm respectively, reflecting comparatively lower solubilization capability. Quantitative assay indicated complete solubilization of Zn salts by the 7th day of incubation. Based on this assay, isolates BR2B and BRC exhibited the highest Zn solubilization activity, reaching 797 mg/L and 784.5 mg/L, respectively, followed by BRS (640 mg/L). These findings are comparable to previous reports, where Pseudomonas species solubilized 625 mg/L of Zn ions from ZnO and 753 mg/L from $ZnCO_3$ (Bapiri et al., 2012), *Pantoea eucrina* solubilized 624 mg/L of ZnO, and Streptomyces spp. solubilized 503 mg/L of

$ZnCO_3$ (Choudhary et al., 2024). Similarly, Streptomyces spp. was reported to solubilize 529.71 mg/L of $ZnCO_3$ (Suriyachadkun et al., 2022).

A notable decrease in pH (from 7.4 to 4) was also observed in all inoculated samples compared to the abiotic control by the end of incubation. This acidification suggested the production of organic acids such as gluconic, lactic, and malic acids. These organic acids chelate Zn ions and enhance its bioavailability (Chanu and Yadav, 2024; Haroon et al., 2021; Vidyashree et al., 2018).

Table 2: ZSB isolated in the study and their efficiency

Sample type	Total	Strain	Solubilization Zone (cm)	SI	SE	Zn Solubilization (mg/L)	pH of the medium
Vermi-compost	4	VC3, VC4	-	-	-	-	-
		VC1	2.333 ± 0.529	2.975 ± 0.672	156.25 ± 8.84	-	-
		VC2	2.2 ± 0.141	2.87 ± 0.061	188.97 ± 3.44	-	-
Rice Fields	6	RIC 1, RIC 2, D1, D4	-	-	-	-	-
		D3	2.45 ± 0.071	3.04 ± 0.057	204 ± 5.66	274	6
		D2	2.25 ± 0.071	2.78 ± 0.028	179.5 ± 4.95	-	-
Banana tree soil	8	BR1, BR3, BR6, BR7	-	-	-	-	-
		BR2A	1.775 ± 0.106	2.675 ± 0.248	190 ± 0	-	-
		BR2B	3.067 ± 0.208	3.71 ± 0.526	241 ± 15.56	797	4.3
		BR3	2.693 ± 0.160	2.52 ± 0.388	202.67 ± 39.02	-	-
		BRS	2.3 ± 0.283	2.99 ± 0.127	199 ± 12.73	640	5.2
		BRC	2.35 ± 0.354	3.355 ± 0.248	201.5 ± 23.33	784.5	4.3
Papaya tree	2	PA1	2 ± 0	2.73 ± 0.187	172.67 ± 18.62	-	-
		PA2	1.85 ± 0.071	2.63 ± 0.240	163 ± 24.04	-	-
Pomegranate tree	2	POM1	-	-	-	-	-
		POM2	2.6 ± 0.3	3.29 ± 0.356	228.87 ± 34.62	339	6.5
Mango tree	1	MAN	-	-	-	-	-
Fig tree	1	FIG	2.433 ± 0.208	2.8 ± 0.246	182.30 ± 23.21	-	-
Moringa tree	1	SHI	2.5 ± 0	3 ± 0.0	200 ± 0	399	6
Control						52.30	7.4

3.2 Determination of the most suitable bacterial consortia

The bacterial isolates BR2B, BRC and BRS exhibited no visible inhibition at the point of intersection during the cross-streak assay, confirming their mutual compatibility (Fig. 1). Such compatibility is critical for establishing a functional bacterial consortium that can enhance their metabolic activity leading to better Zn solubilization and other plant growth-promoting traits (Sarkar et al., 2013; Patowary et al., 2016; Santiago et al., 2017). Hence, different combinations of bacterial isolates were mixed in equal volumes to develop the most suitable consortium (Table 3). The BR2B + BRC combination demonstrated the highest solubilization activity, achieving approximately 1085 mg/L of solubilized Zn within 24 h. This synergistic performance significantly exceeded that of individual isolates, which had previously shown around 700 mg/L solubilization activity. This clearly indicates a synergistic metabolic interaction, likely involving complementary pathways for organic acid synthesis. The pronounced pH drop from 7.5 to 3.8 within 24 h further suggested either an enhanced rate of production, or diversity, of organic acids (Macias-Benitez et al., 2020). The solubilization activity recorded in this study is notably higher than that reported by Upadhayay (2021), where co-inoculation of BMRR126 and BMAR64 resulted in solubilization of 30.08 ± 1.54 µg/mL of Zn with a pH reduction from 7 to 4.38 after 10 days.

