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Isolation, identification of 16S rRNA gene sequences analysis and co-inoculation of *Pseudomonas fluorescens* (LNPF1) and *Bacillus subtilis* (LSBS2) for Plant Growth Promotion and Nutrient Fortification in *Arachis hypogaea*

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Abstract In the Salem area of Tamil Nadu, India, rhizospheres of agricultural soil were found to contain rhizobacteria that promote plant development. These isolates were recognised as *Pseudomonas fluorescens* (LNPF1) and *Bacillus subtilis* (LSBS2) based on their biochemical traits and 16S rRNA gene sequence analysis. The PGP activity, phosphate solubilisation, IAA production, HCN production, ammonium production, and siderophore production of both isolates were examined. Furthermore, in pot experiment was conducted with *Arachis hypogaea* resulted to be the highest yield parameter in co-inoculation of LNPF1+LSBS2 significantly increased in shoot length 39.16±0.37, root length 31.13±0.55, fresh weight 12.5±0.30 dry weight 7.03±0.15, total nitrogen 19.33±0.64 mg N/g , chlorophyll 0.479±0.58 mg/g and carotenoids content 0.573±0.01 mg/g in treated plantsd. Biomass accumulation of root nodules were observed the highest number in LNPF1+LSBS2 53.00±1.00 nodules fresh weight, and dry weight also increased. Nitrogenase activity was more in LNPF1+LSBS2 17.42±1.15 n moles. Leghemoglobin assay with inoculation of LNPF1+LSBS2 1.70mg/g was comparable to those un-inoculated plants of 1.16mg/g. Nutrients content also increased in protein of 15.00±0.13%, carbohydrate 24.75±0.98%, fat 47.69±0.15%, fiber 15.40±0.31%, iron 7.36±0.5mg/g, calcium 16.63±0.20mg/g, and energy 563Kcal when compared to that uninoculated plants. Total seed oil content, bioinoculant-treated plants with 50.06±0.42% un-inoculated plants which found to be 34.93±0.56 % of oil content. Therefore, the strain LNPF1 (*Pseudomonas*) and LSBS2 (*Bacillus*) could be exploited as a potential bioinoculant for plant growth and increased in plant nutrients, oil yielding content, and increased in iron content of *Arachis hypogaea.*

Keywords: Plant growth, Inoculation, *Arachis hypogaea,* Yield parameter

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Introduction

PGPR, as opposed to synthetic fertilisers and pesticides, are known to improve plant development in a variety of ways. They promoted crop growth and can support crop yield and the sustainability of protected areas. Many PGPR community groups may be found in the rhizospheric soil, and these groups have positive effects on crop productivity. Numerous studies are conducted to better understand the dynamics, variety, and importance of soil PGPR populations as well as their suggested and collaborative roles in agricultural production. *Pseudomonas, Azospirillum, Azotobacter, Bacillus, Burkholdaria, Enterobacter, Rhizobium, Erwinia, Mycobacterium, Mesorhizobium,* and *Flavobacterium* are a few PGPR species that often display plant growth promotion activity (Singh, 2013). Between them, *Bacillus* and *Pseudomonas* are better at organic mineralization and assimilating phosphate so that plants can use it, increasing agricultural output (Kaur *et al*., 2016).

Activities that promote plant development include the synthesis of ammonium, siderophores, hydrogen cyanide (HCN), phosphate solubilization, and indole acetic acid (IAA). Through the mobilisation of nutrients in soil, the production of different plant growth regulators, and the inhibition or control of improving soil structure, they protect plants against phytopathogens and promote plant development (Nithyapriya *et al.,* 2021).The most important element for plant nutrition is phosphorus. It is essential to all of the main metabolic functions that plants carry out, including respiration, photosynthesis, sign transduction, energy transfer, and macromolecular biosynthesis (Khan *et al.,* 2010). Only the monobasic and dibasic ions—two soluble forms of phosphate—are absorbed by plants (Bhattacharyya and Jha, 2012).

IAA is an essential plant growth regulator that helps plants defend themselves against a variety of phytopathogenic microbes. (Spaepen and Vanderleyden, 2011). IAA contributes to an increase in the amount of root exudation, which gives rhizosphere microorganisms more nutrients to sustain their development (Glick, 2012). Tryptophan has been identified as a biosynthetic pathway in microorganisms, and IAA play a crucial role in this process (Kamilova *et al.,* 2006; Shilev, 2013).

