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Characterization of Plant Growth Promoting Rhizobacteria and Their Benefits on Soybean Growth

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ABSTRACT

Background: Soybean is the most important legume in the world and its seeds contains 40% protein and 20% oil. In recent years, more attention has been paid to the use of plant growth-promoting (PGP) rhizobacteria as a biofertilizer alternative to chemical fertilizers, which may pose risks to the environment. The rhizobia inoculation of soybean is a sustainable practice to induce atmospheric nitrogen fixation and subsequently improve crop productivity and soil fertility.

Methods: In this study, 19 indigenous rhizobia isolated from soybean were tested *in vitro* for their plant growth-promoting properties (PGPR), while genotypic characterization included sequencing of the 16S rRNA gene.

Result: Morphological characterisation has shown that all of the isolates were rod-shaped gram negative bacteria. The sequencing has shown that 12 out of 19 isolates belong to the genus *Pseudomonas*, four of which belong to *P. fluorescens* species. Isolates which belong to the *Pseudomonas* genus have shown the highest ability of indole-3-acetic acid synthesis, phosphorous solubilization and along with isolates SGN6, SGN7 and SGS4 (*Sphingomonas sanguinis*) potassium solubilisation too. All the isolates from *Pseudomonas* genus as well as those belonging to *B. japonicum* species have shown the protease synthesis abilities while amylase synthesis abilities was observed only in the isolate SAK2 (*P. chlororaphis*). The most efficient strains in in vitro biological nitrogen fixation assay belong to *Pseudomonas* and *Rhizobium genera*. Most of the isolates were positive to the organic acid production while all of them have shown the ability of the exopolysaccharide production.

Key words: 16S rRNA, *In vitro* assay, PGPR, Phenotypic characterization, Soybean.

INTRODUCTION

Soybean (*Glycine max* L.) is one of the most important cultivated legumes in the world due to the chemical composition of its grain; high content of oil (18-22%), protein (17 to 42%), minerals (calcium, zinc and iron) and vitamins (Widawati, 2018). As a legume, soybean has the ability to fix atmospheric nitrogen through symbiosis with efficient root nodule bacteria. This capability provides significant amounts of available nitrogen, making soybean well fitted for crop rotations (Marinkovic *et al.,* 2018). Because of its importance, more research related to the production of soybean in different parts of the world is being conduct. This includes utilization of plant growth promoting rhizobacteria (PGPR) to increase soybean yield and to aid soybean adaptation in extreme environments. Application of PGPR in legume production has been mainly restricted to rhizobia manipulation in studies on increase legume growth and development, specifically by means of nodulation and nitrogen fixation. The main reason for that is because a broad range of soil-borne rhizobia species can establish symbiosis with legumes (Cooper, 2007). Plant growth promoting rhizobacteria (PGPR) include an ample variety of soil bacteria which, when grown in association with host plant stimulate the growth of their host by increasing mobility uptake and enrichment of nutrients in plant (Cakmakci *et al.*, 2006). PGPR also possess lots of mechanisms for the production and regulation of phytohormones and suppression of disease causing organisms (Ngoma *et al.,* 2012). These bacteria are used to sustain agriculture as biofertilizers and biocontrol agents (Babalola, 2010). Plant growth promotion occurs by the alteration of the whole microbial community in rhizospheric region through the production of various substances by PGPR (Vacheron *et al.* 2013). PGPR promote plant growth directly by either enhancing plant's nutritional status facilitating resource acquisition (nitrogen, phosphorus and essential minerals) and stimulating systemic disease resistance mechanisms or modulating plant hormone levels or indirectly by decreasing the inhibitory effects of various pathogens on plant growth and development in the forms of biocontrol agents (Yu *et al.*, 2016; Glick *et al.,* 2007).