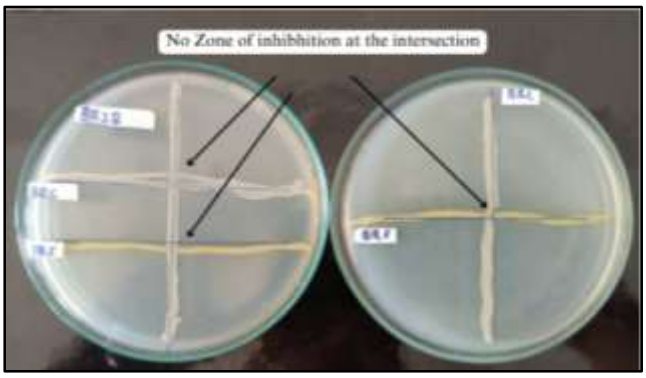

Fig 1: Cross streak assay indicating no zone of inhibition at the intersection

Table 3: Quantitative assessment of Zn Solubilization activity of the consortium

Consortium	Zinc solubilization in (mg/L)	pH
BR2B+ BRC+ BRS	889	4.3
BR2B+ BRC	1085	3.8
BRS+ BR2B	143	6.5
BRC+ BRS	536	4.6
Control	52.30	7

3.3 Plant growth promoting properties of ZSB consortia

Table 4 summarizes the plant growth promoting properties of BR2B and BRC strains. The tests and observations for the same are represented in Fig. 2. It is well documented that ZSB often exhibit additional plant growth-promoting traits (Jalal et al., 2024; Jalal-ud-din et al., 2024; Singh et al., 2024), which were also observed in this study.

As observed from the table, both isolates produced IAA and ammonia, and demonstrated nitrogen-fixation activity. IAA is a key auxin that stimulates rapid responses like cell elongation, and long-term processes such as cell proliferation. They promote enhanced root development and accelerated nutrient uptake. Notably, around 80% of rhizospheric bacteria naturally secrete IAA and previous studies have shown that inoculating plants with IAA producing ZSB can significantly boost plant growth (Patten and Glick, 1996; Saharan and Nehra, 2011). For instance, a Zn solubilizing strain of *Bacillus megaterium* was previously reported to produce 13.8 µg/mL IAA and enhance the yield of *Capsicum annuum* L. (Bhatt and Maheshwari, 2020).

Nitrogen is a fundamental macronutrient for plants. The bacteria convert atmospheric nitrogen into ammonia and other nitrogenous compounds that plants can readily absorb and utilize. Nitrogen is required in large quantities since it is involved in the formation of amino acids, chlorophyll, adenosine triphosphate and nucleic acids. The presence of ammonia suggests efficient nitrogen metabolism by these bacteria contributing to soil fertility and plant nutrient availability (de Andrade et al., 2023; Othman et al., 2022; Singh et al., 2024).

The BR2B and BRC strains also demonstrated potassium solubilization activity, which is essential for converting potassium minerals, such as feldspar and mica, into forms accessible to plants (Yasmin et al., 2021; Shaikh and Saraf, 2017). Besides Zn, phosphorus is another essential micronutrient necessary for fruit development. Unfortunately, the ZSB strains of the consortium did not exhibit phosphorus solubilization activity. Further studies on screening of potential phosphorus solubilization bacteria which is also compatible with the consortia developed in this study, may enhance its effectiveness as bio-fertilizer. The production of amylase by BRC suggests a metabolic advantage in degrading complex organic substrates, which could promote plant-microbe interactions by providing easily accessible carbon sources. This activity may also facilitate the breakdown of plant-derived starches in root exudates, thereby improving soil microbial community dynamics and plant nutrient uptake (Vithoba, 2023; Devi et al., 2022). Cellulolytic activity is equally beneficial in degrading plant cell walls, and hence promotes recycling of plant biomass, which is crucial for maintaining soil organic matter levels and promoting sustainable agricultural practices (Devi et al., 2022; Othman et al., 2022). In addition to these qualities, high tolerance to Zn helps bacteria to withstand the concentrated Zn ions generated during the solubilization process itself (Sultan et al., 2023). It also helps bacteria to function optimally under stress environments with elevated levels of Zn concentrations in environments which are excessively amended with chemical Zn fertilizers or contaminated soils. The ability of BRC to tolerate Zn concentrations up to 1200 ppm suggests strong metal resistance mechanisms, which may include efflux systems, metal-binding proteins or intracellular sequestration (Sultan et al., 2023).

