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Characterization of plant growth-promoting microorganisms from soybean rhizospheric soil and their influence on soybean development

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Abstract

When administered to seeds or crops, plant growth promoting bacteria (PGPB) have the capacity to colonise the rhizosphere, plant roots, or both, enhancing plant growth and production. The motive of this study was to find elite bacteria in the soil and employ them to help soybean plants grow faster. Purified four bacterial strains were tested in vitro for plant growth promoting (PGP) properties. Phosphate solubilizing activity was discovered in 04 isolates, IAA production was detected in 04 isolates, and N2 fixation activity was found in 04 isolates. SJ-5 microbial strains tested positive for all PGP features and were further described using biochemical and molecular methods. Bacillus sp. was discovered using biochemical and 16S rRNA gene sequence analyses. Plant inoculation tests revealed that this PGPB strain increased shoot and root length as well as shoot and root biomass significantly. In comparison to the uninoculated control, there was a considerable increase in the number of lateral roots. The research suggests that this PGPB might be used to make inoculums or as a biofertilizer to boost soybean growth and nutritional content.

Keywords: Plant growth promoting bacteria, Soybean, Phosphate solubilization, 16 rRNA

Introduction

Agricultural products are the main source of proteins, carbohydrates, fats, vitamins, and other elements in the diet, and soybean (*Glycine max* L. Merrill) is one of the greatest sources of 'all in one' with a high nutritional value. Healthy food, which is closely tied to the current agro-ecosystem, is the primary issue for good health. As the world's population grows, so does the need for food, which leads to the usage of various chemicals such as phytohormones and pesticides to encourage plant growth and alleviate infections, respectively, in order to get speedy results. In today's agro-world, the cultivator's main aim is to grow a healthy plant, that is, one that is free of infectious diseases, and to achieve a high yield under any circumstances. As a result, a method for providing plant protection while also improving crop yields in a way that does not disrupt the agro-ecological ecosystem's balance is urgently needed. When administered to seed or crops, soil-borne beneficial bacteria known as Plant growth promoting bacteria (PGPB) have the capacity to aggressively colonise the rhizosphere, plant roots, or both (Ashrafuzzaman et al., 2009 and Kaymak, 2011). The capacity of the PGPB to manufacture and release metabolites that directly stimulate plant development is responsible for its plant growth promoting (PGP) capabilities, and numerous mechanisms have been proposed to explain how it might benefit the host plant. The root is the major portion of the plant that acquires nutrients from the earth and serves as a stable foundation for the plant's upshoot to develop. Plant growth hormone indole acetic acid (IAA) produced by PGPB increased root development and hence nutrient intake (Egamberdieva and Kucharova 2009; Piccoli et al. 2011; Duca et al. 2014). In