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Several different studies have depicted proteobacteria especially those from family *Pseudomonadaceae* or *Burkholderiaceae* as dominant members of rhizosphere microflora (Wei *et al.,* 2017, Uroz *et al.,* 2010). Diverse symbiotic (*Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*) and non-symbiotic (*Pseudomonas*, *Bacillus*, *Klebsiella*, *Azotobacter*, *Azospirillum*, *Azomonas*) rhizobacteria are used worldwide as bio-inoculants in order to promote plant growth and development by various mechanisms, including nitrogen fixation, production of siderophores, solubilization of minerals such as phosphorus and synthesis of phytohormones (Compant *et al.,* 2010). Different PGPR can be administered to crops in some formulations that are commercially available (Lucy *et al.,* 2004) and recently, the popularity of microbial inoculants has substantially increased, facilitated by extensive and systematic research that has enhanced effectiveness and consistency (Berg, 2009). Understanding how plant roots select soil microbes to form the microbial community of the rhizosphere is an important scientific issue when considering the use of rhizobacteria as plant growth promoters (Drogue *et al.,* 2012).

According to Ferreira de Paula *et al.* (2021), the demand for PGPR biofertilizers has risen continuously due to the increasing importance of organic farming with minimal use of chemicals.The effects of the use of biofertilizers on plant development and soil quality are cumulative and long-lasting; they are also less harmful to the ecosystem than those of chemical fertilizers and pesticides.

The objective of work was to characterize bacterial isolates regarding their biological activity and growth promotion of soybean plants (*Glycine max* L.) grown in a controlled environment.

Materials and Methods

Isolation of bacterial strains

Soil samples were collected from the agricultural area of the Faculty of Agriculture (45° 82′ 56.36″ N, 16° 032 31.80″ E). In greenhouse pot experiment, surface sterilized soybean seeds (cultivar Gabriela) were sown directly into soil samples collected. Each experimental pot contained about 3 kg of soil sample. These plants were grown under controlled conditions; day temperature 26°C, night temperature 22°C, moisture was continiously 65%. Daylight period was setup for 16 h and night period for 8 h.

The soybean tissue was collected at the flowering stage. Ten healthy plants were carefully removed, washed under tap water to remove vermiculit and they were then separated into stems, roots and nodules. Stems and roots were cut into sections 2-3 cm long. The tissue was rinsed in 70% ethanol for 30 seconds and then sterilized with 3% NaClO 3 minutes for roots and nodules and 5 minutes for stems. The tissue was then washed ten times with sterile water (Hung and Annapurna, 2004). Surface-disinfected tissue was crushed with a sterile glass rod in a sterile test tube. One loop full of the nodule, stem or root content suspension was streaked on yeast mannitol agar (YEM) plates containing 0.0025% (w/v) Congo red. After incubation for 3 to 5 days at 28°C, single colonies were selected and restreaked on YEM agar for purity (Vincent 1970). Pure cultures were preserved in 20% glycerol at -20°C until further use. The study involved 19 selected isolates and this population was partially characterized on the basis of their morphology, 16S rRNA gene sequencing and screened for different PGP traits and biocontrol properties.

Screening of isolates for potential plant growth promoting properties

Phosphate solubilisation

Cells from each isolate in the LOG growth phase (10^8 CFU/ml) were spotted on Pikovskaya's agar (Bhutani *et al.* 2018). All the bacterial isolates were spot inoculated (15 µl bacterial culture previously grown on Yeast Mannitol Broth medium) at the centre of the Pikovskay agar plates and incubated for 5-6 days at 28°C. After incubation at 30°C a clear zone around the inoculation spot indicated positive result.

Organic acid production

For testing production of organic acids 24-h-old cultures produced in YMB were transferred to Methyl red - Voges-Proskauer (MR-VP) liquid medium (Senko *et al.,* 2024) and incubated for five days at 30°C on a shaker (150 rpm). After incubation for visualization of results Methyl red test according to Senko *et al.* (2024) was performed using 0.02% Methyl red solution.

