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Research Article

IMPACT OF RHIZOSPHERIC, NODULATION AND SOIL MICROBIOME ON SOYBEAN AND RICE GROWTH FOR SUSTAINABLE AGRICULTURE

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Abstract

Microorganisms are critical for the survival of life on Earth, but we know very little about the vast majority of microbes. We studied the culturable soybean root, soil, and nodules microbiome here. Thirty bacterial samples were isolated from different localities of soybean crops, including soil, root, and nodules, to assess their ability for plant growth promotion. To evaluate the richness and diversity in the root and soil, we performed the most probable number **(**MPN) test. Seven of the bacterial isolates were able to produce cellulase, in which JSW1 and JSW6 were most efficient with 2.56 and 2.05 cellulolytic index. All isolates were able to grow and utilized pectin, in which JSW2 and JSW3 were most efficient with 0.8 and 0.66 pectinolytic index. Pikovskaya media modified with TCP JSW1 and JSW2 showed a 3.0 and 2.33 solubility index, and on $CaCO_3$ media, the JSW1 strain produced a 1.58 solubilization index. All bacterial isolates were phylogenetically identified by sequencing and analyzing the 16S rRNA gene. Under controlled conditions, consortia, *Pantoea dispersa*, and *Rhizobium sp.* isolates considerably improved rice root dry weight by 53%, 75%, and 69% and shoot dry weight by 85%, 71%, and 76%. Based on in vitro characterization, the potential bacteria *Pantoea dispersa*, *Pseudomonas koreensis*, and *Rhizobium sp.* were selected to be evaluated for plant growth, nodulation, and grain yield under field experiment. Under field conditions, *Rhizobium sp* and *Pseudomonas koreensis* were found best as single inoculation by improving 53% and 49% grain yield, respectively. These potential bacteria could be used as biofertilizers for soybean crop productivity.

Keywords: Soybean, PGPB, MPN, diversity, abundance, microbiome
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1. INTRODUCTION

Agriculture is the foundation of Pakistan's economy, which depends on its major cereal crops like wheat, rice, and maize to meet basic requirements. Soybean (Glycine max L.) is a valuable and nutritionally important legume grain and oilseed crop grown worldwide. Because of its high protein (36%) and oil (19%) reserves in its seed, soybean has been used as human food, animal feed, edible oil, and industrial products (Ilangumaran, Schwinghamer et al. 2021). Soybeans were planted on 122.68 million hectares worldwide in 2019, yielding 337.41 million metric tons (MMT).

Rice (Oryza sativa) is a secondary staple food in Pakistan, and it is estimated that rice yields 7.4 million metric tons annually, and800 million tonnes of rice will be required globally in 2025 (Kubo and Purevdorj 2004). A significant decline was recently discovered due to the whims of natural climatic variables, including

temperature, rainfall, and CO2, which are crucial determinants of crop productivity (Richards 2018).

Fertilizers play a significant role in developing any country's agriculture industry. It aids in improving yield efficiency, quality, and quantity, thus playing a major role in agricultural development (Savci 2012). Chemical fertilizers have some advantages, but they also have drawbacks that must be evaluated. These negative impacts can include air pollution, water pollution, soil acidification, mineral depletion, plant and microbe harm, death of important microbes, etc (Savci 2012).

The answer to this problem may lie in using biological fertilizers or PGPB inocula in the Soybean creation framework, which comprise living cells of Plant Growth Promoting Bacteria (PGPB). Bio-fertilizers (living bacteria) have the potential to help the environment and soil reestablish their health by fixing nitrogen, mobilizing insoluble minerals or compounds that exist in the soil in a form that cannot be taken up by the plant, delivering beneficial hormones to plant (Tariq, Hameed et al. 2014) (Umesha, Singh et al. 2018). Rhizosphericmicrosymbionts (colonized the plants' root surface (Tariq, Hameed et al. 2014)) and endophytic microsymbionts (inside healthy tissue) are classified as plant growth-promoting bacteria with the potential to act as biofertilizers by forming a symbiotic connection with the plants to help it grow by making nutrients available, Creating phytohormones and controlling illness (Sessitsch, Hardoim et al. 2012).

Soil is a naturally occurring Basel medium in one gram of soil that contains approximately 10 billion microorganisms and thousands of distinct species, whereas the prokaryotic group covers the maximum percentage (Williams, McGinnis et al. 2018) (Richards 2018)

The microbiome (core part of microbial taxa) collects microorganisms inside or outside plant tissue. Soil plant-associated bacteria, including rhizospheric and endophytic culturablemicrobiome and non-culturablemicrobiome (unable to grow on media but functionally viable), are important for ecosystem stability and sustainability because they drive the transformation of organic materials and nutrients.

The valuable food crop soybean (Glycine max L) contributes significantly to soil nitrogen by forming symbiotic relationships with nitrogen-fixing rhizobia. In soybeans, most of the nodule endophytic bacteria showed cellulolytic activity. Cellulase stimulates plant growth by decomposing soil organic matter and reducing stress tolerance (Bhattacharyya, Ros et al. 2022). Previously, (Flores-Duarte, Mateos-Naranjo et al. 2022) showed that cellulase activity is important because it breaks down the vegetal call wall and enables endophytic bacteria to enter the root.

The abundance and richness of root nodules differ from one plant species to another. Among these, root microbiota is considered a critical alternative to chemical fertilizer as it is environmentally friendly, cost-effective, and a viable long-term solution for meeting present and future needs. It has been determined that bacterial strains performed better by colonizing with rhizospheric soil microbiome and root-associated bacteria if appropriate environmental conditions (Berendsen, Pieterse et al. 2012).Additionally, the rhizosphere hosts microbes for various task completions, including disease resistance and growth promotion. The acceptance of microbial inoculants has considerably increased and encouraged due to broad and orderly research that has upgraded viability and consistency (Hayat, Ali et al. 2010).

Keeping this perspective of the developing need for bio-fertilizers as a more secure option alternative to chemical fertilizers, this research work was planned to explore the utilization of soil, root, and root nodules associated with soybean and their

in vitro characterization for plant growth-promoting properties for soybean growth enhancement under field experiments.

2. Materials and Methods

2.1. Sample collection and isolation

The study was conducted at the Directorate of Farms, University of Agriculture Faisalabad, in 2021. Soybean variety AVRD was obtained from the Agriculture Research Service, USDA. Samples including bulk soil, root, and root nodules were collected in mid-October 2021 from coordinates 31.3980 N and 73.025 E.. Samples were of 60-day-old soybean plants, and soil texture was clay with a pH of 7.5.. All samples were selected and subjected after sterilized measures (Tufail, Akhtar et al. 2006) for plant growth-promoting attributes in the Somatic Cell Genetics and Molecular Biology laboratory at the Centre of Agricultural Biochemistry and Biotechnology (CABB), University of Agriculture, Faisalabad.