Table 4: Plant growth promoting properties of BR2B and BRC strains

Isolates	PGP properties
BR2B	IAA production observed in 48h; Nitrogen fixation and ammonia production observed in 24h; potassium solubilization observed in 48h; Zn tolerance up to 800ppm
	No phosphorus or silica solubilization activity; cellulolytic and amylolytic activity absent
BRC	IAA production observed in 48h; Nitrogen fixation and ammonia production observed in 24h; potassium solubilization observed in 48h; cellulolytic and amylolytic activity observed in 48h and 24h respectively; Zn tolerance up to 1200ppm
	No phosphorus or silica solubilization activity

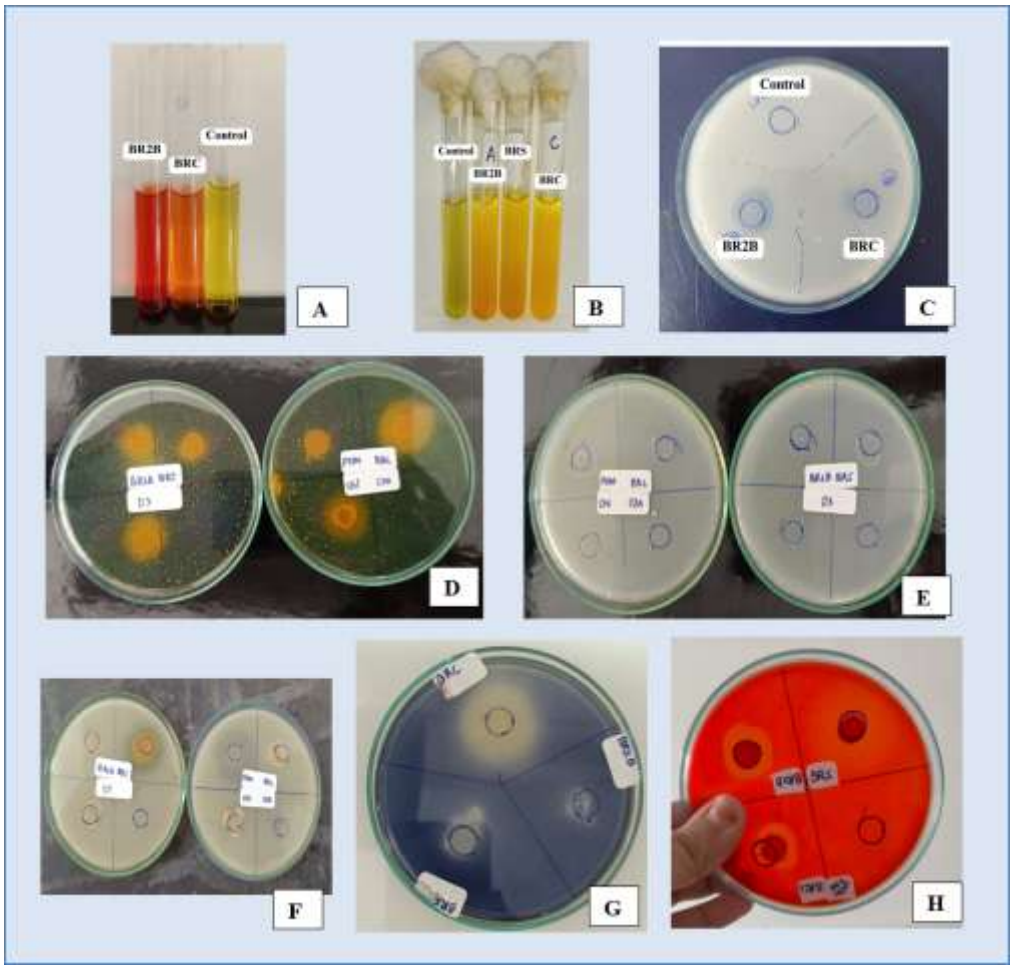

Fig. 2: Plant growth promoting activities of BR2B and BRC strains

The figure shows (a) positive test for IAA; (b) positive test for ammonia production; (c) positive Nitrogen fixing activity on Norris glucose medium (d) positive potassium solubilization activity (e) Negative silica solubilization activity (f) Negative phosphate solubilization activity (g) Positive amylase activity by BRC and (h) positive cellulase activity

3.4 Characteristics of Isolates and their Identification

Table 5 summarises the observed cultural, biochemical and microscopic characteristics of ZSB strains. Fig. 3 represents the observations of BLASTN analysis and Fig. 4 represents the phylogenetic tree for clustering of isolates BR2B and BRC. Based on molecular analysis, BR2B was identified as *Acinetobacter baumannii* (strain ATCC 19606). The BLASTN analysis of the BRC isolate against the NCBI Type strain database (using version 2.12.0) revealed the highest similarity to *Acinetobacter calcoaceticus* (strain NCCB 22016) and *Acinetobacter pittii* (strain DSM 1653), both with a bit score of 2700, alignment score of 1462, and 100% query coverage and identity. The number of taxonomic hits, however, was more for *A. calcoceticus* as compared to *A. pitti*. Hence, BRC was identified most likely as *Acinetobacter calcoaceticus* (strain NCCB 22016) based on BLASTN and phylogenetic analysis.