Indole-acetic-acid (IAA) test

IAA production was detected as described by Sherpa *et al.* (2021). 1 ml of the overnight culture of bacteria inoculated to a 250 ml Erlenmeyer containing 100 ml nutrient broth fortified with L-tryptophan (0.1 mg/ml). After incubation in shaker incubator for 48 h at 30°C with 150 rpm (Biosan ES-20, Latvia), bacterial culture was centrifuged at 10000 rpm for 30 min. Then 2 ml of the supernatant was mixed with 2 ml Salkowski reagent (0.5 M $FeCl_3$ in 35% per chloric acid) and incubated for 30 min at room temperature in darkness. The optical density (OD) was recorded at 530 nm using a UV-VIS spectrophotometer (Lambda EZ 210, Perkin Elmer, USA) and the produced IAA was measured by standard curve graph.

N_2- fixation test

The capability of nitrogen fixation was checked by allowing the bacterial strains to grow in medium devoid of nitrogen. The qualitative estimation of N-fixation was checked using N-free Jensen's agar medium. Jensen's Medium is used for detection and cultivation of nitrogen fixing bacteria. Bacterial strains were streaked on Jensen's medium and incubated at 28±2°C for 4-5 days. After incubation plates were observed for the bacterial growth (Sherpa *et al.*, 2021). The growth of the milk colonies indicates a positive result.

EPS production

EPS producing activity of the bacterial isolates was done according to the method described by Jain *et al.* (2016).

Volume of 200 µl of overnight grown cultures of all bacterial isolates were inoculated in 100 ml of NB medium and incubated at 28±2°C for 72 h at 150 rpm in shaking incubator (Biosan ES-20, Latvia). After incubation, supernatant was collected with centrifugation at 12000 rpm for 10 min at 4°C and pre-chilled acetone was added to supernatant in 3:1 ratio. Formation of precipitation was considered as positive result for EPS production.

Screening bacterial isolates for hydrolytic enzyme production

Bacterial isolates were screened for their hydrolytic enzyme production like protease and amylase.

Bacterial isolates were screened for their ability to produce protease onto skim milk agar or SMA (3% v/v) medium. After 48 hours of incubation at 30°C, bacterial isolates with a clear halo zone on skim milk agar showed a positive result for protease synthesis (Tsegaye *et al.*, 2019).

Amylase production by bacterial isolates was determined using starch agar as described in Mir *et al.* (2021) and incubated at 30°C for 48 h. At the end of the incubation period, the plates were flooded with iodine solution. Iodine reacts with starch to form a blue color compound. Hence the colorless zone surrounding colonies indicates the production of amylase.

Molecular characterization of plant growth promoting bacteria

Total DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, USA) according to manufacturer's instructions. After extraction of genomic DNA, it was stored at -20°C for further studies. The 16S rRNA genes were polymerase chain reaction (PCR) amplified by using two universal bacterial primers fD1 (5′-CCGAATTCGTCGACAACA GAGTTTGATCC TGGCTCAG- 3′) and rD1 (5′- CCCGGGATC CAAGCTTAA GGAGGTGATCCAGCC- 3′) (Sikora and Redzepović, 2003). The reaction mixture with a total volume of 25 µl, is composed of bacterial DNA (100 ng), buffer, dNTP (100 pmol), TaKaRa Taq™ polymerase (1.25U) and sterile Milli-Q water. PCR conditions were: an initial cycle of denaturation at 95°C for 3 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min, 72°C for 1.5 min; and a final extension at 72°C for 10 min (Sikora and Redzepovic). The PCR products were checked by horizontal gel electrophoresis (1% w/v agarose) in Tris-Acetate-EDTA (TAE) buffer. The PCR products were visualised under UV illumination after staining with ethidium bromide and photographed with Cannon Powershot A640 camera. The PCR products were purified and sequenced by Macrogen (Seoul, South Korea) using an ABI3730 XL automatic DNA sequencer and the primers fD1 and rD1. A similarity search for so generated sequence was performed using National Centre of Biotechnology Information (NCBI) BLAST program. The phylogenetic tree was constructed by Neighbour-joining (NJ) method using software MEGA X.

Results and Discussion

Bacterial isolation

For the preliminary characterizations of potential PGPR, 18 pure bacterial colonies were selected and characterized for their morphological traits. All bacterial isolates were Gram negative rods.

