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RESEARCH ARTICLE

PGPR attributes and molecular identification of non-rhizobial Endophytes for growth enhancement of *Sesbania sesban* (L) Merr.

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Abstract

The ongoing research explores the isolation, characterization, identification and plant growth promoting (PGP) functions of selected plant growth-promoting rhizobacteria (PGPR) isolated from the root nodules of a wild leguminous plant *Sesbania sesban* (L) Merr., growing in a plain area adjacent to the Shivalik hill regions of Uttarakhand. From an initial pool of 70 bacterial isolates, 16 gram-negative strains were selected and screened for their potential PGP traits, such as phosphate solubilization, siderophore production, hydrogen cyanide (HCN) and indole-3-acetic acid (IAA) production. Notably, bacterial strains SSP1 and SSP3, demonstrated strong efficacy in these traits. Ribotyping analysis of 16S rRNA genes identified them as *Enterobacter xiangfangensis* SSP01 and *Acinetobacter pittii* SSP03, showcasing their taxonomy as non-rhizobial endophytes. Moreover, these bacterial strains demonstrated significant enhancements in biomass accumulation and vegetative growth parameters in *S. sesban*. In pot trial experiments, the SSP01 and SSP03 consortia displayed better results, highlighting their potential in agricultural applications. By exploiting the beneficial properties of these bacterial strains, our findings offers a promising avenue for sustainable agriculture, presenting an eco-friendly alternative to conventional chemical fertilizers.

Keywords: *Sesbania sesban*, Traditional green manure, PGPR attributes, Molecular identification, Vegetative growth, Pot trial.

Introduction

Excessive utilization of pesticides, fungicides and chemical fertilizers in modern agronomy poses significant risks to animals, humans, the environment, and microbial life forms. The advent of the Green Revolution in 1967 brought about a remarkable transformation in Indian agriculture, resulting in unprecedented levels of crop production. Over the course of the past few years, the rate of growth in crop production has been decreasing continuously. This decline can be attributed to the worsening of soil quality and health, primarily caused by the immoderate utilization of agrochemicals, pesticides, fungicides, and chemical fertilizers in modern farming systems. Pesticides are regarded as an important tool for crop protection and growth, but they are toxic to the environment as well as for all living organisms. overuse of pesticides induces biodiversity loss and destruction. (Ahmed and Turcihi 2021, Khan *et al*. 2023, Shafiq *et al*. 2024). In India, soils typically exhibit poor fertility due to nutrient leaching resulting from intensive cultivation, limited agricultural knowledge, imbalanced use of chemical fertilizers, and inadequate fertilization which have been used more frequently over the past few decades, due to which soil efficiency deteriorates. These factors not only support a decline in crop productivity but also lead to greenhouse gas emissions and water contamination (Aleminew and Alemayehu 2020). The diminishing fertility of the soil also poses a serious challenge to restoring vegetation on degraded lands. One of the key nutrient deficiencies observed in these soils is nitrogen, which performs an imperative role in maintaining soil fecundity. However, nitrogen fertilizer is expensive, scarce, and prone to rapid depletion through leaching, runoff, and evaporation, further contributing to water and environmental contamination. Addressing the increasing reliance on chemical fertilizers necessitates the development

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of sustainable agricultural strategies (Prasad *et al.* 2021, Timmis and Ramos 2021). Previously, the conventional understanding was that rhizobia were exclusively responsible for symbiotic biological nitrogen fixation (BNF) within the microenvironments of root nodules. Several investigations suggest that the root nodules harbor a more diverse array of bacteria associated with nodulation (De Meyer *et al.* 2015). Various non-rhizobial endophytes (NREs) from distinct groups have been documented to play a role in facilitating the root nodulation approach and promoting plant health and growth (Preyanga *et al*. 2021). NREs infiltrate plant tissues and establish residence in the inner compartments of the plant, as documented by Hardoim *et al*. (2008). Several investigations suggest that most of the NREs have the capability to boost plant growth, elevate productivity, and provide protection against pathogens. Consequentially, divergent genera of NREs were recorded from various research, including *Agrobacterium, Acinetobacter, Bacillus, Enterobacter, Klebsiella, Phyllobacterium, Pseudomonas, Pantoea,* and *Serratia* (Saharan and Nehra 2011, Debnath *et al.* 2023). In terms of genera and species, PGPR displays significant diversity, with each plant species hosting a specific set of PGPR in its rhizosphere. PGPR are soil bacteria linked to plant root nodules that confer useful benefits on their hosts. Certain PGPRs are able to establish endophytic populations by penetrating the interior of the root. It is essential to identify the appropriate species of PGPR for artificial inoculation in the rhizosphere of a particular plant spp. to ensure compatibility. Researchers can introduce suitable PGPR species into the rhizosphere to supplement the existing population of naturally occurring PGPRs (Kenneth *et al.* 2019). By identifying and characterizing these new PGPRs, we can unlock their beneficial attributes and explore their use in sustainable agricultural practices. The continuous discovery of PGPRs among different bacterial genera highlights the relevance of this study as well as the microorganisms in the rhizosphere and their potential contributions to soil health and the producibility of agriculture (Chandran *et al.* 2021, Chauhan *et al.* 2021).*Sesbania sesban* (L.) Merrill, frequently known as "Egyptian Sesban" or "Jayanti," belongs to the leguminous plant family Fabaceae. *S. sesban* is an annual crop that can reach heights of 4 to 8 meters and achieve a growth rate of 4-5 meters within six months (Mahamat *et al.* 2021). Choosing this plant for bacterial isolation offers several advantages. *Sesbania* species, known for rapid growth and high biomass production, provide an abundant source of root nodules for successful bacterial isolation. These plants have a cooperative friendship with bacteria that fix nitrogen, offering the opportunity to isolate strains with beneficial traits. *Sesbania*'s adaptation to tropical and subtropical regions ensures the discovery of bacteria adapted to local agricultural conditions. *Sesbania* plants serve as an excellent choice for exploring bacterial diversity and their possible applications within environmentally friendly farming (Nohwar *et al.* 2019, Singh *et al.* 2021; Kumar *et al.* 2024). The aims of the present study was to isolate and screen PGPRs from *Sesbania sesban* , expanding knowledge of their bacterial diversity and potential applications, and identifying novel PGPR species for enhancing plant growth and development by effectively colonizing the rhizosphere and establishing symbiotic relationships with plant roots, while also exploring potential synergistic effects within bacterial consortia.