stressed conditions, the plant's level of ethylene, the only gaseous hormone that inhibits plant growth, increases. Some PGPB lowered this amount via producing the ACC-Deaminase enzyme, which degrades 1-aminocyclopropane-1-carboxylate (ACC), an immediate precursor of ethylene, into 2-oxobutanoate and ammonia, and therefore increased plant growth indirectly (Kumari et al. 2015). Bacterial polysaccharides have the ability to bind soil particles together to create microaggregates and macroaggregates. Plants that have been treated with bacteria that produce exopolysaccharide (EPS) have better soil structure and are more resistant to water stress (Sandhya et al. 2009). Under saline circumstances, EPS may bind to cations such as Na+, rendering it unavailable to plants. Some microbial strains generate cytokinin and antioxidants, which cause ABA to be reduced and reactive oxygen species to be degraded (ROS). Phosphorus, potassium, iron, zinc, and copper, for example, have restricted mobility in the soil and are found in insoluble form. By producing organic acids such as gluconic acid, citric acid, and others, PGPB may solubilize insoluble inorganic phosphate complexes such tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate, converting them into accessible phosphorus (Richardson et al. 2009; Khan et al. 2009; Egamberdiyeva 2007). One of the primary stumbling blocks is plants' inability to utilise atmospheric nitrogen. Some PGPB use a nitrogenase enzyme complex to convert atmospheric nitrogen to ammonia, which plants may use (Santi et al. 2013). Iron, like phosphorus, is found in inaccessible state (Fe3+) in the environment for plants. Through the synthesis of siderophores, PGPB provides this iron to plants by chelating it from the soil (Fouzia et al. 2015; Boukhalfa and Crumbliss 2002). PGPB secretes proteases that break down complex proteins in soil into amino acids that plants can use. They function as degradative enzymes because they catalyse total hydrolysis of proteins to peptides. In addition to these plant growth-promoting activities, some PGPB secrets include cell wall degrading enzymes, antibiotics, and fungicidal compounds that aid in the elimination of fungal pathogens (Dey et al., 2004; Lucy et al., 2004).The capacity of the PGPB to manufacture and release compounds directly encouraging plant development is responsible for its plant growth promoting (PGP) capabilities, and numerous mechanisms have been proposed to explain how it might benefit the host plant. The root is the major portion of the plant that acquires nutrients from the earth and serves as a stable foundation for the growth of the shoot. By secreting the plant growth hormone indole acetic acid (IAA), PGPB increased root development and hence nutrient intake (Egamberdieva and Kucharova 2009; Piccoli et al. 2011; Duca et al. 2014). In stressful circumstances, the amount of ethylene, the sole gaseous hormone that inhibits plant development, rises in the plant. By producing the ACC-Deaminase enzyme, certain PGPB lowered this level and indirectly increased plant development by decomposing 1-aminocyclopropane-1-carboxylate (ACC), an immediate precursor of ethylene, into 2-oxobutanoate and ammonia (Kumari et al. 2015). Bacterial polysaccharides may produce microaggregates and macroaggregates by binding soil particles together. Plants treated with bacteria that produce exopolysaccharide (EPS) have better soil structure and are more resistant to water stress (Sandhya et al. 2009). Because EPS can bind to cations such as Na+, it is unavailable to plants in saline environments. Some microbial strains generate cytokinin and antioxidants, which cause abscisic acid (ABA) to be reduced and reactive oxygen species to be degraded (ROS). Furthermore, nutritional elements including phosphorus, potassium, iron, zinc, and copper have little mobility in the soil and are found in insoluble form. By producing organic acids such as gluconic acid, citric acid, and others, PGPB may solubilize insoluble inorganic phosphate complexes such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate, and thereby convert it to accessible phosphorus (Richardson et al. 2009; Khan et al. 2009; Egamberdiyeva 2007). One of the biggest restrictions is plants' inability to utilise atmospheric nitrogen. Some PGPB use a nitrogenase enzyme complex to convert atmospheric nitrogen to ammonia, making it accessible to plants (Santi et al. 2013). Iron, like phosphorus, is available in the environment in an inaccessible state for plants (Fe3+). By producing siderophores, PGPB provides this iron to plants by chelating it from the soil (Fouzia et al. 2015; Boukhalfa and Crumbliss 2002). Proteases released by PGPB break down complex proteins in the soil into amino acids that plants may use. They serve as degradative enzymes since they catalyse entire hydrolysis of proteins to peptides (López-Otn et al. 2002). In addition to these plant growth-promoting properties, other PGPB secrets include a cell wall disintegrating enzyme, antibiotics, and fungicidal chemicals that aid in the elimination of fungal infections (Dey et al., 2004; Lucy et al., 2004).

Material and methods

Sampling site and soil sample collection

Washim district in the Risod area of (M.H.) India was selected as the sampling location. This is one of India's most notable soybean-rich regions. Healthy soybean plant samples with thick rhizosphere soil were obtained from several locations in the Risod area while wearing pre-sterilized hands and put separately in pre-sterilized zip log bags. Plant samples were collected and then sent to the laboratory for additional bacterial isolation.

Isolation of bacterial strains

To extract bacterial strains from distinct soil samples, scientists utilised a serial dilution method. One gramme of soil was homogenised in 9 mL of sterilised physiological saline water (0.85%) and incubated at 26°C for 5 minutes. 500 µl of various dilute samples were speeded on nutritional agar (NA) plates after serial dilution in saline water up to 10-6. Plates were incubated at 28 2oC in a BOD incubator for 24 hours and bacterial growth was detected. A single colony was selected from several soil sample plates and streaked on NA plates for pure culture.