2.2. Isolation of nodule-associated bacteria

Soybean root nodules were cleaned with sterilization according to the method (Valetti, Iriarte et al. 2018)for bacterial growth observation nodules crushing, suspension prepared, and twenty-six different morphological bacterial colonies on yeast extract mannitol (YEM) Congo red media were picked and sub-cultured until purified by following the method (Tariq, Hameed et al. 2014).

2.3. Isolation of soil-associated bacteria

One gram of soil was placed inside a laminar airflow cabinet to isolate bacteria from the soil, determine the identity of specific species' growth habits, and evaluate diversity and their response to various environmental conditions. The mixture was vortexed for 1 min before serially diluting between $10^{-1}$and 10^{-3}. 100 µl of the soil suspension was then plated onto YEM agar media plates. The plates were then labelled, wrapped with Para film tape, and kept at 28 °C for 48 hours for bacterial growth. After 48 hours, the plates were observed for bacterial growth. Different morphological 314 bacterial colonies were picked and subcultured until purified from Figure 6.

2.4. Isolation of root-associated bacteria

Soybean roots were cleaned with sterilization according to the method (Valetti, Iriarte et al. 2018) (Valetti, Iriarte et al. 2018), and for bacterial growth observation, roots were crushed, suspension prepared, and twenty-four different morphological bacterial colonies were picked and sub-cultured until purified were performed by following method (Tariq, Hameed et al. 2014).

Figure1: Bacterial isolation from bulk soil, root and root nodules of soybean samples. (a) Soybean root nodules (b) Bulk soil (c) soybean root sample

2.5. Bacterial abundance via the most probable number (MPN) counting

Suspension of 1 g of roots, nodules and soil was prepared separately, as mentioned in the isolation procedure, in 1 ml 0.89% saline solution and serially diluted up to 10-8 in glass tubes carrying 9 ml saline solution. 100 µl from all dilutions were plated in YEM Petri dishes and incubated at 28 °C for 48 h. The plate was observed for bacteria growth, and those carrying 20-200 bacterial colonies were used for counting according to the formula given below (Peperzak and van Bleijswijk 2021).-

To calculate the bacterial number, the following formula is useddilution tube x fraction of ml x CFU (Blodgett 2005)

2.6. Enzymatic activity of culturable bacterial microbiome

The enzymatic activity of bacterial isolates was determined for cellulase, pectinase, and chitinaseProduction, phosphate and CaCO3 solubilization.

2.7. Cellulase production assay

Bacterial colonies were spot inoculated on the mid of Carboxy methyl cellulase (CMC) agar media plates g1-¹ of K_2HPO4, 10 g MgSO4 0.25 g, peptone 5 g, Yeast extract 5 g, cellulose 10 g, gelatin 2 g, agar 20 g, pH adjusted to 7.0. Inoculated plates were incubated at 28 °C for seven days (Premalatha, Gopal et al. 2015).(Premalatha, Gopal et al. 2015)Cellulase production ability was confirmed by staining the Petri plates with 1% w/v Congo red solution for 15 min. 1M NaCl solution as a distraining reagent was applied on the plates and for 15 min incubated at room temperature and then discarded. Formation of the halo zone was observed, and the cellulytic index was calculated by using the following formula:

$$Cellulytic\ Index = \frac{Halo\ zone\ diameter}{colony\ diameter}$$

2.8. Pectinase production assay

Bacterial isolates were spot inoculated on the middle of pectin media agar plates NaNO3 1 g, KCI 1 g, K_2HPO4 1 g, MgSO4 0.5 g, yeast extract 0.5 g, pectin 10 g, agar 20 g, pH adjusted to 7.0. The inoculated plates were incubated for seven days at 30°C. Pectinase ability was confirmed by staining the plates with 1% iodine solution for 15 min (Tsegaye, Yimam et al. 2019) Wash the plates with distilled water. Formation of the halo zone was observed, and the pectinolytic index was calculated by using the following formula:

Colony diameter- halozone diameter/ colony diameter.

2.9. Chitinase production assay

Colloidal chitin was prepared using a modified method (Sixto-Berrocal, Vázquez-Aldana et al. 2023). (Sixto-Berrocal, Vázquez-Aldana et al. 2023)At room temperature, 0.5 g of chitin and 100 ml of phosphoric acid were vigorously shaken until a light brown colour was achieved. At this stage, ice-cold distilled water was poured until the mixture turned white.It was kept out at room temperature overnight.The pH of cooloidal solution was lowered to5.5 after washing . Colloidal chitin was stored at 4 °C for further use.Chitinase activity was determined for each bacterial strain by inoculating them on chitin agar plates supplemented with 0.5% colloidal chitin, 0.5% yeast extract, 1.0 % tryptone, and 0.5% NaCI.

2.10. Pikovskaya agar media phosphate solubilization

Bacterial isolates were spot inoculated on the middle of Pikovskaya media agar plates having (TCP) Ca3(PO4)2 0.3 g/L, KCI 0.2 g/L, (NH4)2SO4 0.5g/L, MgSO4 0.1 g/L, yeast extract 0.5 g/L, Sucrose 10 g/L, NaCl 0.2 g/L, MnSO4 0.004 g/L, FeSO4 (Fe-EDTA) 0.002 g/L, pH adjusted to 7.0 (Altinkaynak and Ozkoc 2020). The inoculated plates were incubated for seven days at 28 ± 2°C. Formation of the halo zone was observed, and the Phosphate solubilization index was calculated by using the following formula:

$$PSI = \frac{total\ diameter\ of\ halozone}{colony\ diametr}$$

(Kumar, Chaudhary et al. 2022)

2.11. Pikovskaya agar media CaCO3 solubilization

Bacterial isolates were spot inoculated on the middle of modified Pikovskaya agar plates having CaCO3 3 g/L similar to soil conditions KCI 0.2g/L, (NH4)2SO4 0.5 g/L, MgSO4 0.1 g/L, yeast extract 0.5 g/L, Sucrose 10 g/L, NaCl 0.2 g/L, MnSO4 0.004g/L, FeSO4 (Fe-EDTA) 0.002 g/L, pH adjusted to 7.0(Altinkaynak and Ozkoc 2020). The inoculated plates were incubated for seven days at 28 ± 2°C. Formation of the halo zone was observed, and the CaCO3 solubilization index was calculated by using the following formula:

$$PSI = \frac{Total\ diameter\ of\ halozone}{Colony\ diameter}$$(Kumar, Chaudhary et al. 2022)

2.12. Effective isolates DNA extraction and 16S rRNA gene amplification

Bacteria JSW1 to JSW7 isolated from soybean soil were considered diverse due to microbial richness and excellent performance in plant growth promotion characteristics and selected for understanding soybean microbe interaction vital for nutrient cycling, soil health, and sustainable soybean cultivation practices. In Eppendorf tubes, 50 µl of double distilled water wastaken. With the help of sterilized tips, a single colony was picked carefully and added into the Eppendorf tube and vortex for 1min, resulting in DNA release.For phylogenetic identification,1500 bp of the 16S rRNA gene was amplified by fD1 and rD1 primers (Wang, Han et al. 2022). (Wang, Han et al. 2022)Further purification for sequencing is done by the manufacturer's procedure of thermo scientific Gene JET PCR purification kit and purified amplicons. Sanger sequencing was commercially performed by (Macrogen, Seoul, Korea).