The well-known secondary metabolite hydrogen cyanide, which is generated by Pseudomonas fluorescens, plays a function in suppressing infections that are carried by soil. Plant diseases are biologically controlled by a large number of rhizospheric bacteria that are generated by HCN (Kesawlya, 2015). By increasing the amount of iron in the soil around the roots, PGPR can release a siderophore that helps plants overcome their iron deficiency and develop more rapidly (Vejan *et al*., 2016).

The *Pseudomonas fluorescens* group includes many species, including *P. brassicacearum, P. protegens, P. chlororaphis,* and *P. fluorescens*. These species are classified as PGPR because of their ability to use aggressive tissue colonisation and metabolite reorganisation to inhibit plant illnesses caused by pathogens. (Garrido-Sanz *et al*., 2016). Because *Pseudomonas fluorescens* is widely present in soil and has high rhizosphere colonisation capabilities, it has been the subject of several PGPR experiments (Novinscak and Filion, 2019). In addition to producing siderophores, phytohormones, and antibiotics, *Pseudomonas fluorescens* may also actively compete with other microbes (Santoro *et al.,* 2015).

The formation of IAA, phosphate solubilization, nitrogen fixation, and biocontrol of phytopathogens such as HCN, siderophores, hydrolytic enzymes, and antibiotics are among the effective growth-protective characteristics of *Bacillus* species (Wang *et al.,* 2018). Previous studies have demonstrated that PGPR contributes to biocontrol in *Bacillus* species against *Fusarium,* which is linked to plant root rot disease (Singh, 2013). *Bacillus* species are rod-shaped bacteria that are ubiquitous in nature and members of the phylum Firmicutes. They can be facultative anaerobes, meaning they can be either aerobic or anaerobic, or obligatory aerobes, meaning they need oxygen to survive. Research has shown that B. subtilis is a good model (Khan and Bano, 2016).

Arachis hypogaea L., a member of the *Fabaceae* family, is also known as the poor man's nut. It is cultivated as the nation's first oil seed plant and is recognised by Indians as a vegetable oil seed crop. Globally, 26.4 million hectares are used for peanut farming, and 39.46 million metric tonnes are produced annually. An important oilseed crops those accounts for 45–50% of India's oil output is *Arachis hypogaea.* High-quality edible oil extraction accounts for almost half of the world's groundnut production; the other 30% is used to make confections, and the remaining 20% is used to produce feed and seeds (Dwivedi *et al*., 2003). The most significant of these peanut illnesses, including *Fusarium oxysporum's* leaf blight, are caused by fungus. This fungus can afflict seedlings as well as leaves, roots, fruits, and seeds. (Sobolev *et al*., 2018). Therefore, a greenhouse experiment was conducted with *Arachis hypogaea* L. plant. The objectives were done as isolation and characterization of *Pseudomonas* and *Bacillus* ,. molecular identification of bacteria, pot culture studies on *Arachis hypogaea* L. and post-harvest studies.

Materials and methods

Gram staining test

A sterile slide was filled with a thin layer of bacterial culture, which was then heated to fix it. One drop of crystal violet per minute was used to cleanse the slide under running water. After adding gramme iodine and waiting two minutes, 95% ethyl alcohol was used to colour the strain. After adding a few drops of safranine and washing the slide with tap water for two to three minutes, the slide was mounted under a microscope (Vincent, 1970).

Test of indoleproduction

A test tube was filled with 5 ml of bacterial culture, 2 ml of peptone water, and the mixture was cultured for 48 hours. In a test tube, one millilitre of Kovac's solution was then added. After 10 minutes, the tube developed a red colour on top of the test tubes, indicating that indole was being produced. Negative evidence that they did not develop a red colour (Morello *et al.,* 2002).

Test for methyl red

By dissolving 0.1 mg of bacto methyl red in 100 millilitres of 95% alcohol and diluting with 100 millilitres of distilled water, the phenyl red solution was created. A 0.5ml sterile glucose phosphate broth and a bacterial culture were inoculated to conduct the test. One drop of methyl red solution was added after a 24hour. The outcomes demonstrate that the methyl red test was positive when a bright red colour appeared.

Test for vogas proskauver

2 ml of sterile glucose phosphate peptone water was added and incubated culture tube at 48 hours at 37°C next added to 3ml of sodium hydroxide well shaken, and allowed to incubate at room temperature. It was absorbed the present of pink colour the culture was positive and negative in case they do not form pink colour.

Test for citrate utilization

Simmons citrate agar slants was streaked with the bacteria and incubated at 30°C for 42h and colour changes on slants were recorded. After incubation the isolate was development of deep blue colour indicates a positive result.