Screening of potential isolates through characterization of plant growth promoting properties

Phosphate solubilization

Phosphate solubilize medium were used in the phosphate-solubilizing test (PSB). All ten bacterial cultures were injected in the middle of PSB agar plates and incubated at 28 2°C for 5-6 days. Plates were examined after the incubation time to look for a clear halo zone that had developed around the bacterial colony, and the colony's diameter and clearing zone were measured. The following formula was used to determine the P-solubilization index:

$$\text{P-solubilization index} = \frac{\text{Colony diameter} + \text{Clearing zone}}{\text{Colony diameter}}$$

Effect of bacterial isolates on plant growth

To investigate whether the screened isolates had any influence on plant development, soybean seeds were surface sterilised for 3 minutes with 0.1 percent HgCl2 and 70% ethanol, then rinsed several times with Milli Q water (Millipore, Germany) before planting. Bacterial cultures that had been cultivated overnight were centrifuged at 10,000 rpm for 20 minutes and the pellets were washed in 10 mM phosphate buffer saline (PBS, pH 7.2). The OD600 was adjusted to 0.2 by resuspending the pellets in PBS containing 0.1 percent carboxymethylcellulose (CMC) (108 CFU ml-1). Surface sterilised seeds were soaked for 1 hour in PBS with 0.1 percent carboxymethylcellulose (CMC) as a binder, then dried in a laminar dryer. Throughout the experiment, sterile conditions were maintained. Control seeds were soaked simply in autoclaved Milli Q water (non-microbiolized). Soybean seeds were planted in tiny polypropylene cups containing autoclaved soil and kept at 26 °C for 15 days in a plant development laboratory with a 16 h/8 h light/dark photoperiod and 80% humidity.

Sample collection and plant growth parameter studies

Plants were carefully pulled 15 days after seeding without causing any harm to root tissues. From each replication of the treatment, three plants were chosen at random and washed with Milli Q water. Different plant growth metrics were examined, including root and shoot length, root and shoot fresh weight, and the number of lateral roots.

Biochemical characterization of plant growth promoting bacterial isolate

Gram reaction, catalase responses, endospore and capsule staining, motility test, mannitol fermentation test, urease activity, and amylase test were used to assess biochemical properties of the powerful isolate SJ-5. Standard kits were used for the Gram reaction, endospore staining, and capsule staining (Himedia, India). The catalase test was carried out by dissolving 100 l of overnight developed bacterial culture in 1 ml of

H2O2 and looking for gas bubbles. Inculcating bacteria on 0.35 percent NA media, phenol red mannitol broth medium (pH 7.3), urea broth, and starch minimum medium completed motility, mannitol fermentation, urease activity, and amylase tests, respectively.

Molecular characterization of plant growth promoting bacteria

Bollet et al. (1991) approach was used to isolate bacterial genomic DNA with minor modifications. The quantitative estimate of DNA was carried out using a biophotometer (Eppendorf, India) that is based on Beer Lamberts law, whilst the qualitative estimation was carried out using agarose gel electrophoresis and inspection of the DNA band using the UVITECH gel doc system. Using universal 16S rRNA primers: 27F-AGAGTTTGATCMTGGCTCAG and 1492R- CGGTTACCTTG TTACGACTT in a Thermal cycler, PCR amplification of the 16S rRNA gene from genomic DNA of the bacterial isolate SJ-5 was performed (Kyratec, Australia). 100 ng genomic DNA, 1 l Taq polymerase (3U/ul), 2 l 10 buffer, 2 l dNTP mix (10mM), 2 l MgCl2 (25mM), and 1 l each primer (10 pmol/l) were included in the 25 l reaction mixture. The following PCR conditions were used: initial denaturation at 94°C for 4 minutes, with subsequent 30 cycles of denaturation for 94°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 1.30 minutes, and a final extension at 72°C for 10 minutes. The PCR result was put onto a 1 percent agarose gel (wt/vol) containing EtBr and run for 45 minutes at 85 Volts in 1X TAE buffer for gene evaluation. Visualizing the gel for the DNA band with the UVITECH gel doc system was used to see the DNA band.