2.13. Pot experiment: Co-inoculation of soil crop microbiome

Plant growth-promoting attributes of potential isolates were evaluated under a controlled condition experiment. Rice Kissan Basmati seeds sterilization was performed according to the (Razinataj and Khodakaramian 2021)(Razinataj and Khodakaramian 2021) procedure using 10 seconds of 70% ethanol and 30 seconds of 5% bleach and series washing with autoclaved sterilized water. Surface sterilized seed germination was allowed at 25 ± 2 °C in a dark room on water agar from Fig 4. Uniformly sized germinated seedlings were planted in sterilized sand pots containing 1g/kg TCP. 100 µl of seven treatments of bio inoculum were applied in four replicates, including consortia, diammonium phosphate (DAP) chemical-based fertilizers and five potential bacterial isolates. The experiment

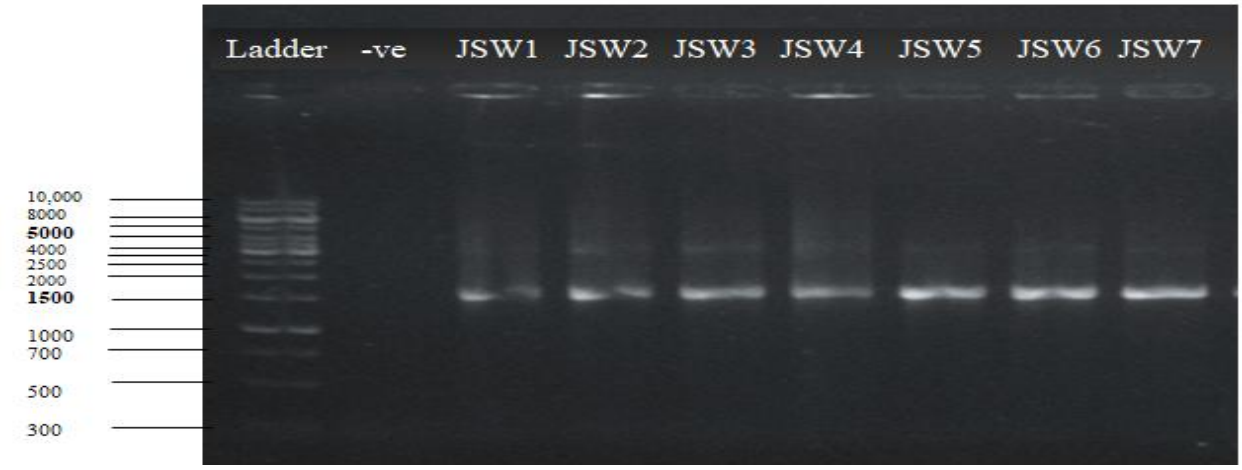

Figure 2: Amplification of 16S rRNA gene of soil-associated bacterial isolates of soybean Ladder, negative control (did not receive any template), JSW1, JSW2, JSW3, JSW4, JSW5, JSW6, and JSW7

was arranged in a growth chamber (day/night temperature 30/ 20 ± 2°C, light/dark periods 16/8). Pots were watered with 10 ml of Hoagland's solution and distilled water on alternate days (Mony, Gaudu et al. 2021). (Mony, Gaudu et al. 2021)A randomized design-based experiment was conducted with four replicates, and agronomic parameters were recorded and statistically analyzed.

Figure3: Seed germination on water agar

2.14. Field experimental site and growth conditions

The site for the field experiment was located at the University of Agriculture Faisalabad (GPS coordinates at 31.3980 N and 73.025 E). The soil type at the experimental sites was clay soil with a composition of 2.5% sand, 65% clay, 4.8% organic matter, 31% silt, and a pH 7.3. The experimental field was disc-ploughed approximately ninety days before the soybean crop sowing date. A randomized complete block design (RCBD) based experiment was conducted with six treatments, including (DAP) Diammonium phosphate as a phosphorus fertilizer source and five potential bacterial isolates in five replicates in winter conditions. The inoculum of each treatment was prepared using 1 mL 0.5 OD600 in 1 L nutrient broth from Figure 5. The seed varieties AVRD were dipped in the inoculum for 30 minand sown in a triplicate plot. Based on the soil analysis results, 320 kg ha−1 of the 0-20-20 (DAP) was applied to the soil inside each crop row. During crop growth, 120 g ha−1clethodim was used to manage grass weeds. The few weeds with wide leaves were pulled out by hand. To prevent interfering with the establishment of the seedlings, the fields received irrigation as needed. Ten plants were randomly selected and uprooted from each replicate at the nodulation stage (after eight weeks of germination). Data on the parameters was recorded, including shoot length, plant fresh weight, plant dry weight, leaves per plant, root and shoot diameter, and number of nodules. At the harvesting stage (after 120 days of germination), ten plants from each replicate were harvested to determine yield-related components (plant height, pods per plant, number of seeds per plant and weight of seed) of soybean plants (Razinataj and Khodakaramian 2021).

2.15. Statistical analysis

All In vitro and controlled condition pot experiment data were presented as means in three replicates on MS Excel. Letters indicate post hoc significance testing outcomes, categorizing factors based on similarity. If factor A shares a letter with another in the same treatment, it represents their similarity, signifying no differences. Significance difference was identified by the least significant difference (LSD) and ANOVA Statistix 10.0 software.

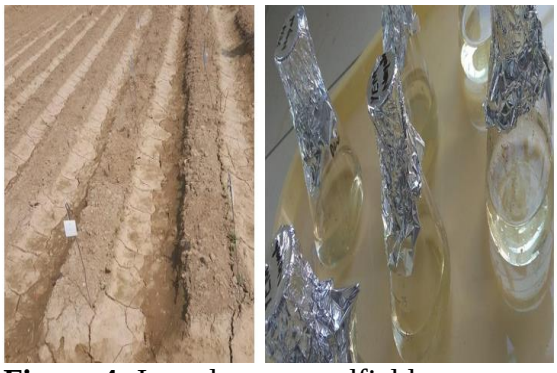

Figure 4: Inoculum-treatedfield

3. RESULT

3.1. Root nodule bacterial diversity

Six isolated soybean nodule endophytic bacteria on YEM agar media indicated diversity and variability in morphology characteristics and incubation period in Table 1and Fig 5.

Table 1: Diversity and morphological variation in soybean nodule endophytic bacterial isolatesonYEM agar media

Isolate	Colony morphology	Pigments	incubation time
JNW1	Round, rough	Pink	23 hours
JNW2	doted, smooth, hyphae	light Pink	24 hours
JNW3	Round, circular, smooth	Pink	23 hours
JNW4	Irregular, smooth hyphae	Creamy	35 hours
JNW5	Irregular, circular, wrinkled	Light Pinkish/White	35 hours
JNW6	Irregular, smooth	Reddish Pink	24 hours

3.2. Bacterial diversity in soil

A total of 10 bacteria were isolated from the soybean soil on YEM agar media, indicating that bacteria were diverse and variable in morphology characteristics and incubation period (see Table 2).