Screening of isolates for potential growth promoting properties

Testing the phosphate solubilization ability, the results showed that two isolates (SAN1 and SAK2) were able to solubilizate phosphate. Both isolates belong to the genus *Pseudomonas*, one of the most potent phosphomobilizers in addition to the genera *Bacillus*, *Rhizobium*, *Enterobacter*, *Aspergillus* and *Penicillium* (Xiao *et al.,* 2011).

In a search for organic acids synthesize ability, it was found that seven isolates produced ring on the top of test tubes *i.e.* were able to synthesize organic acid. Glulati *et al.* (2010) demonstrated good results by application of *P. fluorescens* in soils with a high tricalcium phosphate content and concluded that the decomposition of phosphate was directly related to the production of organic acids.

Nitrogen fixation ability was demonstrated for 12 isolates belonging to the genera *Pseudomonas*, *Rhizobium* and *Bradyrhizobium*. All tested isolates had the ability to produce exopolysaccharides which is very important trait while inoculation of plants with exopolysaccharide-producing PGP bacteria results in decreased salt uptake and growth stimulation (Haggag *et al.,* 2014).

Different bacteria possess the ability to produce IAA. Ahmad *et al.* (2008) found that more than 80% of the tested *Azotobacter* and *Pseudomonas* strains as well 20% of the *Bacillus* strains synthesized indole-3-acetic acid. Kumar *et al.* (2012) found that PGPRs synthesize indole-3-acetic acid and other metabolically active substances which leads to an increase in root length, height of the shoot of the plant and the yield. The results in Table 1 show that all isolates have the ability to synthesize IAA. The amount of IAA produced ranges from 6.83-95.30 µg ml^{-1}. Isolates SGS1 and SAN1 showed the highest concentration of IAA produced.

Screening bacterial isolates for hydrolytic enzyme production

The results in Table 2 show that 63% of the isolates exhibit protease synthesis capability, which was evident as the clear zone was formed around the colony. These isolates could play a significant role in the biocontrol of a large number of plant pathogens such as *Botrytis cinerea*, *Pythium ultimum*, *Fusarium oxysporum*, *Phytophthora* spp., *Sclerotium rolfsii*, *Rhizoctonia solani* (Glick, 2012). The ability to synthesize amylase was demonstrated only by the SAK2 isolate belonging to *P. chlororaphis*.

The obtained results present valuable data about strains which should further be analyzed in order to replace