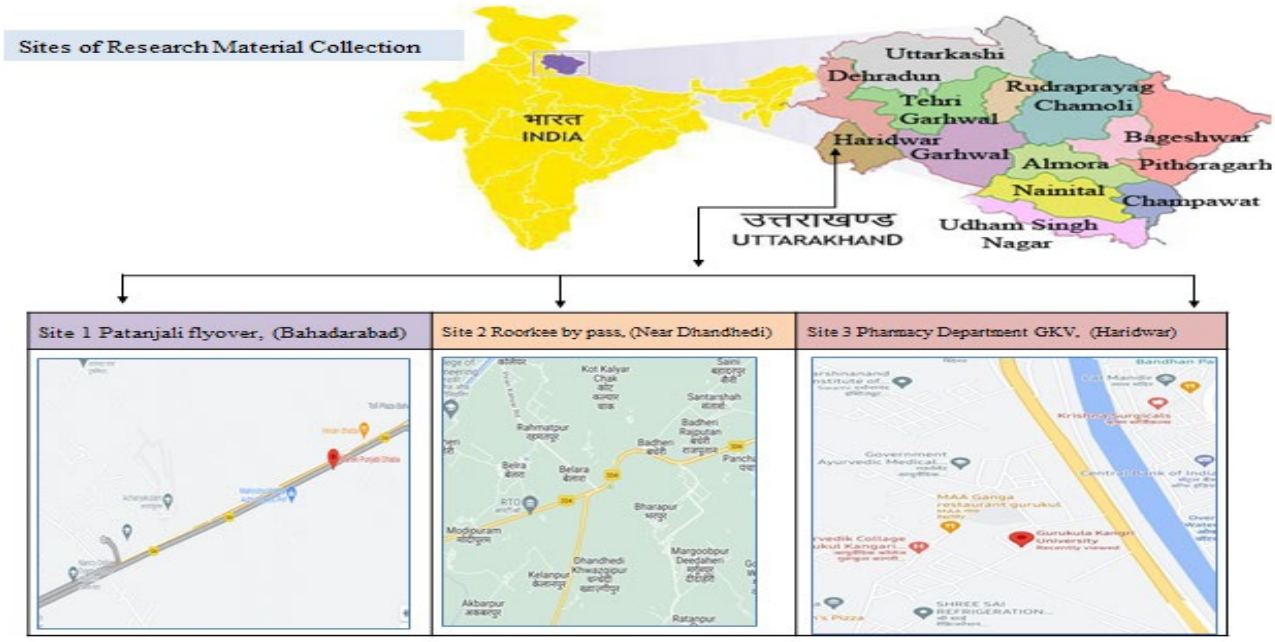


Figure1: Geographical representation of the bacterial isolation experimental sites

Materials and Methods

Collection of Root Nodules

Healthy, fresh, and young nodules grew at different locations in the district of Haridwar, India, and were detached from the root of *S. sesban*. Nodules were collected from three different regions: (i) Near Patanjali flyover (29.905804N, 78.002941E); (ii) Village Dhandhedi Near Roorkee bypass (29.877135N, 77.959460E); (iii) Department of Pharmaceutical Sciences, Gurukula Kangri (D.U.), Haridwar (29.91884N, 78.11771E) (Figure 1). Nodules were surface sterilized to eliminate debris, soil particles, and microbes that had attached to the outer surface. During the collection, plants at location 1 were not in the flowering stage, while those at locations 2 and 3 were flowering. Plant height and the number of nodules were recorded and are presented in Figure 2.

Isolation and Screening of Rhizobia

Root nodules were first cleaned with distilled water (DW) that had been sterilized by dipping in 4% sodium hypochlorite (NaClO) for 5 minutes, after which they were rinsed multiple times with sterile DW. After rinsing the nodules 4-5 times with sterilized distilled water to banish any remaining material, they were blot-dried and kept for later use (Jildeh *et al.* 2020). Sterilized nodules were transferred into a test tube, that had 10 ml of sterile DW. To produce a cloudy bacteroid suspension, the nodules were crumbled by using a sterile glass rod. These were streaked on Congo Red YEMA medium plates. The streaked plates were tightly sealed by using parafilm to avoid contamination, and they were incubated for a duration of 24 to 72 hours at 28 to 30°C. After 24-72 hours, bacterial colonies appear white, translucent, elevated, and mucilaginous, after being picked up, the unique colonies were moved to the YEMA plate for purification and further evaluation. The isolation of pure culture was facilitated by additional streaking, spreading, and visual analysis of colony morphology. In order to preserve and conduct further investigations, pure isolates were streaked on YEMA slants. All experiments were conducted in triplicate (Dhiman *et al.* 2019(a); Kumar and Dubey, 2022).

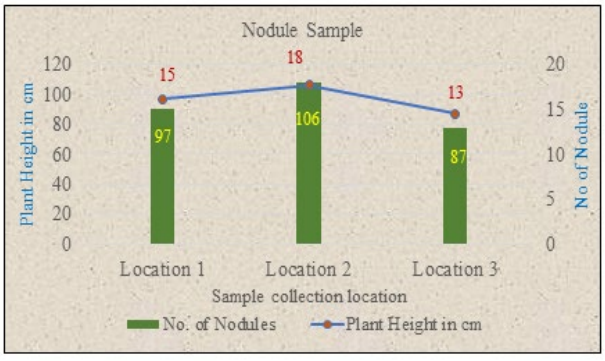


Figure 2: Graph represents plant height and no of nodules of different locations

Colony Morphology

The handpicked bacteria were streaked on nutrient agar medium (NAM) petri plates using the pour plate procedure and stored in an incubator at 30°C for 24 hours. Subsequently, different bacterial samples were examined for colony characteristics, including color, shape, surface features, and opacity (Kalaivanan and Mohan, 2017).

Gram Stains

Gram staining was performed by preparing smearing on the glass slide. The culture was 24h old, after it had been fixed with heat. Then after rinsing with running water, crystal violet solution was dropped on the glass slide and stood for 2 minutes. Followed by aquadest and the alcohol 95% wash for 30 seconds, it was dipped in Gram iodine solution for 2 minutes. We poured safranin over it, rinsed it with aquadest, air-dried it, and investigated it under the microscope. Only Gram-negative isolates were used for further research work (Layly *et al.* 2021).

Motility Test

Agar slants containing the preserved bacterial isolates were used for inoculation on a cover glass with the aid of an inoculum loop. The cover glass is secured onto a concavity glass slide and brought into focus under 45x objectives to examine the motility of bacterial isolates (Legesse, 2017).

Effect of Temperature, pH and Salt (NaCl) Concentrations

Different studies have been carried out that included temperature, pH, and NaCl (sodium chloride) concentrations to maximize the growth conditions of selected rhizobacterial strains. All isolates had been incubated at temperatures of 15°C, 30°C and 40°C. The medium had been prepared in a buffer to measure the effects of different pH levels (4, 7, and 9). The NAM medium was supplemented with different concentrations of salt 1%, 2%, 4%, and 5% (Basbuga *et al.* 2021).

Biochemical Assessment of a Selected Isolate

Each of the isolates undergoes a battery of biochemical tests, including, catalase, citrate, oxidase, nitrate MR, VP, hydrolysis of gelatin, starch and urea.

Catalase Test

Heme enzyme catalase breaks down the hydrogen peroxide (H_2O_2) into oxygen and water. 48-hour-old bacterial culture was placed on a slide with 1 ml of a 3% H_2O_2 solution. Bubbles signify a positive response (Wadhwa *et al.* 2017).