3.4. MPN(most probable number)

Twenty-six bacterial colonies were found on the plate in the dilution plates of nodules. Back calculation showed that nodules have 2.6 x 105 cfu/ml. Roots

Table 2: Diversity and morphological variation in soil-associated rhizospheric bacterial isolates on YEM agar media

Isolate	Morphology	Pigments	Incubation time
JSW1	Irregular, wavy	light Pink	35 hours
JSW2	Irregular, rough	Reddish	35 hours
JSW3	Irregular, round	Reddish	35 hours
JSW4	Irregular, circular	pinkish white	35 hours
JSW5	Filamentous, Round, regular	Reddish	35 hours
JSW6	Irregular, smooth	ReddishPink	35 hours
JSW7	Filamentous,wrinkledsmooth	WhiteRed	35 hours
JSW8	wavy,rough,	PinkWhite	35 hours
JSW9	Irregular, smooth	White	35hours
JSW10	Round, medium, wrinkled	White Red	35 hours

3.3. Bacterial diversity in root

Twelve bacteria were isolated from the soybean roots on YEM agar media, indicating that bacteria had diverse and variable morphological characteristics and incubation period see Table 3.

dilution plates represented 24 bacterial colonies. Root as 2.49x104 cfu/ml confirmed by Back calculation. On soil dilution plates, 314 bacterial colonies were found. Back calculations showed that soil

Table 3: Diversity and morphological variation in soybean root-associated rhizospheric on YEM agar media

Isolate	Colony morphology	Pigments	Incubation time
JRW1	Irregular, medium, lobate	Reddish pink	36 hours
JRW2	Irregular, small, rough	Pinkish White	36 hours
JRW3	Irregular, small, wavy	Reddish	36 hours
JRW4	Irregular, large, wavy	Red	36 hours
JRW5	Round, large, Regular	Red	36 hours
JRW6	Round, small, rough	Reddish Pink	36 hours
JRW7	Round, large, smooth	pinkish white	36 hours
JRW8	Round, medium, Smooth	Red	36 hours
JRW9	Irregular	Red	36 hours
JRW10	Irregular, circular, small	White	36 hours
JRW11	Round, smooth	Red	36 hours
JRW12	Round, irregular, smooth	White	36 hours

has 3.14x106 cfu/ml. Our study discovered the highest population of bacteria was found in soil compared to the root and nodules.

Table 4: Result from the soil, nodule and root sample of Soybean

Serial dilution	Soybean soil sample	Soybean nodule sample	Soybean root sample
Serial dilution 1	Bacterial loam	Bacterial loam	$2.1x10^4$/ml
Serial dilution 2	2.5×10^6/ml	2.4×10^5/ml	$2.44x10^4$/ml
Serial dilution 3	3.14×10^6/ml	2.6×10^5/ml	$2.49x10^4$/ml

3.5. Cellulase Screening

The cellulase production assay results indicate that among the seven isolates, JSW1 and JSW6 showed a greater diameter of a zone with 2.56 and 2.05 cellulytic index on cellulose agar plates see Fig 7. JSW3, JSW4 and JSW5 showed moderate ability with 0.47, 0.92 and 0.61 cellulytic indexes. The appearance of the halo-zone indicates that these isolates can hydrolyze the cellulose by reacting with Congo red dye.

3.6. Pectinase production assay

All bacterial isolates showed a positive response by forming a clear halo zone. JSW2 and JSW3 showed the highest pectinase activity with 0.8 and 0.66 pectinolytic indexes, while JSW5 showed the lowest pectinase activity with 0.59 pectinolytic index compared to negative control (see Fig 8).

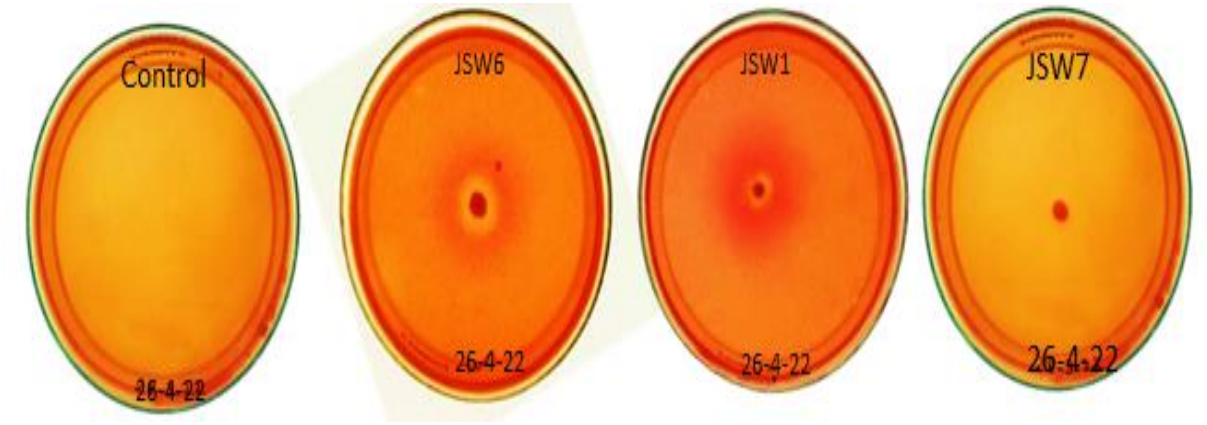

Figure 7: Halo-zone formation observed in JSW1 and JSW6; no zone observed in JSW7 as compared to negative control, which did not receive any strains

3.7. Chitinase production assay

Seven bacterial strains were tested on chitin agar plates having insoluble colloidal chitin for chitinolytic activity. Out of seven, only three strains (JSW1, JSW5 and JSW6) were chitinase-positive and produced a clear zone around the growing colonies in Table 5.