16S rRNA gene sequence homology and phylogeny analysis

The bacterium SJ-5 was identified by homology analysis of the 16S rRNA gene sequence acquired from sequencing, which was done using the NCBI's BLAST programme, which can be found at http://blast.ncbi.nlm.nih.gov/. The top 10 sequences were chosen for further examination based on their greatest identity score. Clustal W was used to align selected sequences and check for gaps. MEGA 6 was used to undertake phylogenetic and molecular evolutionary analyses (Tamura et al 2013).

Sequence submission and culture deposition

The 16S rRNA gene nucleotide sequence was submitted to NCBI Genbank and assigned the accession number KJ 184312. Bacterial culture was also donated to the Microbial Culture Collection (MCC) in Pune, with the accession number 'MCC-2069.'

Results

Bacterial isolation

To conduct a preliminary investigation into possible plant growth-promoting bacterium SJ-1, SJ-2, SJ-3, SJ-4, SJ-5, SJ-6, SJ-7, SJ-8, SJ-9, and SJ-10 are the bacterial strains that were identified from the soil sample.

Plant growth promoting activities

Functional characterization of direct plant growth promoting activities was done by growing all bacterial isolates on different specified medium. Except SJ-4, all isolates were found with P-solubilizing activity while few isolates were found positive for other properties. Among the all isolates SJ-2 and SJ-5 were found most potent as both of these possess all the PGP properties. Based on the plant growth promoting activities shown by all isolates, SJ-2 and SJ-5 bacterial isolates were selected for further to check effect on soybean plant growth (Fig 1). The solubilization index and presence and absence of functional characteristics measured are shown in Table 1.

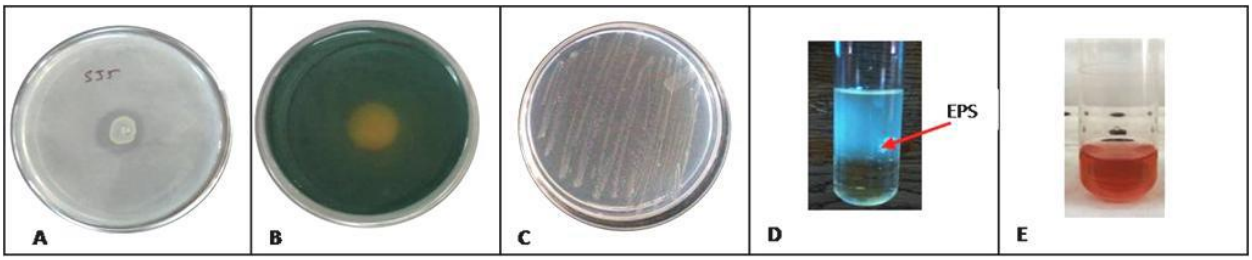

Fig. 1 PGP properties of *Bacillus* sp. SJ-5 **A.** Phosphate solubilization **B.** Siderophore production **C.** Nitrogen fixation **D.** EPS production **E.** IAA production

Table 1 Plant growth promoting traits of the bacterial isolate

Bacterial strain	Phosphate solubilization (SI)	Siderophore production (SPI)	EPS production (gm /100ml)	IAA production (µg/ml)	ACC deaminase (+/-)	N2 fixation (+/-)
SJ-1	1.2±0.18	Nil	Nil	Nil	+	-
SJ-2	1.9±0.65	2.87±0.12	1.2±0.11	15.3±0.58	+	+
SJ-3	1.32±0.37	4.2±0.19	Nil	Nil	-	+
SJ-4	Nil	Nil	0.8±0.09	11.2±0.43	+	-
SJ-5	2±0.25	3.42±0.42	1.5±0.21	19±0.6	+	+
SJ-6	2.41±0.26	3.2±0.32	Nil	Nil	+	-
SJ-7	1.52±0.41	Nil	Nil	8.7±0.29	-	-
SJ-8	2.3±0.14	3.14±0.3	1.6±0.14	9.7±0.32	-	-
SJ-9	1.08±0.23	Nil	0.5±0.17	Nil	+	-
SJ-10	2.2±0.11	1.5±0.17	1.2±0.2	Nil	-	-