Starch hydrolysis test

The isolated bacterial culture was inoculated on starch agar plates and incubated at 30°C for 48h. After the growth of bacterial culture, it was flooded with iodine solution using dropper for 30sec. Where the positive results showed clear zones, which were formed around the bacterial culture.

Catalase activity

In catalase test 48h bacteria culture were placed on a clean slide and one drop of hydrogen peroxide was added, after 5 min bubbles were formed. The results showed that positive for catalase test (Kumar *et al.,* 2012).

Oxidase production

The isolated bacteria were streaked on nutrient agar and kings B medium. Plates were incubated for 3 days at 28ºC. After incubation a loopful of culture was placed on oxidase disc development of blue colour oxidase production was positive.

Test for urease activity

Bacterial culture was streaked on urea agar slants and incubated for 3 days at 28ºC. Positive results show that bright pink colour was formed in negative results they didn't form pink colour (Mac Faddin, 2000).

Antibiotic susceptibility test

The antibiotics were examined based on the inhibitory zone widths, which were documented in relation to clinical microorganisms (Bauer *et al.,* 1996). The following discs were employed as antibiotics: Chloromphencol$^{-25,}$ Chloromphencol-^{50}, and streptomycin-^{25}. Following a three-day incubation period at 37°C, antibiotic discs (three discs per plate) were dispensed onto the plates using a dispenser.

Production for Hydrogen Cyanide

The isolated bacteria were streaked on nutrient agar plates and kings B medium. After that Whatman filter paper was soaked in 0.5% picric acid and 1% Sodium carbonate and were placed in the upper lid on a plate. The plates were incubated at 28 °C for 48h. The results showed that development of yellow to light brown colour, moderate brown or strong brown.

Production for Ammonia

After being injected with 10 millilitres of peptone water, a freshly produced culture was cultured for 72 hours at 28°C. In the test tube culture, 0.5 ml of Nessler's reagent was then introduced. Ammonia production is positively correlated with the brown to yellow colour development (Cappuccino and Sherman, 2002).

Production for Indole Acetic Acid

For the IAA performing cultures, tryptophan was utilised. The cultures were centrifuged for 30 minutes at 3000 rpm, and the cell-free supernatant was combined with 2-3 drops of orthophosphoric acid and 4 millilitres of Salkowski's reagent. They spent 35 minutes being incubated at 28°C. Following the formation of a pink hue, there was a favourable development in the production of IAA (Ahmad *et al.,* 2005).

Production for Ammonia

After being injected with 10 millilitres of peptone water, a freshly produced culture was cultured for 72 hours at 28°C. In the test tube culture, 0.5 ml of Nessler's reagent was then introduced. Ammonia production is positively correlated with the brown to yellow colour development (Cappuccino and Sherman, 2002).

Phosphate solubilisation

Pikovskayas agar plates were injected with cultures of isolated bacteria. For three days, the plates were incubated at 28°C. Following that, a clear zone began to form surrounding the colonies as a result of their production of organic acid, which solubilised inorganic phosphate (Sharma *et al.,* 2013).

Production of siderophore

For the qualitative examination, the Chrome Azurol Sulfonate (CAS) test (Schwyn and Neilands, 1987) was employed. The cultures were placed on the blue agar, spot-inoculated, and then incubated for 24 to 48 hours at 37 °C. Results were seen on a culture that was actively developing when orange-halo zones appeared after a 72-hour incubation period at 28°C.

16S rRNA gene identification and sequencing

Through polymerase chain reaction amplification of the 16S RNA gene, the possible isolates were molecularly characterised. Using the isolated DNA and universal primers 27 F (5'-AGA GTT TGATCM TGG CTCA-3') and 1492 R (5'-GGTTACCTTGTTACGACG ACT T-3'), the 16S rRNA gene sequence of the bacterium was amplified.Following the purification of the PCR products, the sequence was assembled using homology, which was verified using the BLASTN tool. The National Centre for Biotechnology Information (NCBI) has access to the sequencing data that was collected (Tamura *et al*., 2013).