Figure 8: Representing halo-zone formation observed in JSW2 & JSW3

Table 5: Index of the Cellulolytic, Pectinase and Chitinase bacterial isolates

Isolate	Cellulolyticindex	Pectinaseindex	Chitinaseindex
JSW1	2.56 ± 0.3	0.56 ± 0.12	0.40 ± 0.22
JSW2	0	0.8 ± 0.13	0
JSW3	0.47 ± 0.12	0.66 ± 0.16	0
JSW4	0.92 ± 0.05	0.64 ± 0.2	0
JSW5	0.61 ±0.13	0.59 ± 0.12	0.35 ± 0.24
JSW6	2.05 ± 0.08	0.4 ± 0.25	0.33 ± 0.41
JSW7	0	0.53 ± 0.11	0

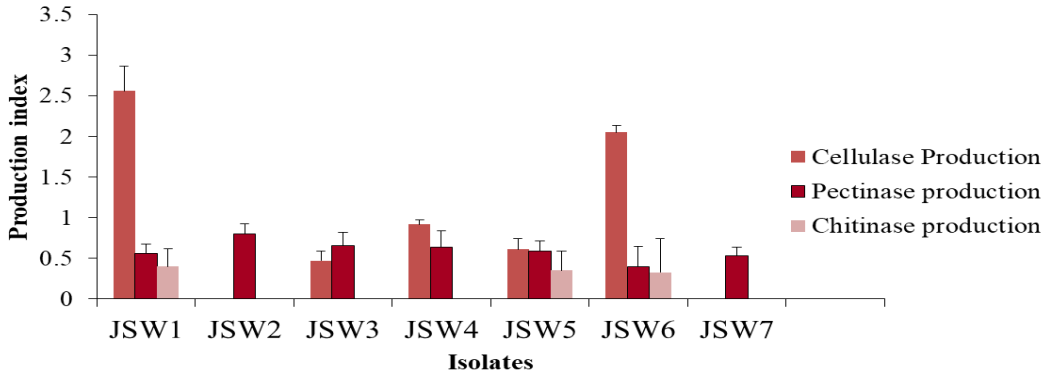

Figure 9: Representing Cellulolytic, pectinolytic and Chitinase index in bacterial isolates. Cellulose production in JSW1 and JSW6 was highly significant ($p<0.001$). Cellulose production was not observed in JSW2 and JSW7 isolates. Similarly Chitinase prod

3.8. Bacterial pikovskaya agar media (TCP) solubilization

All bacterial isolates responded positively by forming a clear halo zone around the growing colonies. JSW2, JSW1and JSW6 showed the highest TCP solubilization activity with 3.0, 2.33 and 2.44 solubility index, while JSW3 and JSW4 showed moderate solubilization activity with 1.81and 1.62 index. However, JSW5 exhibited minor solubility see Fig 10.

3.9. Bacterial pikovskaya agar media CaCO3solubilization

Seven bacterial strains were tested on CaCO3 pikovskaya agar media for solubilization activity. JSW1strain showed highest solubility index (1.58). JSW2, JSW3, JSW4 and JSW5 were also found positive with average solubility index ranging from (1.1, 1.13 and 1.15) and produced a clear zone around the

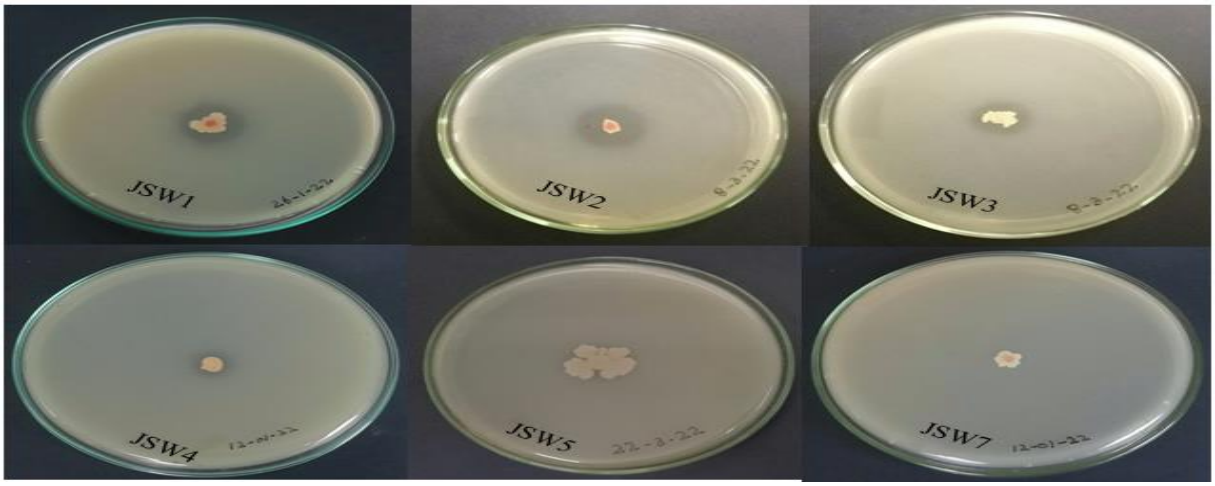

Figure 10:TCPsolubilizationzoneformationobservedinJSW1, JSW2, JSW3, JSW4& JSW5

growing colonies (see Table 6 and Fig. 11).

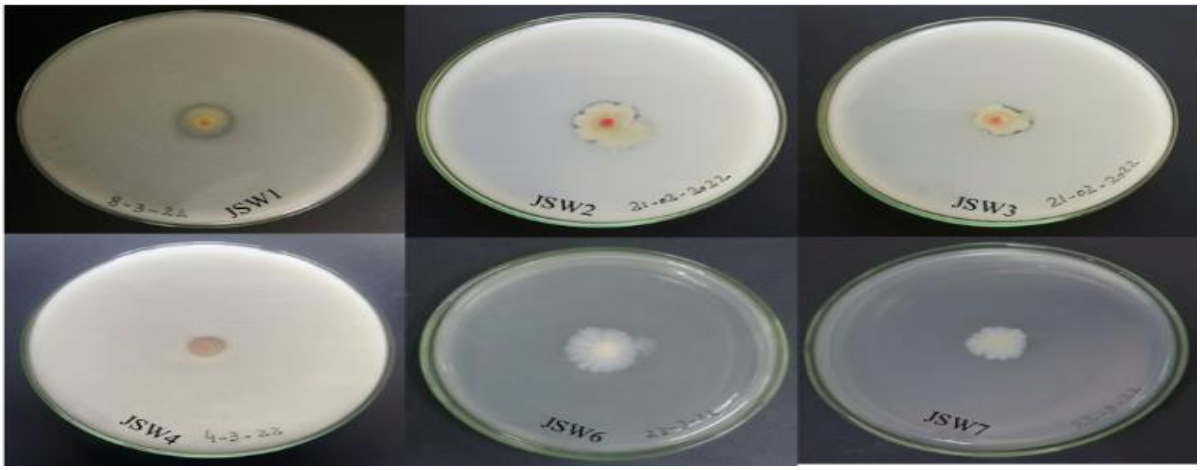

Figure 11: CaCO3 solubilization zone formation observed in JSW1, JSW2, JSW3 and JSW4

Table 6: index of the Phosphate and $CaCO_3$ solubilizing bacterial isolates

Isolate	Phosphate solubilization (TCP) index	$CaCO_3$ solubilization index
JSW1	2.33 ± 0.23	1.58 ± 0.18
JSW2	3.0 ± 0.12	1.1 ± 0.07
JSW3	1.81 ± 0.14	1.13 ± 0.02
JSW4	1.62 ± 0.177	1.15 ± 0.03
JSW5	1.27 ± 0.02	1.13 ± 0.03
JSW6	2.44 ± 0.3	0
JSW7	0	0