Values are the means of three replications ± S.E

Effect of bacterial isolates on plant growth

In the rhizosphere, plant-microbe interaction is critical for plant development. Bacillus sp. SJ-5 demonstrated a substantial increase in percentage of healthy plants and growth-promoting properties such as root, shoot length, and number of lateral roots in a plant growth chamber experiment when compared to other treatments. SJ-2 exhibited plant growth-promoting properties as well, albeit to a lesser extent than SJ-5. SJ-5 increased shoot length by 26.7 percent and root length by 292 percent above control plants, whereas SJ-2 increased shoot length by 11 percent and root length by 211 percent. Similarly, the number of lateral roots in SJ-5 treated plants (20) was found to be 300 percent more than in control plants (5), whereas it was shown to be 180 percent higher in SJ-5 (14) plants. Similarly, the SJ-5-treated plant's root and shoot length and fresh weight were determined to be much larger than the control plant's (Table 2). SJ-5 was discovered to be a powerful growth promoter based on comparative plant growth parameter data.

Table 2 Effect of SJ-2 and SJ-5 on soybean plant growth promoting parameters

Treatment	Shoot length (cm)	Root length (cm)	Root fresh weight (g)	Shoot fresh weight (g)	Lateral roots
Control	4.5±0.27	2.6±0.2	0.045±0.003	0.425±0.03	5±0.21
SJ-2	5±0.18	8.1±0.11	0.082±0.001	0.45±0.02	14±0.17
SJ-5	5.7±0.3	10.2±0.31	0.093±0.002	0.493±0.02	20±0.28

Values represent the mean of 3 replications

Biochemical characterization of plant growth promoting bacterial isolate

For preliminary identification of bacterial isolate SJ-5, biochemical characterisation was performed, and it was discovered to be a rod-shaped, gram-positive, endospore and capsule-forming bacterium. SJ-5 tested positive for catalase, indicating that it belongs to the Bacillus genus. The parameters of endospore and capsule development were checked for further confirmation.