Table 5: Cultural, biochemical and microscopic characteristics of ZSB strains

Characteristics	BR2B	BRC
Cultural	Circular, white, low convex, translucent and butyrous colony of 1mm size	Circular, white, low convex, translucent and butyrous colony of 1mm size
Biochemical	Catalase positive; No haemolytic activity	Catalase positive; No haemolytic activity
Microscopic	Gram negative coccobacilli arranged singly; capsule present	Gram negative coccobacilli arranged singly; capsule present

BLAST ANALYSIS RESULT — A

Program Name: BLASTN Program Version: 2.12.0 Database used: NCBI Type strain

qseqid	sseqid	bitscore	score	qcovs	pident
24P110_414_BR2B	NR_117620.1_Acinetobacter_baumannii_strain_ATCC_19606_16S_ribosomal_RNA,_partial_sequence	2699	1461	100	100.000
24P110_414_BR2B	NR_148847.1_Acinetobacter_vivianii_strain_NIPH_2168_16S_ribosomal_RNA,_partial_sequence	2555	1383	100	98.224
24P110_414_BR2B	NR_117623.1_Acinetobacter_junii_strain_ATCC_17908_16S_ribosomal_RNA,_partial_sequence	2538	1374	100	98.020
24P110_414_BR2B	NR_148843.1_Acinetobacter_courvalinii_strain_ANC_3623_16S_ribosomal_RNA,_partial_sequence	2532	1371	100	97.951

BLAST ANALYSIS RESULT — B

Program Name: BLASTN Program Version: 2.12.0 Database used: NCBI Type strain

qseqid	sseqid	bitscore	score	qcovs	pident
25A110_060_BRC	NR_042387.1_Acinetobacter_calcoaceticus_strain_NCCB_22016_16S_ribosomal_RNA,_partial_sequence	2700	1462	100	100.000
25A110_060_BRC	NR_117621.1_Acinetobacter_pittii_DSM_21653_strain_ATCC_19004_16S_ribosomal_RNA,_partial_sequence	2700	1462	100	100.000
25A110_060_BRC	NR_102814.1_Acinetobacter_oleivorans_strain_DR1_16S_ribosomal_RNA,_partial_sequence	2695	1459	100	99.932

C

Taxonomy	Number of hits	Number of Organisms	Description
⊟Pseudomonadota	101	72	
. ⊟Acinetobacter	100	71	
. . ⊟Acinetobacter calcoaceticus/baumannii complex	21	8	
. . . ⊟Acinetobacter calcoaceticus	7	2	Acinetobacter calcoaceticus hits
. . . ⊞Acinetobacter pittii	1	2	Acinetobacter pittii hits
. . . Acinetobacter seifertii	1	1	Acinetobacter seifertii hits
. . . Acinetobacter lactucae	2	1	Acinetobacter lactucae hits
. . . Acinetobacter nosocomialis	1	1	Acinetobacter nosocomialis hits
. . . Acinetobacter baumannii	6	1	Acinetobacter baumannii hits
. . Acinetobacter oleivorans	2	1	Acinetobacter oleivorans hits

Fig. 3: Identification of ZSB

Figure shows BLASTN analysis of (a) BR2B and (b) BRC isolates, and (c) Taxonomic hits observed for BRC