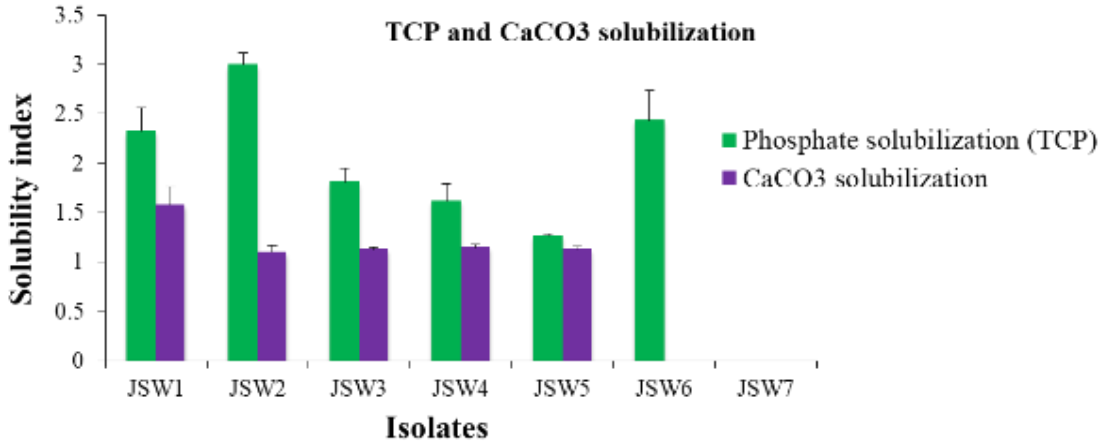

Figure12: Representing TCP and CaCO3 solubility index in bacterial isolates

3.10. Phylogenetic analysis

The accession no. of bacterial isolates KF465842.1, KX353756.1, MT86230.1, GU29031.1, EF125933.1, MT577595.1, MT383661.1 were compared withusing the BLAST program. *Pseudomonas sp.* PT19 (JSW1) showed 100% similarity to *Pseudomonas koreensis* strain NB2 (JSW2). However these two bacterial isolates showed 95% similarity with *Pantoea dispersa* strain ITI (JSW3) and JSW5. JSW4 and JSW7 showed 100% similarity with JSW6 as seen in Fig 14.

3.11. Soil, nodule and root-associated microbes for rice yield analysis

Under controlled conditions, bio-inoculum treatments in tricalcium phosphate amended sand showed variability in rice growth promotion due to the absorption superiority of nutrients (TCP). Pots treated with consortia exhibited prominent

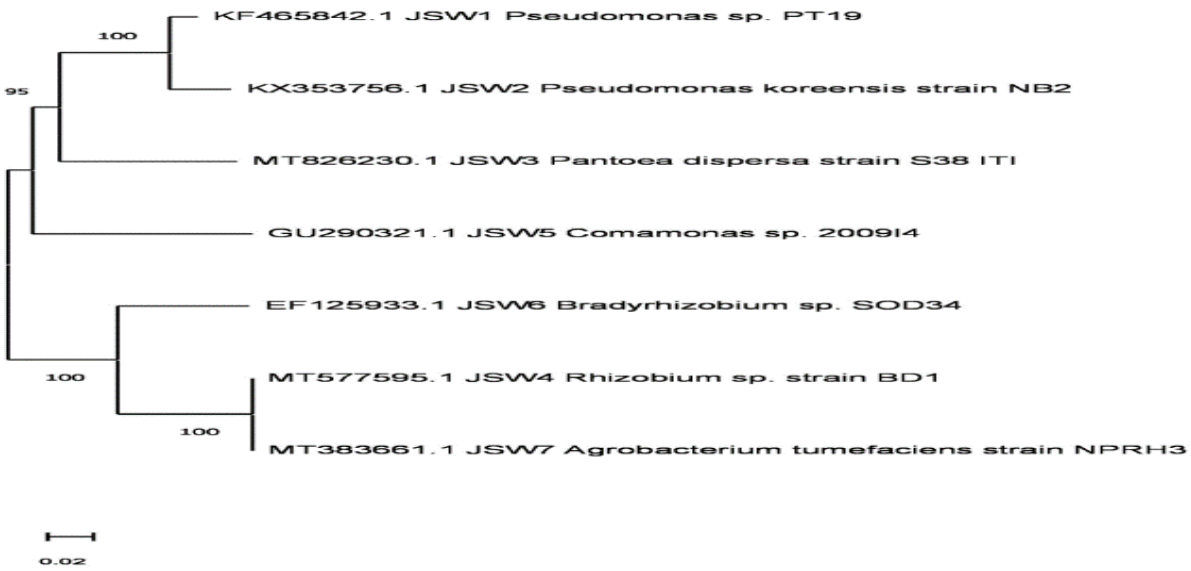

Figure 14 The phylogentic tree was constructed by using MEGA 11 software by neighbor joining method and the Bootstrap value was 100. The bacterial isolates were claded with Pseudomonas sp., Pantoea dispersa, Comomonas sp., Bradyrhizobium sp., Rhizobium s

Table 6: NCBI Blast results showing highly similar sequences

Description	Scientific name	Per. Identity	Accession
Rhizobium sp. strain BD1 16S ribosomal RNA gene, partial sequence	Rhizobium sp.	100.00%	MT577595.1
Rhizobium sp. HGR13 16S ribosomal RNA gene, partial sequence	Rhizobium sp. HGR13	100.00%	GQ483459.1
Agrobacterium pusense strain 76 chromosome R76C2, complete sequence	Agrobacterium pusense	99.93%	CP053857.1
Agrobacterium pusense strain 76 chromosome R76C1, complete sequence	Agrobacterium pusense	99.93%	CP053856.1
Agrobacterium pusense strain FDAARGOS_633 chromosome 3	Agrobacterium pusense	99.93%	CP050899.1
Agrobacterium pusense strain FDAARGOS_633 chromosome 1	Agrobacterium pusense	99.93%	CP050898.1
Agrobacterium pusense strain CFBP5875 chromosome linear, complete sequence	Agrobacterium pusense	99.93%	CP039895.1
Agrobacterium pusense strain CFBP5875 chromosome circular, complete sequence	Agrobacterium pusense	99.93%	CP039894.1
Agrobacterium sp. 33MFTa1.1 chromosome linear, complete sequence	Agrobacterium sp. 33MFTa1.1	99.93%	CP036359.1
Agrobacterium sp. 33MFTa1.1 chromosome circular, complete sequence	Agrobacterium sp. 33MFTa1.1	99.93%	CP036358.1
Rhizobium sp. strain CPAO 8.2F3 16S ribosomal RNA gene, partial sequence	Rhizobium sp.	99.93%	KY971001.1
Rhizobium sp. Y9 chromosome 2, complete sequence	Rhizobium sp. Y9	99.93%	CP018000.1
Rhizobium sp. Y9 chromosome 1, complete sequence	Rhizobium sp. Y9	99.93%	CP017999.1
Rhizobium sp. isolate Moz90 16S ribosomal RNA gene, partial sequence	Rhizobium sp.		

enhancement in shoot length (46%), root length (37%), dry weight of shoots (85%**),** and dry weight of roots (53%**)** in plants as compared to control. In the case of single inoculums amended pots, *Pantoea dispersa* and *Rhizobium sp.* increased impact on all aspects of plant's physical attributes like shoot length (45% and 35%), root length (21% and 17%), root fresh weight (99% and 97%), root dry weight (75%, 69%), shoot fresh weight (86% and 91%), shoot dry weight (71% and 76%). *Comomonas sp.* and *Pseudomonas Koreensis* strains also considerably improved plant growth parameters but less than consortia, *Pantoea dispersa* and *Rhizobium sp. Pseudomonas sp.* presented less potential for growth enhancement in contrast control. Our findings revealed that PGPRs, individually or in combination, had significantly impacted rice growth. Using bacterial-based biofertilizers in this study to promote plant development is strongly recommended for increasing rice yields (see Table 7 and Fig 15).