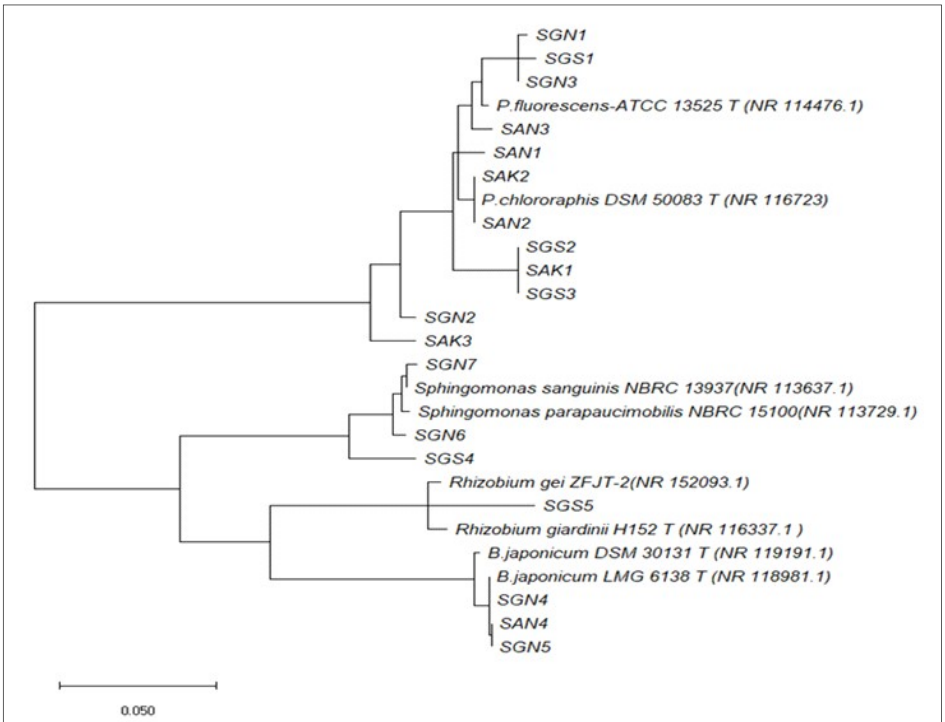


Fig 1: Phylogenetic tree showing the relationship among 19 bacterial isolates of soybean endophytes, 16S rRNA gene sequences with reference sequences obtained through BLASTn analysis.

Table 1: Plant growth promoting characteristics of bacterial isolates.

	Plant growth promoting properties				
Bacterial strains	Phosphate solubilization (+/-)	EPS production (µg/ml)	IAA production (+/-)	N_2 fixation (+/-)	Organic acid production (+/-)
SGN1		+	31.30	+	-
SGN2	-	+	32.90	-	+
SGN3	-	+	25.20	+	-
SGN4	-	+	20.90	+	-
SGN5	-	+	18.30	+	-
SGN6	-	+	19.20	-	+
SGN7	-	+	15.16	-	-
SGS1	-	+	95.30	-	+
SGS2	-	+	21.40	-	+
SGS3	-	+	26.56	+	+
SGS4	-	+	19.25	+	+
SGS5	-	+	30.30	+	-
SAN1	+	+	84.26	+	+
SAN2	-	+	68.46	+	+
SAN3	-	+	54.53	-	-
SAN4	-	+	15.06	+	-
SAK1	-	+	21.66	-	-
SAK2	+	+	29.50	+	-
SAK3	-	+	24.86	+	-

Table 2: Biochemical caracterization of plant growth promoting bacterial isolate.

	Hydrolytic enzime production	
Bacterial strains	Protease (+/-)	Amylase (+/-)
SGN1	+	-
SGN2	+	-
SGN3	+	-
SGN4	+	-
SGN5	+	-
SGN6	-	-
SGN7	-	-
SGS1	-	-
SGS2	+	-
SGS3	+	-
SGS4	-	-
SGS5	-	-
SAN1	-	-
SAN2	+	-
SAN3	+	-
SAN4	-	-
SAK1	+	-
SAK2	+	+
SAK3	+	-

environmentally harmful mineral fertilizers. Among them, two strains deserve special attention; SAN1 for its ability to produce indole-3-acetic acid, organic acids and to solubilize phosphates and SAK2 which was also able to solubilize phosphates, fix nitrogen and demonstrated protease activity.

Molecular characterization of plant growth promoting bacteria

According to the results of 16S rRNA gene sequencing (Fig 1), 12 of 19 isolates (63%) belong to the genus *Pseudomonas,* four of which were identified as *P. fluorescens* (21%) and eight isolates (42%) belong to *P. chlororaphis* species. Three out of 19 isolates (16%) were identified as *B. japonicum* and three as *S. sanguinis*. Only one isolate (0.05%) belongs to the genus *Rhizobium* but it was not possible to identify it at species level only by sequencing 16S rRNA gene.

Conclusion

Inoculation with rhizobacteria to promote soybean growth is useful because they efficiently mineralize and make nutrients available. In addition, many of them have an antagonistic effect against pathogens and produce phytohormones for growth. The isolates SAN1, SAN2 and SAK2 were highlighted for all PGPR traits and show biotechnological potential. However, the efficiency and biotechnological potential of these bacterial isolates should be confirmed in the field to evaluate the effects of these microorganisms in combination with biotic and abiotic factors.

Conflict of interest

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educatioanl grants; participation in speakers' bureaus; membership, empolayment, consultancies, stock ownership, on other equity interest; and expert testimony or petent-licensing arrangements), or non-financial interest (such as personal or professional relationship, affiliation, knowledge or beliefs) in the subject matter or meterials discussed in this manuscript.

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