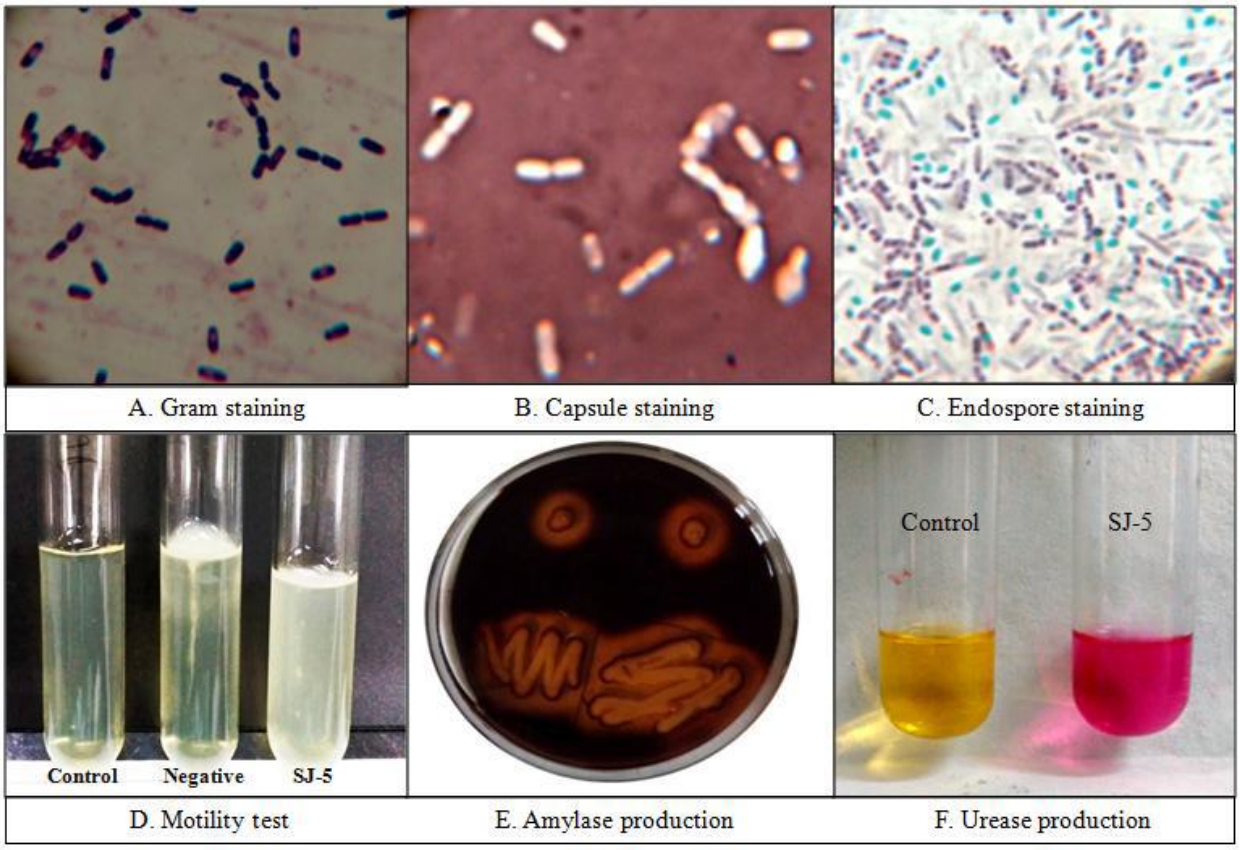

Fig. 2 Biochemical characteristics of *Bacillus* sp. SJ-5

It was shown to be motile on the motility test media because growth was detected through the medium rather than in the inoculation line. It also tested positive for the enzymes amylase and urease (Fig 2). As a consequence of the biochemical findings, the stain was recognised as Bacillus sp., and molecular characterisation was used to validate the identification.

Molecular characterization of plant growth promoting bacterium and phylogeny analysis

The 16S rRNA gene was amplified from genomic DNA using universal 16S rRNA primers to characterise the plant growth promoting bacteria SJ-5. A sharp band approximately 1.5 kb was seen on the agarose gel as standard for DNA quality. The bacterium was verified as a member of the genus Bacillus and submitted to the NCBI Genbank under the name Bacillus sp. SJ-5 after sequence homology and phylogenetic analysis revealed similarities with *B. cereus* and *B. thuringiensis*. KJ 184312 is the NCBI accession number for this bacterium. A bacterial culture was also donated to the Microbial Culture Collection (MCC) in Pune, with the accession number MCC-2069.

Discussion

Beneficial bacteria in the rhizosphere interact with plant roots to boost plant growth and productivity. Four different bacterial isolates were found in soil samples collected in the Indian districts of Mandsaur (MP) and Kota (Rajasthan). Phosphate solubilization, siderophore production, nitrogen fixation, IAA production, ACC deaminase production, and EPS generation were all present in all of the isolates, albeit with varying PGP activity. P-solubilizing activities were discovered in 90% of isolates, whereas siderophore and EPS generation activities were observed in 60%. Only 30% of isolates were found to be able to grow on nitrogen-free media. The first step in any plant-microbe interaction study is to isolate and characterise the PGP activities of beneficial bacteria. Yadav (2013) identified 185 bacterial isolates from V. angularis, V. aconitifolia, V. mungo, and V. radiata rhizosphere related soils and investigated their capacity to synthesise IAA. For the in vitro plant growth promoting traits analysis, about 86.46 percent of the 266 bacterial isolates isolated from the 24 rhizospheric soil samples of Ocimum sp.l showed ammonium production, 89.09

percent exhibited phosphate solubilization, 87.59 percent catalase production, and 7.14 percent showed positive reaction for HCN production (Saharan and Verma 2015).