Citrate Test

Modifies Simmon's citrate broth media by substituting the carbon base by sodium citrate ($Na_3C_6H_5O_7$) and bromothymol blue (pH indicator). Streak the plates containing modified media with log phase culture of isolated root nodulating

bacterial strains and incubate for 48 h. Observe the plate after incubation to see that the color changes from green to blue (Hayward, 1960; Wadhwa *et al.* 2017).

Oxidase Test

The oxidase test was conducted on 24h old bacterial cultures grown on nutrient agar, where a 1 percent glucose smear was applied to Whatman's No. 1 filter paper saturated with a newly prepared tetramethyl-p-phenylenediamine dihydrochloride ($C_{10}H_{18}C_{l2}N_2$) 1% aqueous solution. A positive outcome is denoted by the emergence of a purple hue within 5-10 seconds. A response between 10-60 seconds is categorized as a delayed favorable result, while any response after 60 seconds or the absence of a reaction is labeled as negative (Singh and Singh, 2015).

Nitrate Reduction Test

For nitrate reduction, the bacterial inoculated suspension into the nitrate broth at 30°C for 24h. After incubation look for nitrogen gas first then add 6-8 drops of nitrite reagents A (sulfanilic acid) and 6-8 drops of reagent B (ά-naphthylamine). Observe color change within a minute, the appearance of a red color upon the add-on reagents A and B signifies the existence of nitrite, if color is no change means lack of nitrite can imply no reduction, and this can be illustrated by adding reagent C (zinc powder) to generate a red colour in the presence of nitrate. If there is no color change when reagents A, B, and C are present, it means that the nitrate has been reduced beyond nitrite and changed into different nitrogen compounds (Bhusal and Muriana, 2021).

Methyl Red-Voges Proskauer (MR-VP) Test

The selected isolates were added to sterile MR-VP broth tubes, which were afterward placed in the incubator for a duration of 2 days at 30°C. Five drops of methyl red ($C_{15}H_{15}N_3O_2$) were appended to the tubes after incubation, and any changes in color were noted. Within 10 to 15 seconds, the color changes from yellow to red, indicating a favorable result (Mazotto *et al*. 2010). After autoclaving, the Voges-Proskauer broth was cold at 25-30^{0}C temperature. A sterilized loop was applied to inoculate the 24 hours old cultures of the selected isolates, and the medley was stored for 48 hours at 30°C. After the incubation duration, 1 ml alpha-naphthol was assorted to the broth and shaken, and then 0.5 ml of 40% potassium hydroxide (KOH) was appended and agitated. A positive outcome was considered to be the development of a red color within one hour after the reagents were added (Han *et al.* 2012; Van Thuoc *et al.* 2019).

Gelatin Hydrolysis Test

Inoculate the solid medium plates with 0.4% (w/v) gelatin using a drastically grown culture of isolated bacterial strains. Petri-plates were cultured at 28-30°C for the duration of 7 days to facilitate optimal growth. Ice bath is given to tubes and immersed tilted in an ice bath and exposed to cold treatment, liquification of gelatin occurs which is a positive indicator of gelatin hydrolysis (Bhattacharya *et al.* 2013).

Starch Hydrolysis Test

Starch agar media pour in petri plate and inoculate spot of suspended bacterial culture on media and incubate at 24–48h at 35±2°C. After proper incubation, gram iodine solution is flooded on media, immediately blue–black color formed with starch may fade. A distinct zone surrounding bacterial colonies signifies a positive starch hydrolysis and the absence of a clear zone recorded as a negative result (Wadhwa *et al.* 2017).

Urea Hydrolysis Test

Christensen's urea agar (urea 20g, dextrose 1g, NaCl 5g, peptone 1g, phosphate monobasic 2g, phenol red 0.012g + 100 ml, DW) filter with 0.45 mm pore size filter paper and add (agar 20g+900ml, DW) prepared slants and stored at 4–8°C. Use 18–24h old fresh bacterial culture streak on media incubate at 35°C. Observe color change from 6 hours to 24 hours and every day up to 6 days. Color change 1-6 hours is rapidly positive, color change up to 6 days delayed positive and no color change is negative (Rai and Sen, 2015).

PGPR Attributes of Selected Isolates

Indole Acidic Acid (IAA) Production

Fresh nutrient broth with 24 hours old bacterial isolates (2ml) supplemented with L-tryptophan (0.2%), two blobs of orthophosphoric acid (H_3PO_4) and 4ml Salkowski reagent incubated at 25^{0}C in the dark as long as 30 minutes. IAA production was evidenced due to the emergence of a pink colour on the basis of the incubation timing (Bhutani *et al.* 2018).

Siderophore Production

The chromium azurol-S (CAS) agar medium was utilized to production of siderophores as follows: universal method by Schwyn and Neilands, (1987). All selected isolates were growing in a succinate medium and incubated for 24 hours at 37°C with 120 rpm in a shaking incubator. The bacterial sample was centrifuged for ten minutes, at 4°C temperature, and 10,000 rpm. CAS (0.5 ml) solution (cell-free extract) was blended with an amount of 0.5 ml supernatant. The UV-Vis spectrophotometer (UV-1601) was used to measure the results at 470 nm after 20 minutes of incubation. The relation of (D/d) is the average diameter (of orange (D) to the colony (d)) was measured. The following formula was used for estimation of the percent siderophore unit (PSU):

$$\text{Siderophore Production (psu)} = \left\{\frac{Ar - As}{Ar}\right\} 100 = \%\ \text{siderophore}$$

where Ar represents the absorbance of the sample (cell free supernatant of the sample and CAS solution), and As is the absorbance of the reference (uninoculated broth and CAS solution) (Payne, 1994).