Figure 15: Substantialeffect of bio-inoculum on rice growth

lateral roots. Different treatments had a non-significant effect on shoot length, root length, root diameter, stem diameter and leaves per plant. Selected treatments increased shoot length from 3% to 26%, in which JSW5 performed the best. There was an increase ranging from 7% to 37% in root length upon applying selected treatments, in which JSW5 showed maximum potential. After applying the chosen treatment, plant fresh weight increased from 7% to 34%. Rhizobium sp.and Pseudomonas koreensis showed maximum increase in parameter. A significant increase in plant dry weight was observed after the treatment chosen, ranging from 41% to 99%. Rhizobium sp.was efficient among all. Stem and root diameter increased from 9% to 26% and 8% to 37%, respectively, compared to control (see Table 9). Pseudomonas koreensis performed the best. The number of nodules was observed only in treatments Pantoea dispersa(2%), Pseudomonas koreensis (3%), and Rhizobium sp. (13%). Rhizobium sp. was best among all other isolates, showing a significant improvement in all parameters see Table 8.

3.12.2. Harvesting stage parameter

There was a significant effect of different treatments on the number of pods, number of seeds and weight of seeds per plant.

Table 7: Effects of selected treatment on yield parameter of rice

	Shoot length (cm)	Root length (cm)	Shoot fresh weight (mg)	Root fresh weight (mg)	Shoot dry weight (mg)	Root dry weight (mg)
Control	25.400 ^{d}	13.875 ^{a}	480.53 ^{g}	162.03 ^{f}	81.000 ^{f}	22.100 ^{c}
JSW1	37.125 ^{a}	16.750 ^{a}	897.23 ^{c}	323.30 ^{b}	139.30 ^{b}	38.550 ^{ab}
JSW2	29.000 ^{cd}	14.750 ^{a}	727.98 ^{e}	216.22 ^{d}	127.58 ^{c}	24.350 ^{c}
JSW3	30.250 ^{bcd}	15.500 ^{a}	829.98 ^{d}	217.20 ^{d}	119.23 ^{d}	33.250 ^{b}
JSW4	30.750 ^{bc}	19.500 ^{a}	588.00 ^{f}	177.10 ^{e}	97.750 ^{e}	24.600 ^{c}
JSW5	34.250 ^{ab}	16.250 ^{a}	917.58 ^{bc}	320.65 ^{b}	142.77 ^{b}	37.225 ^{ab}
Consortia	37.500 ^{a}	19.000 ^{a}	1176.3 ^{a}	384.00 ^{a}	150.48 ^{a}	40.250 ^{a}
DAP	31.750 ^{bc}	18.300 ^{a}	949.55 ^{b}	285.63 ^{c}	140.18 ^{b}	38.250 ^{ab}
LSD	5.1838	9.8492	43.677	8.1605	6.6432	6.0137
ANOVA	*	Ns	**	**	**	*

3.12. Effect of isolates on plant growth in a field experiment

3.12.1. Nodulation stage parameter

There was a significant effect of different treatments on plant fresh weight, plant dry weight, number of nodules and number of

There was a non-significant effect of different treatments on plant height. The plant height was increased by 3%. There was a significant increase in pods per plant, ranging from 7% to 46%, in which Rhizobium sp. showed the highest

potential. Selected treatment showed an increase in seed per plant from 10% to 31% .Rhizobium sp. performed the best. The seed weight per plant was increased from 7% to 61% after the selected treatment compared to the control. Rhizobium sp. was the best isolate for improving the grain yield plant.

nodule. The diversity and abundance of bacterial communities in soil and root were checked using the MPN test, in which the highest population of bacteria was found in soil-associated bacteria. These findings agree with (Mansfeldt, Achermann et al. 2019) who reported that soil contains more organic matter and is richer in the

Table 8: Effect of selected treatment on yield parameter of soybean at nodulation stage under field experiment

Treatment	Shoot length (cm)	Root length (cm)	Plant fresh weight (g)	Plant dry weight (g)	Leaves per plant	Stem diameter (mm)	Root diameter (mm)	Number of lateral roots	Number of nodules	Active nodules
JSW1	36.8±1.3^{ab}	15.1±0.68^{ab}	47.5±1.95^{ab}	12.01±0.91^{ab}	35.1±2.56^{ab}	0.57±0.02^{ab}	0.52±0.01^{a}	6.33±0.61^{bc}	9.2±2.07^{d}	+
JSW3	37.1±1.3^{ab}	17.5±1.24^{a}	50.6±1.70^{a}	13.01±0.94^{ab}	40.2±5.46^{ab}	0.61±0.03^{ab}	0.6±0.04^{a}	8.33±0.9^{ab}	11.11±0.29^{d}	+
JSW5	40.5±1.8^{a}	18.2±0.95^{a}	53.1±1.65^{a}	15.08±0.60^{a}	46.1±3.54^{a}	0.66±0.03^{a}	0.56±0.04^{a}	8.66±0.26^{a}	12.77±0.43^{a}	+
Consortia	33.4±0.7^{b}	14.2±1.04^{ab}	46.3±1.36^{bc}	10.80±0.84^{bc}	31.2±1.91^{b}	0.58±0.02^{ab}	0.57±0.02^{a}	5.88±0.45^{c}	7.1±0.4^{c}	+
Control	32.2±1.09^{b}	13.2±0.40^{b}	39.6±1.73^{c}	7.56±0.50^{c}	26.2±1.80^{b}	0.52±0.02^{b}	0.48±0.02^{a}	4.77±0.29^{c}	5.2±0.46^{c}	+
DAP	32.6±1.05^{b}	13.3±0.4^{b}	42.6±0.94^{c}	10.67±0.47^{bc}	30.6±2.25^{b}	0.53±0.02^{ab}	0.46±0.03^{a}	5.33±0.28^{c}	8.2±0.43^{b}	+
LSD	6.09	4.03	7.56	3.50	15.10	0.14	0.15	2.47	2.07	
ANOVA	ns	ns	**	**	Ns	ns	ns	*	***	

Table 9: Effect of selected treatment on yield parameter of soybean at harvesting stage under field experiment.