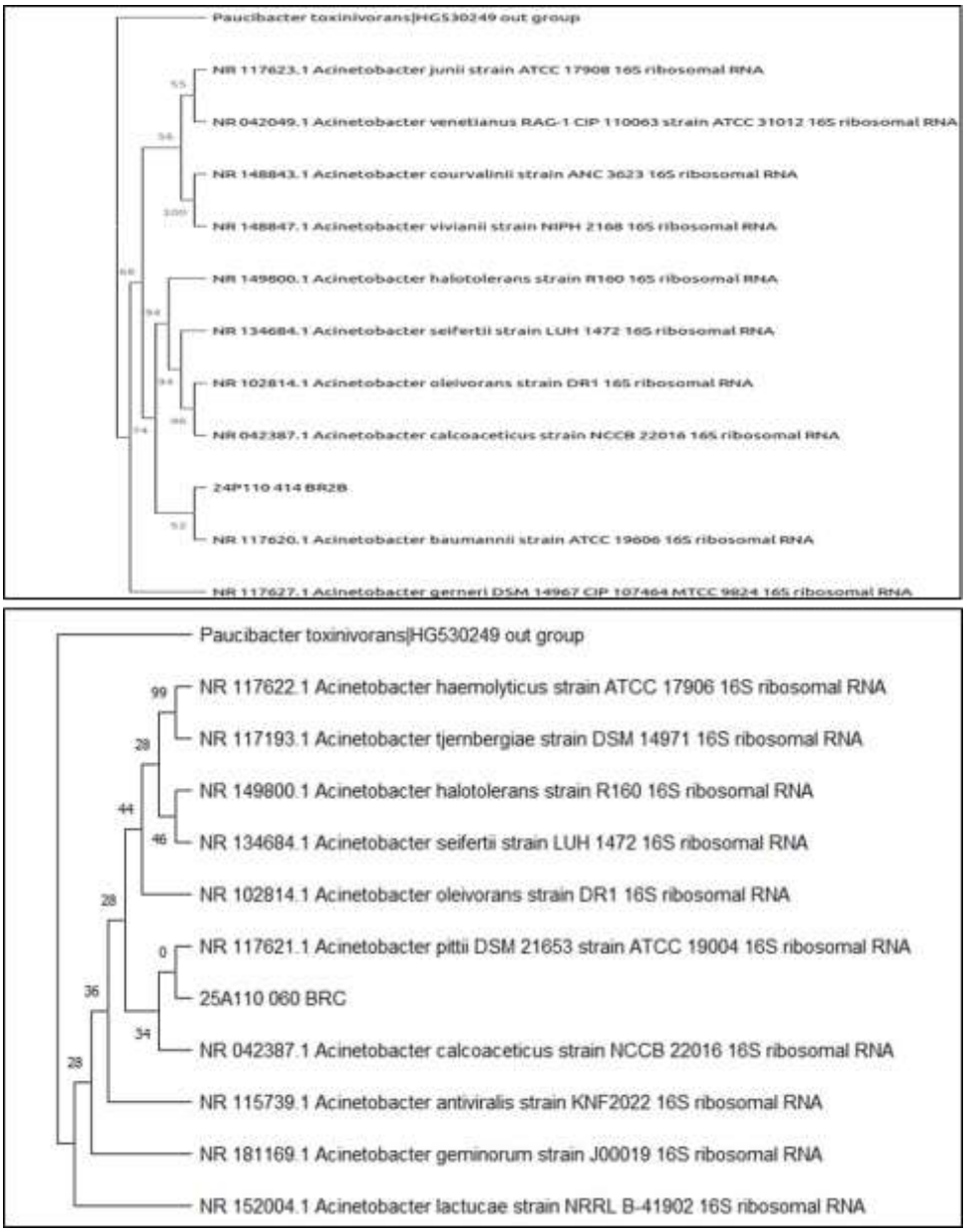

Fig. 4: Phylogenetic tree for BR2B (above) and BRC (below)

3.5 Optimization of Zn solubilization potential of consortia

Fig. 5 represents the optimum parameters for Zn solubilization by the consortium. Optimum solubilization was achieved on inoculation of 1 ml (0.5 $O.D_{540nm}$) consortia in modified St. Pikovskaya's broth adjusted to pH 6 and supplemented with 0.15% ZnO, 1% glucose and 1% yeast extract. Optimum incubation temperature was RT (~28ºC-30ºC). A similar observation was reported by Shaikh and Saraf, (2017) where the isolates MSSZB4 and MSS-ZF3, with biofortification and bioremediation potential, showed optimum activity at pH 6 - 6.5 and temperature 28 - 30˚C in media containing 0.1% ZnO, dextrose and ammonium sulphate.

An interesting observation was noted in a study by Khanghahi et al., (2018) where optimum Zn solubilization (50-72mg/L) by *Agrobacterium tumefaciens* and Rhizobium sp. occurred in presence of 0.8% NaCl in media adjusted to pH 8–10, while no solubilization was observed at pH 6 and 7 at 29°C. This finding indicates that these bacteria may use mechanisms other than production of organic acids for Zn solubilization including proton extrusion, secretion of siderophores or chemical transformations such as redox reaction (Upadhayay et al., 2025). In a more recent study by Khanghahi et al., (2021), optimum growth conditions were determined for Zn solubilizing *Acinetobacter calcoaceticus*. Maximum activity was observed at 0.64% NaCl concentration and 27.4°C. In this study too, optimum activity was observed at pH 9. In another study, Aspergillus strain solubilized Zn most efficiently in presence of 4.6 g/L fructose, 10 g/L $(NH_4)_2SO_4$, and 15 g/L $Zn_3(PO_4)_2$. The study also demonstrated that Zn solubility was directly proportional to the concentrations of $Zn_3(PO_4)_2$ and $(NH_4)_2SO_4$ (Barin et al., 2022).