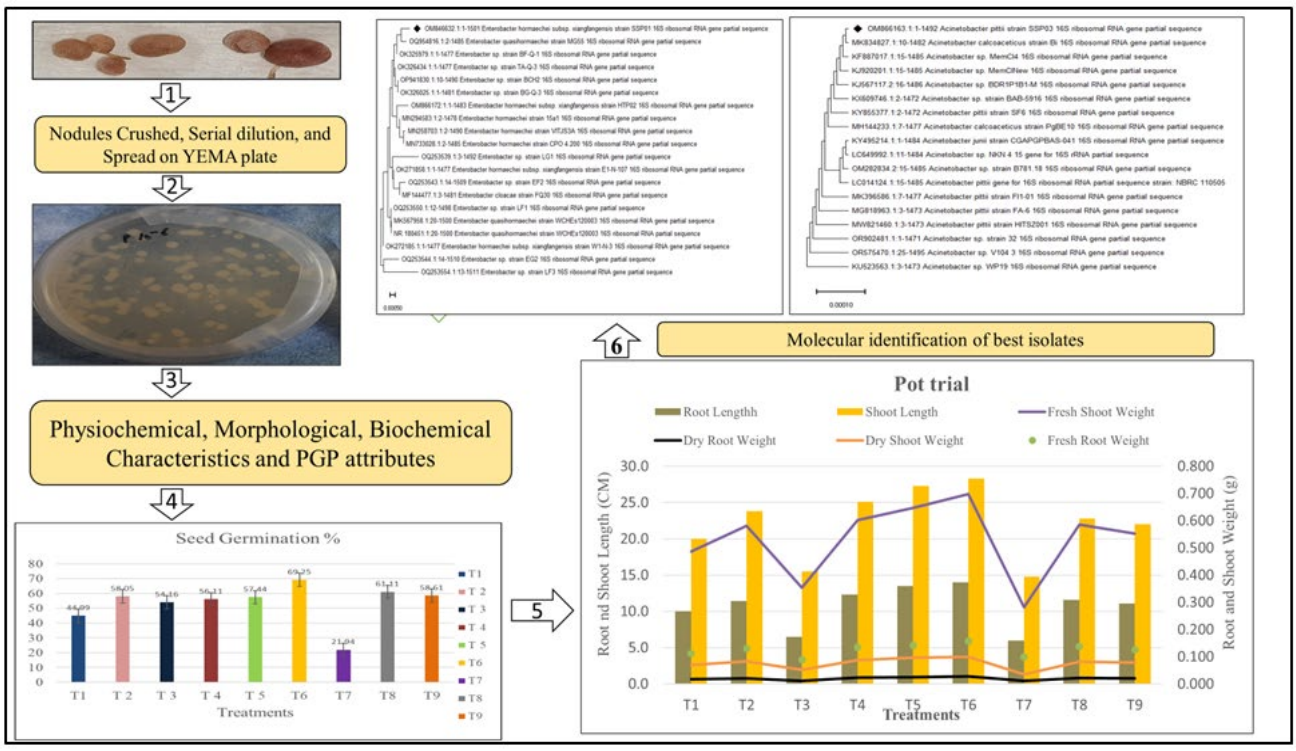


Figure 3: Shows graphical representation of research work

Solubilization of Phosphate

Pikovskaya's agar plates, inoculate with the preferred bacterial samples, were positioned in an incubator for 48–72 hours at 37°C to evaluate phosphate solubilization. Phosphate solubilization was demonstrated by the bacterial colony surrounded by a transparent halo zone. The calculation of phosphate solubilizing index (PSI) was done via the formula (Adhikari and Pandey, 2019).

$$\text{PSI} = \frac{Colony\ diameter + Holo\ zone\ diameter}{Colony\ diameter}$$

Production of Hydrogen Cyanide

Bacterial samples were streaked on the glycine-supplemented with a nutritive agar medium in order to qualitatively determine hydrogen cyanide (HCN). After being soaked in a picric acid (0.5%) solution with sodium carbonate (2%) added, Whatman filter paper No. 1 was put in the lid of the petri plate and incubated for 4 days at 37°C. After 4 days of incubation, the color changed from yellow to brown indicating the formation of volatile HCN (Bakker and Schippers, 1987).

Nitrogen Fixation Activity

Jensen's media (Nitrogen free) was utilized to confirm the nitrogen fixation ability of selected isolates. The isolates were successively spot inoculated onto the Jensen agar plate at an interval of 24 h. The formation of characteristic pellicles was observed (Jensen, 1951).

Molecular Identification of Isolates

16S rRNA gene sequencing was used for the identification of bacterial isolates SSP01, and SSP03, which were the most promising. The polymerase chain reaction (PCR) was employed to amplify the 16S rRNA gene using primers 27F (5′-AGA GTT TGATCMTGG CTC AG-3′) and 1492R (5′-TAC GGY TAC CTT ACG ACT T-3′), which were sourced from Macrogen South Korea. The GenBank database (NCBI) BLAST program was operated to analyze the sequence, and ClustalW was used to align it. MEGA X (10.2.4) was applied to create an evolutionary tree in order to confirm the isolate's identity (Kumar *et al.* 2018).

Seed Bacterization

Healthy seeds were collected from wild plants growing in different regions of the Haridwar district in order to perform seed bacterization (Bahadarabad, Roorkee and Haridwar). The healthy seeds of *S. sesban* were selected for study. The bacterization of seeds was put into practice as described by Weller and Cook, (1983). The seeds were treated for 1 minute with 95% ethanol and 3 minutes with 4% NaOCl, for surface sterilization and washed several times with sterile deioninzed water. Cell biomass of a 24 hours old bacterial cultures was used for making a slurry that prevented bacteria from adhering to the surface of seeds when combined with 1% carboxymethyl cellulose (CMC). The control group consisted of cmc seeds coated without bacteria.

Table 1: Growth of isolates on NaCl concentration, pH, and temperature tolerance (+=less growth, ++=good growth, +++=best growth, and "-" = no growth)

Physiological Characters										
Characteristics	*Growth in NaCl (%)*				*Growth at pH*			*Growth at temp (°C)*		
Isolates	1%	2%	4%	5%	4	7	9	15	30	40
SSP01	+++	+++	+++	++	++	+++	++	+++	+++	+++
SSP02	+++	+++	++	+	+	+++	+	++	+++	+++
SSP03	+++	+++	+++	++	++	+++	+++	+++	+++	+
SSP04	+++	+	-	-	+	+	-	++	++	+
SSP05	+++	+++	++	++	+	+++	-	+++	+++	+++
SSP07	+++	+	+	+	-	+	+	++	+++	++
SSP08	+++	++	+	+	+	+	+	+	+++	+
SSP09	+++	++	-	+	+	+	-	+	++	+
SSP10	+++	++	++	+	-	++	-	+	++	+
SSP12	+++	+	+	-	+	++	+	++	+++	++
SSP18	+++	++	++	+	-	++	-	+	++	+
SSP21	+++	+++	++	-	+	+	+	+	+	+
SSP22	+++	++	+	+	-	+	-	+	++	+
SSP23	+++	+	++	-	+	+	+	++	+++	++
SSP25	+++	++	+	+	+	+	-	+	++	+
SSP30	+++	+	++	-	-	+	-	+	+	+

Pot Trial Experiment

Pot trial was conducted in triplicate of each treatment. The experiment was carried out using sandy clay soil, and pots were maintained in the Department of Botany and Microbiology. Treatments involving bacterized seeds were arranged and planted in pots according to the following: T1 (control), T2- (SSP01), T3- (SSP02), T4- (SSP03), T5- (SSP05), T6- (SSP01 + SSP03), T7- (SSP02 + SSP03), T8- (SSP03 + SSP05) and T9- (SSP01 + SSP02 + SSP03). The growth parameters, such as root and shoot length and weight (both fresh and dry), were assessed by oven-drying root and shoot samples at temperatures ranging from 25 to 30°C. The treatments were formulated through microbe-microbe interactions. However, during these interactions, combinations such as SSP01+SSP05 and SSP01+SSP03+SSP05 did not exhibit synergistic growth. The effect of each isolate and consortia on seed germination was measured after 15 days of sowing (Dhiman *et al.* 2019b).