Treatment	Plant height (cm)	Pods per plant	Seed per plant	Weight of seed per plant (g)
JSW1	29.8 ± 1.04^{a}	60 ± 2.23^{b}	93.2 ± 3.22^{abc}	14.5 ± 1.4^{abc}
JSW3	30.6 ±1.79^{a}	64 ± 2.60^{b}	104.1 ± 2.85^{ab}	16.5 ± 0.6^{ab}
JSW5	32.2 ± 0.66^{a}	81.1 ± 3.94^{a}	106.8 ± 4.62^{a}	17.0 ± 0.4^{a}
Consortia	31.2 ± 1.14^{a}	47.2 ± 3.37^{c}	72.7 ± 3.81^{d}	11.13 ± 0.6^{cd}
Control	31 ± 0.75^{a}	55.4 ±1.16^{bc}	80.6 ± 3.13^{cd}	10.5 ± 0.6^{d}
DAP	29.6 ± 0.66^{a}	59.6 ±1.45^{bc}	89 ± 1.10^{bc}	13.27 ± 0.57^{bcd}
LSD	5.14	12.56	15.6	3.61
ANOVA	ns	***	***	**

4. Discussion

Understanding the composition and function of the bacteria associated with the soil, root and nodule is important for sustainable agricultural production so that the agriculture system can be less dependent on chemical fertilizers. Thirty culturable microbiome bacterial samples were isolated from the different localities of soybean, including soil, root, and

microbial community because it provides the nourishment necessary for microbial survival and growth. Bacteria were further characterized in vitro, including cellulose, pectinase and chitinase production. Previously, (Deng, Zhang et al. 2020)reported that endophytic bacteria are involved in nodulation and can promote plant growth through various mechanisms. A full understanding of the interconnection

and possible function of entire soil microbiomes is limited due to the lack of awareness about soil microbes' taxonomic and functional variety (Zhang, Wang et al. 2023)

The 16S sequences of the bacterial isolates were compared using BLAST function in NCBI tool. The results showed relationship among the isolates. Pseudomoas sp. (JSW1) showed 100% similarity with Pseudomoas korensis (JSW2) revealing that these 2 isolates were genetically similar. However, these isolates showed 95% similarity with Pantoae dispersa (JSW3) and Comamonas sp. (JSW5). This showed divergence in genetic makeup of the isolates showing the existence of distinct species or strains in Pantoea genus. Similarly, Rhizobium sp. (JSW4), Bradyrhizobium sp. (JSW6) and Agrobecterium tumefaciens (JSW7) showed 100% similarity indicating these sequences are 100% conserved. The observed variations and similarities in the 16S DNA sequences highlight the genetic diversity among the bacterial isolates. These findings could have implications for understanding the ecological roles, functional differences, and evolutionary relationships of these bacteria, emphasizing the importance of molecular characterization for precise taxonomic classification.

In this study, microbial strains (PGPB) mineral mobilization and enzymatic production differences in various plant parts substantially impact nutrient availability, effectiveness, agricultural practices precision, and strategic shaping application to boost overall agriculture productivity (Olanrewaju, Ayangbenro et al. 2019). This finding is consistent with (Peng, Qiao et al. 2020)} that microbial variety in roots and soil has a strong colonization filtration capacity that allows the plant to determine the composition of the bacterial community. The microbial community in the rhizosphere produces various hydrolytic enzymes used to degrade the fungal cell wall (Sharma, Sayyed et al. 2013). Several microorganisms have a variety of cellulases for the degradation of cellulose. Cellulose is present in plant cell walls. (Gao, He et al. 2019) () demonstrated that 70% of nodule endophytic bacteria of soybeans produce cellulase. This study also related to (Mohammadipour, Enayatizamir et al. 2021)in which they tested the isolates by forming a halo zone surrounding the colonies, which determined the ability of isolates to hydrolyze CMC in solid media. Pectinase is also a common name for a commercial enzyme that breaks down pectin, a polysaccharide substrate present in plant cell walls (Olanrewaju, Ayangbenro et al. 2019). The entire nodule-associated bacteria showed positive results for pectinase production. Previously, (Gao, He et al. 2019) (Gao, He et al. 2019) investigated that 33% of the bacteria isolated from the root and nodule of soybean were able to produce pectinase. Chitinase plays an important role in the biocontrol of phytopathogenic fungi since it is a prominent component of the membrane of insect gut, about 3-13% and cuticles up to 40% of dry mass (Akram, Zhang et al. 2022). Five soybean-associated bacteria were able to produce chitinase. Previously, it was reported that bacterial isolates hydrolysis area by chitinase secretion, which rapidly acts on colloidal chitin, the only carbon source in the defined Media, confirming bacterial isolates positive for chitinase (Dhole, Phuge et al. 2022). These potential bacteria effectively release phosphorus (TCP) and CaCO3 and can produce acid.

PGPB has been applied to various agricultural plant crops to improve productivity. Under pot conditions, Pantoea dispersa, Rhizobium sp., and consortia significantly improved rice plant height and dry weight. The Pseudomonas koreensis and Rhizobium sp. Consortia can potentially improve soybean production. At the stage of harvesting, plants show a significant increase in

growth. This research study indicates that bacterial isolates have the potential to enhance plant growth as compared to control. Previously, (Mirskaya, Khomyakov et al. 2022) (Mirskaya, Khomyakov et al. 2022)reported that different PGPBs from the wheat crop showed significantly increased plant height, grain yield, straw yield, and weight under pot and field conditions. Our findings agree with (Zhang, Chen et al. 2023) that applying bacterial consortium to soybean crops improves seedling radicle length, shoot length, shoot dry weight, and total dry weight by 44%, 30%, 33%, and 29%, respectively.

After successful field trials, these PGPRs can be used as biofertilizers. For the maintenance of agriculture sustainability, the usage of biofertilizers should be increased to meet future demands.

5. Conclusion

The outcomes of this study demonstrate that biofertilizers based on certain strains of plant growth-promoting (PGP) bacteria application can eliminate the use of DAP and reduce the use of urea. These bio-inculants are organic and have no harmful impact on the human body. Significantly reducing fertilizer imports can save billions of rupees in country spending and generate employment opportunities for the national labour force. This study's anticipated impact has replaced chemical fertilizers with organic farming, stored the nitrogen cycle and other biological processes, and reduced soil acidification. Soybean soil microbiome-based fertilizers are a cost-effective and environmentally friendly technology and enhance agricultural productivity and sustainability by producing phyto-hormones, availability of limiting nutrients (phosphorus) to host plants by colonizing with root zone, organic acid production, reduction in necessary nutrients fixation in soil and sustainably produce healthy and nutritious food, improved crop production. To meet the growing human population's food needs, soil microbiome biofertilizers should be increased, eliminating social, environmental and economic costs.

6. Authors contribution:

All authors contribute equally in this paper

7. Conflict of interest:

All authors have no conflict of interest

8. Data availability statement:

Data will be available on request by corresponding author.

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