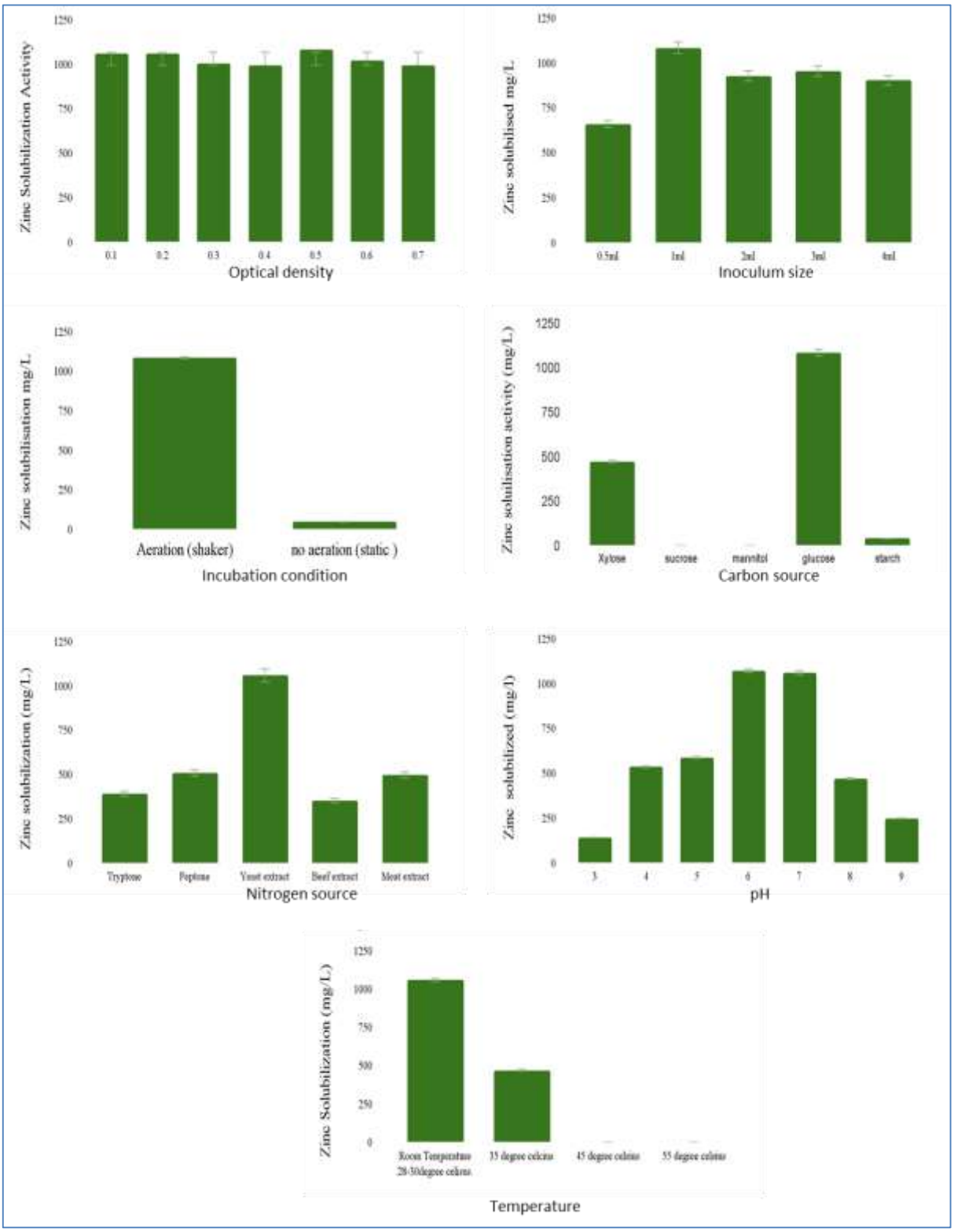

Fig. 5: Optimization of Zn solubilization by consortia

3.6 Application of consortia in plant growth promotion

The observations for pot assay are represented in Fig. 6. The ZSB consortia improved all the studied growth parameters in this study including seed germination rates, leaf length and width, stem and root length. Both individual strains and their combinations with ZnO achieved a shortened germination period of 3 days, indicating an early vigour effect. Also, although $ZnSO_4$ positively influenced leaf width, the overall improvements were substantially greater in treatments involving ZSB. A representation of effect of different treatments on plant and root growth after 30 days is presented in Fig. 7.