Result and Discussion

Isolation of Rhizobia

Rhizobia were detached from the root nodules of *S. sesban* grown under wild conditions. There was a significant diversification in the rhizobial spp. colonizing the roots of *S. sesban*. The current study sought to identify PGP rhizobacteria from the root nodule of *S. sesban*. Root nodulating bacteria living in symbiotic relationships can produce valuable bioactive metabolites that are likely used to improve plant growth and fitness. In a single nodule a variety of nodulating bacteria were associated. (Diagrammatic representation of research work given in Figure 3.)

Physiological Characterization of Selected Isolates

Table 1 demonstrates the growth capabilities of selected isolates across various pH levels (ranging from 4.0 to 9.0), temperatures (15 to 40 degrees Celsius), and NaCl concentrations (1% to 5%). While the isolates generally exhibited growth within the pH range of 4.0 to 9.0. SSP01, SSP02, SSP03, SSP07, SSP08, SSP10, SSP021 and SSP23 isolates displayed particular adaptability to extreme pH conditions, thriving at both pH 4.0 and pH 9.0. Notably, bacterial isolates SSP04, SSP05, SSP07, SSP09, SSP10, and SSP18 were unable to grow at pH levels 4.0 and 9.0. Additionally, bacterial isolates SSP04, SSP09, SSP12, and SSP21 failed to grow under NaCl concentrations of 4% and 5%. Bacteria grew at temperatures ranging from 15 to 40^{0}C. Pawar *et al.* (2014) found that at 36°C and pH 7.0, nodulating bacteria that were isolated from soybean root nodules grew well. Datta *et al.* (2015) discovered that *Rhizobium* strains isolated from *Vigna radiata*, *Glycine max* (soybean), and *Lens culinaris* (lentil) root nodules grew well at pH values of 6.0 and 7.0, a temperature of 34°C, and a concentration of 4% salt. Earlier various researches reported the morphological, physiological, and biochemical properties of rhizobial spp. and these characteristics are confirmed by Bergey's Manual of Systematic Bacteriology (Claus and Berkeley, 1986; Holt

et al. 1994; Lalitha and Immanuel, 2013; Rasool *et al.* 2015).

Validation of Rhizobia Isolated from Root Nodules

Seventy rhizobial isolates were isolated from *S. sesban* root nodules, all the isolates were sorted and tested to a series of authentication, such as the Congo red test, catalase, citrate, oxidase, MR-VP, nitrate reductase, and hydrolysis of gelatin, starch, and urea. On Congo red YEMA medium, 16 isolates tested, which were indicate negative results for Congo red medium (Mahmood and Athar, 2008).

Biochemical Characterization and Gram's Reaction of Selected Rhizobial Isolates

Results of Gram staining, and cell shape, of particular rhizobial isolates. It was determined that the isolates were rhizobia spp. by the production of opaque, gummy colonies, nearly round, with smooth surfaces and raised elevation. They are Gram negative rods. The rhizobial isolate passed through the catalase, citrate, oxidase, nitrate reductase, methyl red test, voges proskauer test and hydrolysis of starch, gelatin, and urea (Table 2).

Nitrogen Fixation Activity

Selected isolates were spot inoculated on the Jensen agar plate sequentially of 24 hours old culture. On Jensen's media (Nitrogen free) SSP01, SSP02, SSP03, SSP05, SSP07, SSP8 and SSP10 were able to grown and SSP04, SSP09, SSP12, SSP18, SSP21, SSP22, SSP23, SSP25 and SSP30 do not able to grow on same media.

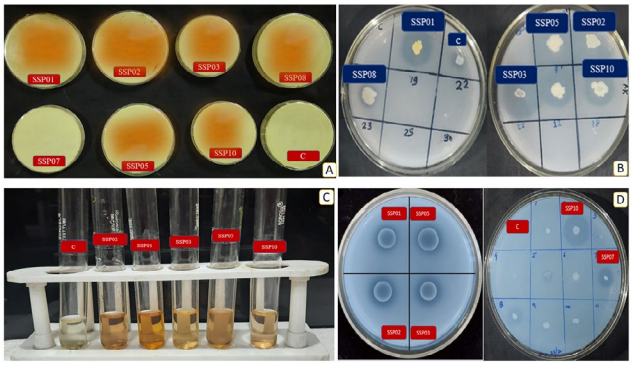


Figure 4: Shows HCN production (A), Phosphate production (B), IAA production (C), Siderophore production (D)

Indole Acetic acid (IAA) Production

IAA synthesis was positively performed with 5 endophytic bacterial isolates (SSP01, SSP02, SSP03, SSP05, and SSP10) shows in Figure 4 (C). After completing 120 hours of incubation, the isolate SSP01 produce the highest amount of IAA (90.8 µg/ml), followed by SSP03 producing 78.6 µg/ml while SSP10 synthesize the least quantity of IAA (46.3 µg/ml). It has previously been reported that the native isolates can produce IAA when tryptophan is present (Da Silva *et al.* 2020). By increasing in the formation of IAA, *Enterobacter cloacae*, which was reported from *Ocimum sanctum*, additionally stimulate plant growth (Panigrahi *et al.* 2020). *E. xiangfangensis* (BWH6) and *E. asburiae* (STY10) synthesize IAA

Table 2: Biochemical characterization, Gram's reaction, shape and motility (R = rod, C = cocci, M = motile, NM = non motile, + = less growth, ++ = good growth, and - = no growth)

Biochemical characterization and Gram's reaction												
Isolates	*Gram's Reaction*	*Shape*	*Motility test*	*Catalase*	*Citrate*	*Oxidase*	*Nitrate reductase*	*MR*	*VP*	*Hydrolysis of*		
										Gelatin	*Starch*	*Urea*
SSP01	-	R	M	++	++	-	+	+	+	-	-	++
SSP02	-	R	M	++	++	++	-	-	-	-	-	-
SSP03	-	R	NM	++	+	-	-	-	-	-	-	-
SSP04	-	C	NM	-	-	+	-	-	-	++	++	++
SSP05	-	R	NM	++	++	++	-	+	+	-	++	-
SSP07	-	R	NM	+	+	-	-	-	-	+	-	-
SSP08	-	C	NM	+	+	-	-	-	+	-	-	+
SSP09	-	C	M	-	+	+	+	+	-	+	-	+
SSP10	-	R	M	+	+	+	-	+	+	-	+	-
SSP12	-	C	NM	+	-	-	+	-	-	++	+	+
SSP18	-	C	NM	-	++	+	-	-	+	-	+	-
SSP21	-	R	M	-	+	-	-	+	-	+	-	++
SSP22	-	C	NM	++	-	-	+	-	-	-	+	-
SSP23	-	C	NM	-	+	-	-	+	-	+	-	+
SSP25	-	C	NM	+	-	++	-	-	+	-	-	-
SSP30	-	R	M	+	-	-	+	-	-	+	++	-

Table 3: Qualitative and quantitative analysis of selected isolates (- = absence, + = presence, ++ = good, +++ = best)

Qualitative analysis					*Quantitative analysis*		
Isolates	*Siderophore*	*HCN*	*IAA*	*Phosphate*	*Phosphate (mg/ml)*	*IAA (µg/ml)*	*Siderophore %*
SSP01	+++	++	+++	+++	19.3	90.8	64
SSP02	++	+	++	++	13.6	70.3	45
SSP03	++	+	++	+++	14.8	78.6	43
SSP05	++	+	+	++	15.7	60.6	51
SSP07	+	-	-	-	-	-	39
SSP08	-	++	-	++	13.1	-	-
SSP10	+	++	+	+	11.9	46.3	41

in the existence of L-tryptophan were also reported (Zang *et al.* 2021). *E. hormaechei* also promote plant growth by synthesizing the IAA, ammonia, N_2 fixation, and solubilization of phosphate and potassium (Ranawat *et al.* 2021).