Tan et al. (2015) isolated 13 bacterial strains from surface-sterilized soybean root nodules based on their phosphate and potassium-solubilizing abilities, while Bai et al. (2002) isolated 14 putative endophytic bacteria strains from surface-sterilized soybean root nodules, excluding endosymbiotic Bradyrhizobium strains. Root and shoot length, root and shoot fresh weight, number of leaves, and lateral roots are examples of plant growth characteristics that may be used to compare treatments. PGPB employs a variety of techniques to promote direct plant growth. PGPB produces organic acid to solubilize insoluble phosphate in the soil, fix atmospheric nitrogen, and supply it to plants; siderophores to chelate iron from the soil and provide it to plant cells; auxins, cytokinins, and gibberellins to enhance plant growth; and ACC deaminase to lower plant ethylene levels. At the seedling stage, the PGPB may assist the plant get enough iron and phosphate from the soil, as well as accelerate cell division with the right phytohormone levels. PGPB produced diverse cell wall degrading enzymes against phytopathogens and evoked induced systemic resistance to boost plant development in an indirect manner (Glick 2015). SJ-5, which was proven to be Bacillus sp. in the current investigation, showed the most promising outcomes across all PGP activities. Many research have been conducted in recent years to evaluate bacteria's plant growth-promoting capabilities and their involvement in boosting plant development in both normal and stressed conditions (Belimov et al. 2009; Heidari and Golpayegani 2012; Nadeem et al. 2010; Tank and Saraf 2010; Glick 2012). Three P-solubilizing isolates had a solubilization index of greater than two in our analysis. Illmer and Schinner (1995) and Hwangbo et al. (1999) both found an inverse association between pH and soluble phosphate (2003). Phosphate-solubilizing Pseudomonas spp. boosted maize growth as well as its phosphorus content, according to Vyas and Gulatti (2009). Phosphate solubilization by B. subtilis and P. fluorescens was reported by Kannahi and Kowsalya (2013). Phosphate, which is essential for plant and bacterial metabolism, is present in the soil in largely insoluble ferric form, making it unavailable to bacteria, and many host species actively prevent bacteria from infecting them (Ratledge and Dover 2000). In CAS agar plates, we discovered SJ-5 producing siderophores, which is necessary for scavenging iron from the soil and making it accessible to the host organism. Only under iron-limited circumstances can the bacterium create siderophore. Many harmful bacteria and fungi may be suppressed as a result of the PGPRs' high-affinity iron absorption mechanisms, which are mediated through siderophore secretion (Sharma et al. 2003). Many researches have documented Bacillus species to produce siderophores (Zhu and Yang 2015; Xu et al. 2014; Jikare et al. 2013; Luo et al. 2012). P. fluorescens and B. subtilis, both isolated from paddy rhizosphere soil, were found to produce siderophores, according to Sivasakthi et al. (2013).

IAA might be a key signal molecule in the control of plant development, making it a crucial plant growth hormone. According to Jangu and Sindhu (2011), IAA generated by bacteria influenced plant development and nodulation in green gramme (V. radiata) and black gramme (V. sativa) (V. mungo). Idris et al. (2007) discovered that B. amyloliquefaciens FZB42 affects plant development by producing IAA in a tryptophan-dependent way, while Mishra and Kumar (2012) looked at the plant growth enhancing and phytostimulatory capabilities of B. subtilis and B. amyloliquefaciens. Bacillus sp. JH 2-2 promotes mustard plant development by producing IAA, according to Shim et al. (2015). Many additional findings were also reported on the IAA generation activity of Bacillus sp. (Boiero et al. 2007; Ab Aziz et al. 2015; Meng et al. 2016). (Boiero et al. 2007; Ab Aziz et al. 2015; Meng et al. 2016). The importance of ACC deaminase in the control of the plant hormone ethylene and the improvement of plant growth and development was described by Glick (2005). In our research, we discovered that SJ-5 creates 19 g/ml IAA, which is a large amount. SJ-5 growth on DF medium reveals its ACC deaminase generating ability, which has previously been described for Bacillus and other PGPR such as Enteobacter cloacae, Klebsiella pneumonie sp., Pseudomonas sp. ACP, Pseudomonas putida strain UW4, and S. quinivorans SUD165 (Shah et al. 1998; Belimov et al. 2005). Bacterial isolate SJ-5 also boosted shoot and root length, dry weight, and lateral root number considerably. The positive actions of applied PGPR isolates, such as plant growth hormone synthesis, nitrogen fixation, and P solubilization, might be the cause of plant growth promotion.

Conclusion

This is a fundamental research that sheds light on the function of bacterial isolates in the stimulation of soybean plant development. These experiments demonstrate that the bacterium SJ-5 has all of the PGP qualities and may boost soybean plant development while also inhibiting fungal infections. This bacteria might be employed as an efficient PGPB in farmer's fields for soybean production. It is a cost-effective and environmentally beneficial technique.

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