Overall, the enhanced vegetative growth observed in ZSB inoculated plants can be attributed not only to Zn solubilization but also to the additional plant growth promoting traits of the isolates, including amylase and cellulolytic activity, nitrogen fixation, ammonia production and IAA synthesis. These mechanisms collectively support nutrient mobilization, root development and early seedling establishment (Yasmin et al., 2021; Shaikh and Saraf, 2017; Vithoba, 2023; Devi et al., 2022; Othman et al., 2022). Zinc supplementation further complements these effects by boosting photosynthetic capacity through increased chlorophyll

content. One study reported that Zn supplementation can boost photosynthesis up to 133.4% and also enhance gas exchange rates (Ojeda‐Barrios et al., 2012).

Our findings were further supported by statistical analysis. One-way ANOVA showed significant differences among treatments for stem height ($F = 9.56$, $p = 0.00024$) and root length ($F = 596.55$, $p = 3.25 \times 10^{-20}$), confirming that Zn supplementation alone is insufficient to drive notable plant growth. Instead, the combined application of both strains with ZnO exhibited a clear synergistic effect, leading to markedly improved Zn bioavailability and plant development. Precisely, root length increased by up to 1.94-fold in ZSB inoculated seeds compared to the un-inoculated control, and stem height increased by up to 1.78-fold with the bacterial consortium.

These findings align with previous studies in which a 2.72-fold increase in canola plant height was reported following ZSB inoculation (Jalal-Ud-Din et al., 2024). Similarly, Yasmin et al. (2021) observed enhanced root and shoot biomass in chickpea plants inoculated with Zn solubilizing *Pseudomonas protegens* RY2. This growth was attributed to phytohormone (IAA) production and improved nutrient availability. In a study similar to the present set-up, the combined application of Zn solubilizing Acinetobacter sp. strains AGM3 and AGM9 significantly enhanced growth and yield parameters in rice genotypes BPT5204 and IR64 under field conditions. The study reported 6.62% and 5.15% increase in plant height of BPT5204 and IR64 cultivars respectively. The grain yield also improved by ~9% (Gandhi and Muralidharan, 2016). Also, Zn solubilizing *A. calcoaceticus* reported by Sultan et al. (2023) improved shoot length, fresh weight, and dry weight of maize. Studies on Zn solubilizing actinobacteria have also shown encouraging results in promoting plant growth. In one study, Streptomyces strain CME34 increased shoot length by 27.98%, root length by 24.09%, plant dry weight by 45.34%, the number of pods per plant by 153.97% and the number of seeds per soybean plant by 121.01%, compared to the control (Suriyachadkun et al., 2022).