Siderophore Production

Six isolates (SSP01, SSP02, SSP03, SSP05, SSP07, and SSP10) produced siderophore, proved by the halo zone that has developed around the colonies (Figure 4(D)). During the growth phase of incubation, siderophore production commenced in all isolates between 24 and 120 hours. In qualitative terms, the most substantial siderophore ring formation was exhibited by SSP01 and SSP03 at 120 hours of incubation. In quantitative terms, isolate SSP01 displayed the highest siderophore production at 64%, followed by SSP03 at 51%. Isolate SSP07 exhibited the lowest siderophore production, recorded only 39%. *Enterobacter* Sp. PR14 was previously publish for synthesis of siderophore (Sagar *et al.* 2020). The production of siderophores by microbes' aids plant growth by chelating iron and facilitating the absorption of soluble compounds by the plants. *Erwinia, Enterobacter, Pseudomonas, Rhizobium,* and *Serratia,* has been documented as a siderophore forming bacteria (Kour *et al.* 2020). Endophytic *Bacillus, Enterobacter,* and *Klebsiella* were also reported to synthesize siderophore (Mowafy *et al.* 2021).

Phosphate solubilization

Six isolates (SSP01, SSP02, SSP03, SSP05, SSP08, and SSP10) exhibited phosphate solubilization, creating a halo zone with solubilization indices ranging between 1.4 to 2.0 cm. The most substantial phosphate utilization was achieved by SSP01, recording 19.3 mg/ml, followed by SSP05 with 15.7 mg/ml (Figure 4 (B), Table 3 and Figure 5). In our investigation, six isolates (SSP01, SSP02, SSP03, SSP05, SSP08, and SSP10) demonstrated phosphate solubilization. Insoluble phosphate could be dissolved in the medium by the endophytic bacteria SSP01, which is the member of *Enterobacter* genus. (Aminu *et al.* 2019). It has been documented that phosphate-solubilizing bacteria constitute a beneficial group capable of converting insoluble phosphate in the soluble form, which plants can absorb as a nutrient for their better growth. Additionally, previous reports indicate that *Enterobacter* spp. also possess the ability for phosphate solubilization at wide range of pH and temperature (Borham *et al.*, 2017). *Acinetobacter pittii* also help in the enhancement of soybean by solubilizing phosphate in both inorganic and organic p-cycling (He and Wan, 2021).

Hydrogen Cyanide Production

For the production of volatile HCN, six isolates (SSP01, SSP02, SSP03, SSP05, SSP08, and SSP10) were cultivated in exponential growth cultures (Table 3). The isolate's color changed from yellow to light brown following a full

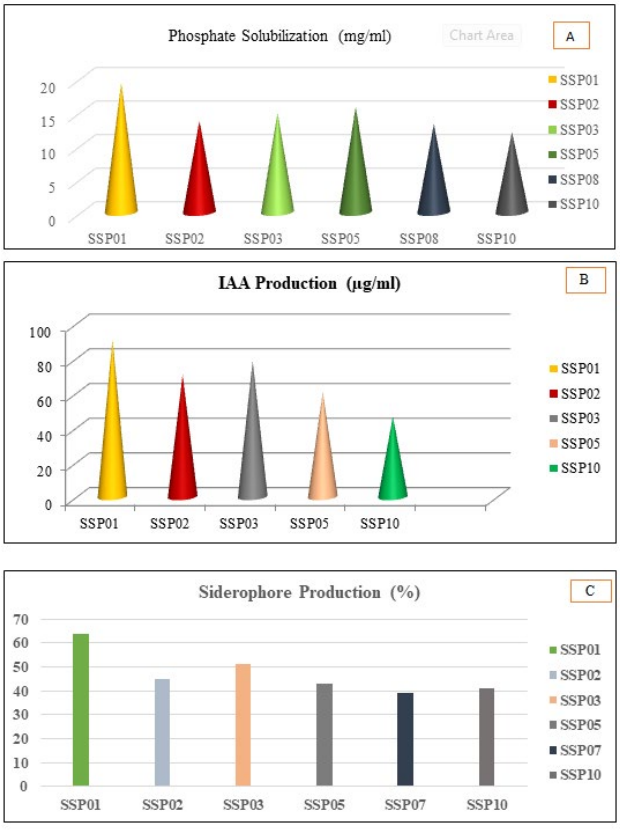


Figure 5: Graph shows quantitative analysis, A=phosphate solubilization, B=IAA production, and C=Siderophore production

incubation period at 30°C. The synthesis of substances that aid host plants in preventing infections through the formation of hydrogen cyanide, a secondary metabolite created when glycine is decarboxylated, is one indirect method of promoting plant growth. It works well as a biological control agent to prevent plant diseases (Admassie *et al.* 2020).

Molecular Identification of the Isolates

The most auspicious bacterial isolates were chosen to be sequenced for the 16S rRNA gene. According to Sambrook and Russell, (2001), the DNA was isolated. PCR was used in the amplification of the 16S rRNA gene by using primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). The sequence was subjected to analysis through the BLAST within the GenBank database (National Center for Biotechnology Information), and alignment was performed via the Clustal W program. To confirm the identification of the isolates, an evolutionary tree was generated by operating MEGA 11 (Kumar *et al*., 2018). Accession numbers *Enterobacter xiangfangensis* (SSP01) OM846632 and *Acinetobacter pittii* (SSP03) OM866163 were given to the identified bacteria. All of the chosen isolates' phylogenetic trees are displayed in Figures 6 and 7. A common species of soil bacteria called *Enterobacter* encourages the growth of plant root systems and shields host plants from harmful infections (Jha *et al*. 2011). *Enterobacter* sp. PR14 exhibits a diverse array of biocontrol traits, producing NH_3, chitinase, siderophore, HCN, and ACC deaminase. Moreover, its ability to tolerate elevated levels of various heavy metal ions positions it as a promising biocontrol agent, an excellent candidate for stress tolerance, and a valuable tool for the bioremediation of heavy metal in contaminated soil. Consequently, it serves as an ideal sustainable alternative to persistent inorganic fertilizers and fungicides (Sagar *et al.* 2020). *Acinetobacter* spp. can be found across various places and frequently occurs in the rhizosphere in numerous plants. It is important in agriculture because it acts as PGPR activities like, phosphate, potassium, siderophore, ammonia, phytohormone production, catalase assay, antibiotics, biosurfactants. Similarly, *A. pittii* has been evaluated and concluded to exhibit PGP potential and can be used alone or in combination in the sustainable cultivation of agricultural crops (Kumari *et al.* 2018; Mujumdar *et al.* 2023).

Seed Germination and Pot Trial Experiment

The maximum seed germination percentage was recorded in T 6 (69.25%), followed by T 8 (61.11%), T 9 (58.61) while minimum was recorded in T 7 (21.94%), shown in (Figure 8). Seeds bacterized with isolates SSP01 (T 2), SSP03 (T 4), SSP05 (T 5), SSP01+SSP03 (T 6), SSP03+SSP05 (T 8), and SSP01+SSP02+SSP03 (T 9) exhibited significant improvements in various plant growth parameters. The following percentage increases were recorded in contrast to the control group; The most substantial increase was

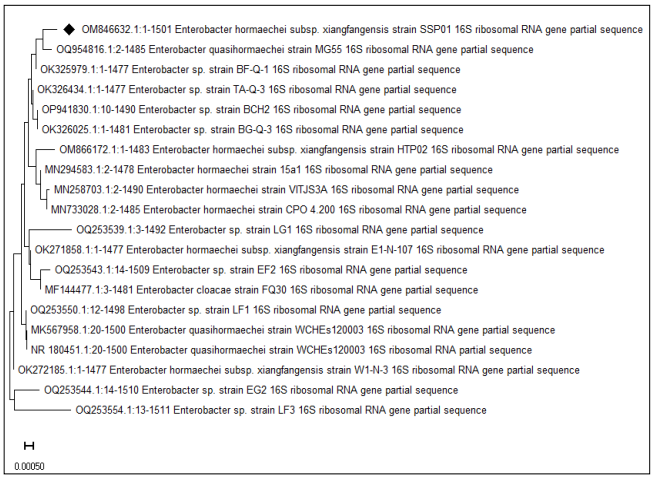


Figure 6: Phylogenetic tree of SSP01 isolate

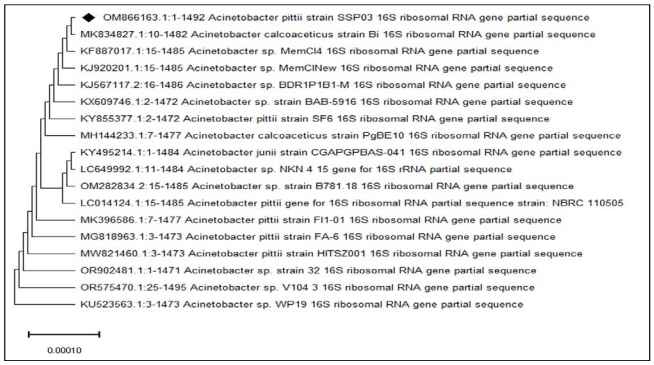
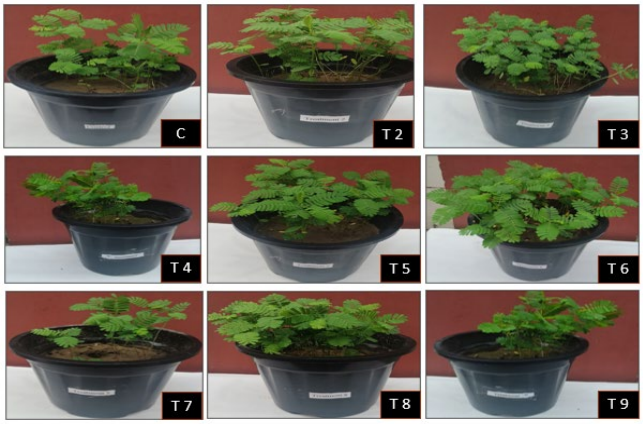


Figure 7: Phylogenetic tree of SSP03 isolate



Photoplate 1: Pot trial experiment

observed in root length is T 6 (40%), followed by T 5 (35%), T 4 (23%), T 8 (16%), T 2 (14%) and T 9 (11%). Shoot Length: T 6 demonstrated the highest increase (41.5%), followed by T 5 (37.5%), T 4 (25.5%), T 2 (19%), T 8 (14%), and T 9 (10%). Fresh weight of root and shoot: The seeds inoculated with T 6 (39.82%) exhibited the maximum fresh weight of root and shoot, followed by T 5 (27.67%), T 4 (20.53%), T 8 (18.75), T 2 (16.07%), and T 9 (12.5%) respectively, in comparison to

the control. Dry weight of plant root and shoot: Similar to fresh weight, the highest dry weight of root was recorded in seeds inoculated with T 6 (44.44%) with the order of effectiveness being T 5 (38.88%), T 4 (27.77%), T 8 (22.22%), T 2 (16.66%) and T 9 (11.11%), in comparison to the control. The highest dry weight of shoot was recorded in seeds

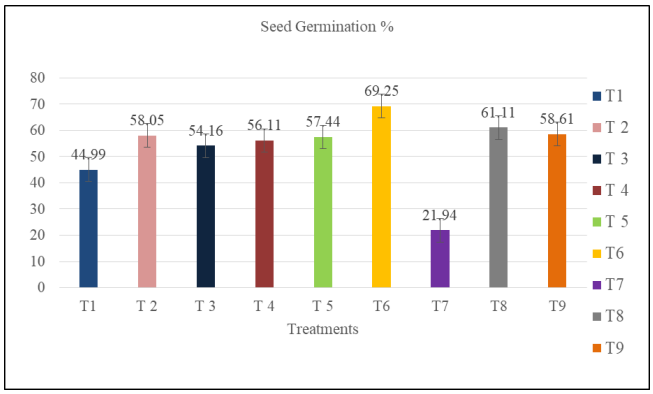


Figure 8: Graph effect of bacterial isolates with consortium on seed germination % parameters of *S. sesban*