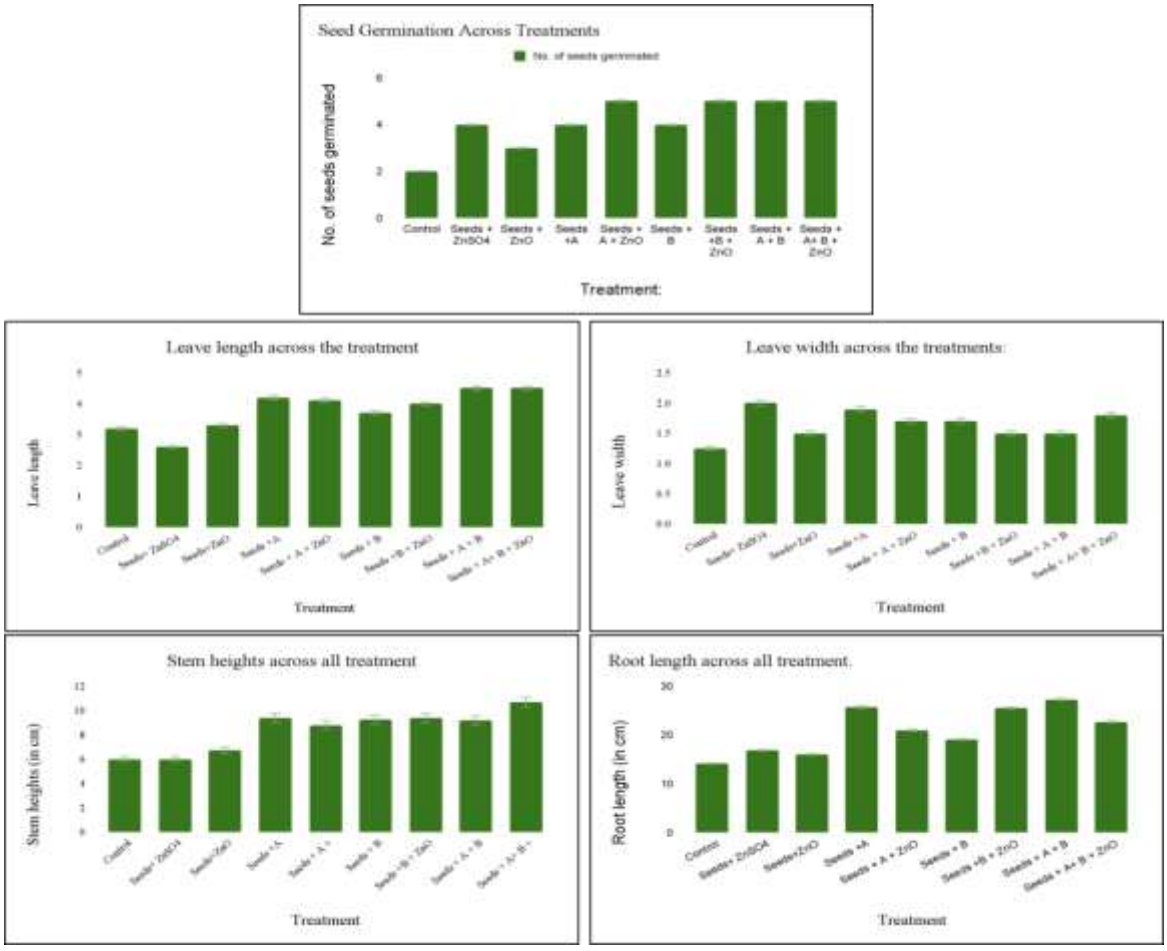

Fig. 6: Effect of Different set-ups on growth of horse gram

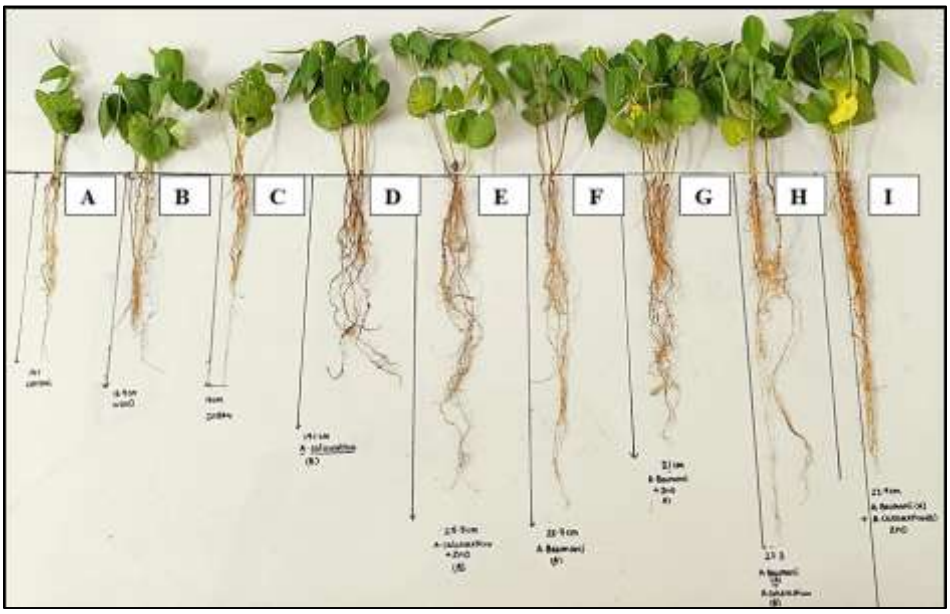

Fig. 7: Comparative representation of effect of different set-ups on growth of horse gram in 30 days

The figure shows plant growth in the following pot set ups: (a) Control (b) ZnO as Zn supplement (c) $ZnSO_4$ as Zn supplement (d) *A. calcoaceticus* (e) *A. calcoaceticus* + ZnO supplement (f) *A. baumanii* (g) *A. baumanii* + ZnO supplement and (h) *A. calcoaceticus* + *A. baumanii* + ZnO supplement

IV. CONCLUSION

The present findings demonstrate the significant potential of ZSB as a sustainable and eco-friendly alternative to chemical fertilizers. By enhancing Zn solubilization and plant nutrient uptake, ZSB inoculation can promote healthier plant development and greater biomass accumulation. Overall, the results of this study highlight the ability of ZSB consortia, particularly when combined with ZnO, as effective bio-inoculants for enhancing plant growth and Zn nutrition.

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