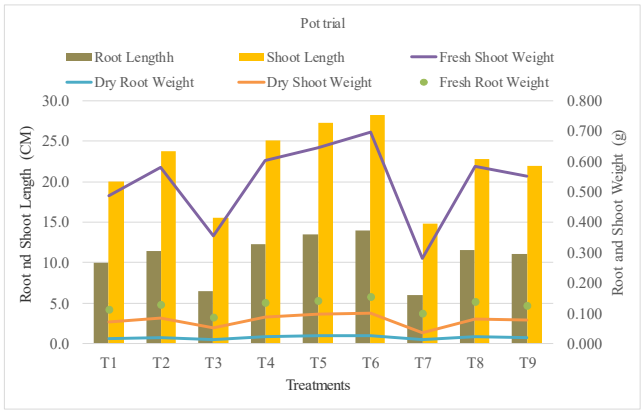


Figure 9: Graph represents pot trial data of all six parameters at 7th week

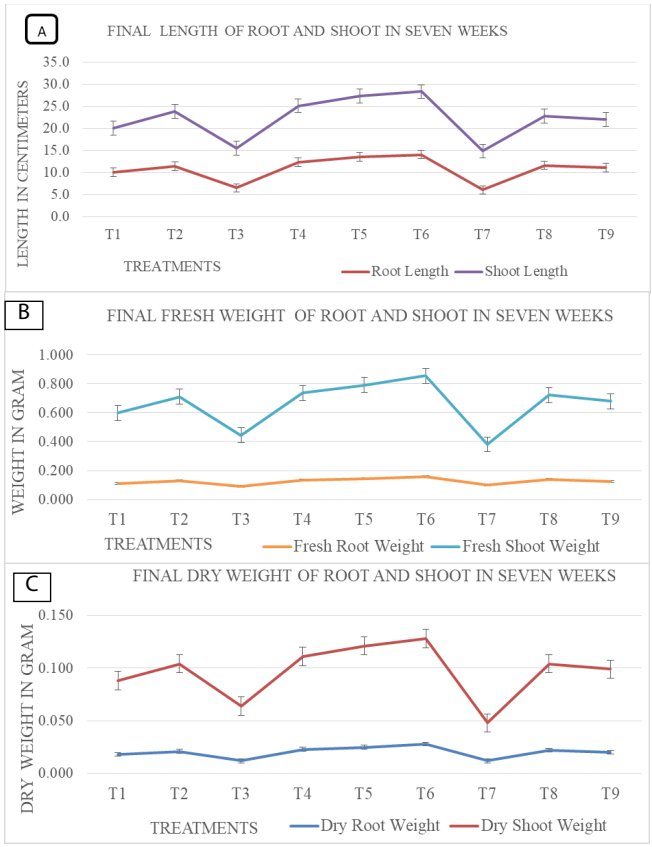


Figure 10: Graph show, length (A), fresh weight (B), dry weight (C) of root and shoot

Table 4: Impact of bacterial isolates with consortium on vegetative growth parameters of *S. sesban*

Treatments	*Root Length (cm)*	*Shoot Length (cm)*	*Fresh Root Weight (g)*	*Fresh Shoot Weight (g)*	*Dry Weight Root (g)*	*Dry Weight Shoot (g)*
T1 (Control)	10 ± 0.28	20 ± 0.29	0.112 ± 0.004	0.487 ± 0.0121	0.018 ± 0.0011	0.07 ± 0.0115
T2 (SSP01)	11.4 ± 0.69**	23.8 ± 0.75**	0.13 ± 0.003**	0.581 ± 0.0012*	0.021 ± 0.0023**	0.0863 ± 0.0002**
T3 (SSP02)	6.5 ± 0.23*	15.5 ± 0.20^{ns}	0.089 ± 0.006*	0.354 ± 0.0031*	0.012 ± 0.0019*	0.052 ± 0.0016**
T4 (SSP03)	12.3 ± 0.24**	25.1 ± 0.25**	0.135 ± 0.0023**	0.602 ± 0.0027**	0.023 ± 0.0023**	0.088 ± 0.0023*
T5 (SSP05)	13.5 ± 0.27**	27.3 ± 0.24*	0.143 ± 0.0012*	0.647 ± 0.0035**	0.025 ± 0.0023*	0.096 ± 0.0046**
T6 (SSP01+SSP03)	14 ± 0.11**	28.3 ± 0.80**	0.157 ± 0.0017**	0.698 ± 0.0058*	0.028 ± 0.035**	0.1 ± 0.0061*
T7 (SSP02+SSP03)	6 ± 0.17*	14.8 ± 0.29**	0.099 ± 0.004*	0.282 ± 0.0017**	0.012 ± 0.0021*	0.036 ± 0.0023**
T8 (SSP03+SSP05)	11.6 ± 0.30*	22.8 ± 0.51*	0.138 ± 0.005*	0.585 ± 0.0035**	0.022 ± 0.0029**	0.082 ± 0.0036**
T9 (SSP01+SSP02+SSP03)	11.1 ± 0.40**	22 ± 0.69*	0.126 ± 0.0029*	0.553 ± 0.0116**	0.02 ± 0.0064^{ns}	0.079 ± 0.0052*
CD at 1%	1.81	1.23	1.51	1.62	0.08	0.05
CD at 5%	1.13	0.89	1.23	1.17	0.73	0.65

Control (non-bacterized seeds) values are mean of ten plant samples from each treatment; significance at 0.01 and 0.05 levels of analysis of variance (ANOVA),

CD, critical difference, ^{ns} Not Significant at 0.05 level of LSD as compared to control

** Significant at 0.01 level of LSD in comparison to control

* Significant at 0.05 level of LSD in comparison to control

inoculated with T6 (42.85%) with the order of effectiveness being T 5 (37.14%), T 4 (25.71%), T 2 (18.53%), T 8 (17.14%), and T 9 (12.85%), as compared to the control. Notably, the most significant enhancement was observed in SSP01+SSP03 (T 6) while the least effect was seen in SSP02+SSP03 (T 7). Pot trial experiment is shown in photoplate 1, Figures 9 and 10 (A, B, and C) and statistically in Table 4. *Enterobacter* sp. and *Acinetobacter* sp. were previously reported from *S. sesban* (Jobby *et al*. 2016; Fanuel *et al*. 2022) both bacterial spp. show PGP attributes (Kumar and Dubey, 2022; Kour and Yadav, 2023). Microbial consortiums have been shown to be as effective as versed chemical fertilizers (e.g., urea (NH_2CONH_2), diammonium phosphate (DAP, $(NH_4)_2HPO_4$)), muriate of potash (MoP), and so on) in raising crops under abiotic stress situations of soil salt concentration (Maheshwari *et al*. 2023).

Conclusion

In conclusion, the selected bacteria in both forms, individual and in consortium, had a weighty sequel on the growth of *S. sesban*. The outcome of the pot trail shows that the plant height and weight (fresh & dry) of root & shoot of *S. sesban* increased observable due to inoculation with the multi-strain bacterial consortium. The potential of the microbial consortium to exert diverse biological effects and trigger the growth of *S. sesban* was recorded. Treatment 6 (SSP01+SSP03) possesses the potential to effect plant growth by various PGP mechanisms. The findings of our research also suggest valuable insights into the diversity and potential applications of beneficial bacteria from *S. sesban* root nodules. We observed, that bioinoculants enhance plant growth and can reduce reliance on chemical fertilizers. However, further investigations were needed to elucidate the specific mechanisms by which these bacteria promoted plant growth and optimize their application techniques in different agricultural systems.

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Author contribution

P.K. executed the experiments and drafted the manuscript. H.V. collected data and planned research work and S.K. corrected, and finalized the manuscript, and read and approved the final manuscript.

Conflict of interest

The authors declare no competing interests.

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