Pot studies

After being submerged in water for the whole night, *Arachis hypogaea* seeds were triple-washed, surface-sterilized for five minutes using 0.1% mercury chloride, and then allowed to air dry at ambient temperature. After carefully combining sterilised garden soil, sand, and manure in a 2:1:1 ratio, the bioinoculant—100 ml of LPNF1 and LSBS2 (1 x 108 CFU ml-1)—was kept at room temperature for subsequent use. Following that, the ten sterile seeds were placed in an earthen pot of 12 to 15 cm in diameter, which was then filled with garden soil that had been treated with bioinoculant and sand (2:1) w/w, as was previously indicated. In a greenhouse, each treated pot was arranged on a slab. One application of bacterial suspension (25 ml per plant in pot assays 200) was given to each plant. directly into the ground in the vicinity of the roots both before and after flowering and during germination. The plants' fresh weight, dry weight, and root and shoot lengths were measured after harvest. The roots and shoots were separated, then dried in an oven at 68 ± 2 °C for 48 hours before being weighed. Four pots, each holding three seedlings, made up each treatment.

Estimation of photosynthetic pigments

Ninety days after planting, the *Arachis hypogaea* leaves from the treated seedlings were taken, and the photosynthetic pigment content was determined using the Wellburn technique. The total carotenoids and the levels of chlorophyll a and chlorophyll b were then determined.

Arachis hypogaea nodules number (nodule/plant)

Using a digging fork, plants from each treatment that still had their roots whole were removed. After the root containing root nodules was carefully

removed from the soil and completely cleaned, the fresh and dried weight of the nodules as well as the number of nodules per plant were counted and recorded.

The activity of nitrogenase in root nodules

Conveniently sized glass tubes were used for the assay, and they were subsealed to make it possible for a hypodermic needle containing a syringe to penetrate them. 10% of the air in the sample container was removed, and the same amount of acetylene gas was added back in. To find the ethylene gas generated, 0.5 cc of gas was extracted after an hour using a syringe and quickly injected into a gas chromatograph fitted with a flame ionisation detector. The outcomes were reported in ethylene nanomoles that were generated (Hardy and associates, 1968).

Leghaemoglobin content of root nodules

In a microphage tube, 50–100 mg of root nodules was gathered and crushed in nine litres of Drabkins solutions. The tubes were centrifuged for 15 minutes at 12,000 rpm.Using a 0.2 µm syringe filter, the supernatant was filtered. In a spectrophotometer set to measure absorbance at 540 nm, the filter was placed in a micro cuvette (Wilson and Reisenauer, 1962).

Nutrient estimation and seed analysis

From bioinoculant-treated and untreated control plants, Arachis hypogaea seeds were collected and allowed to cool at room temperature. After being finely ground, the dry seed samples were treated with 25 millilitres of strong sulphuric acid. After being digested, the residue was poured into a volumetric flask and filled with deionised water to reach a volume of 50 ml. Once the concentrated digests were diluted with deionised water, they were analysed for total fat, carbohydrate, protein, energy, calcium, and iron content. An atomic absorption spectrophotometer was used to record the concentration.

Determination of oil content in seeds

In order to estimate the oil content of peanut seeds, 100 g of dried, powdered seeds were first extracted using chloroform, and then the oil was extracted using a Sokule apparatus. The following method was then used to calculate the oil content as a percentage:

Ether extract (%) is equal to (flask weight plus extract) - sample weight × 100 - flask weight

Results

Gram staining

The LNPF1 bacterial isolate has weak cell walls. The pink rod-shaped, motile pigment produced by the cells indicates to their gram-negative nature (Figure 1b). Because of the gram-positive bacterium's thick layer of cell wall, LSBS2 cells have a purple (Figure 2b). The bacterial isolates biochemical characteristics are listed in (Table 1).

Table 1. Biochemical identification of bacterial isolation

S. NO	Test	LNPF1	LSBS2
1	Gram staining	-	+
2	Indole production test	+	+
3	Methyl Red test	+	-
4	Voges-proskaur test	_	+
5	Citrate utilization test	+	+
6	Starch hydrolysis test	+	+
7	Catalase activity	+	+
8	Oxidase production	+	+
9	Urea’s activity	+	-
10	Hydrogen Cyanide production	+	+
11	Ammonium production	+	+
12	Indole Acetic Acid production	+	+
13	Phosphate solubilisation	+	+
14	Siderophore production	+	+

Note:(+) Positive response, (-) Negative response

Indole production test

The purpose of the indole production test was to determine whether any particular species of bacteria could create indole. Following incubation, a few drops of Kovac's reagent were applied; this produced a hazy, red colour zone. Due to the conversion of the amino acid tryptophan into indole, LNPF1 had a positive response to the indole test (Figure 1c), but LSBS2 displayed a negative reaction since no red colour was generated (Figure 2c).

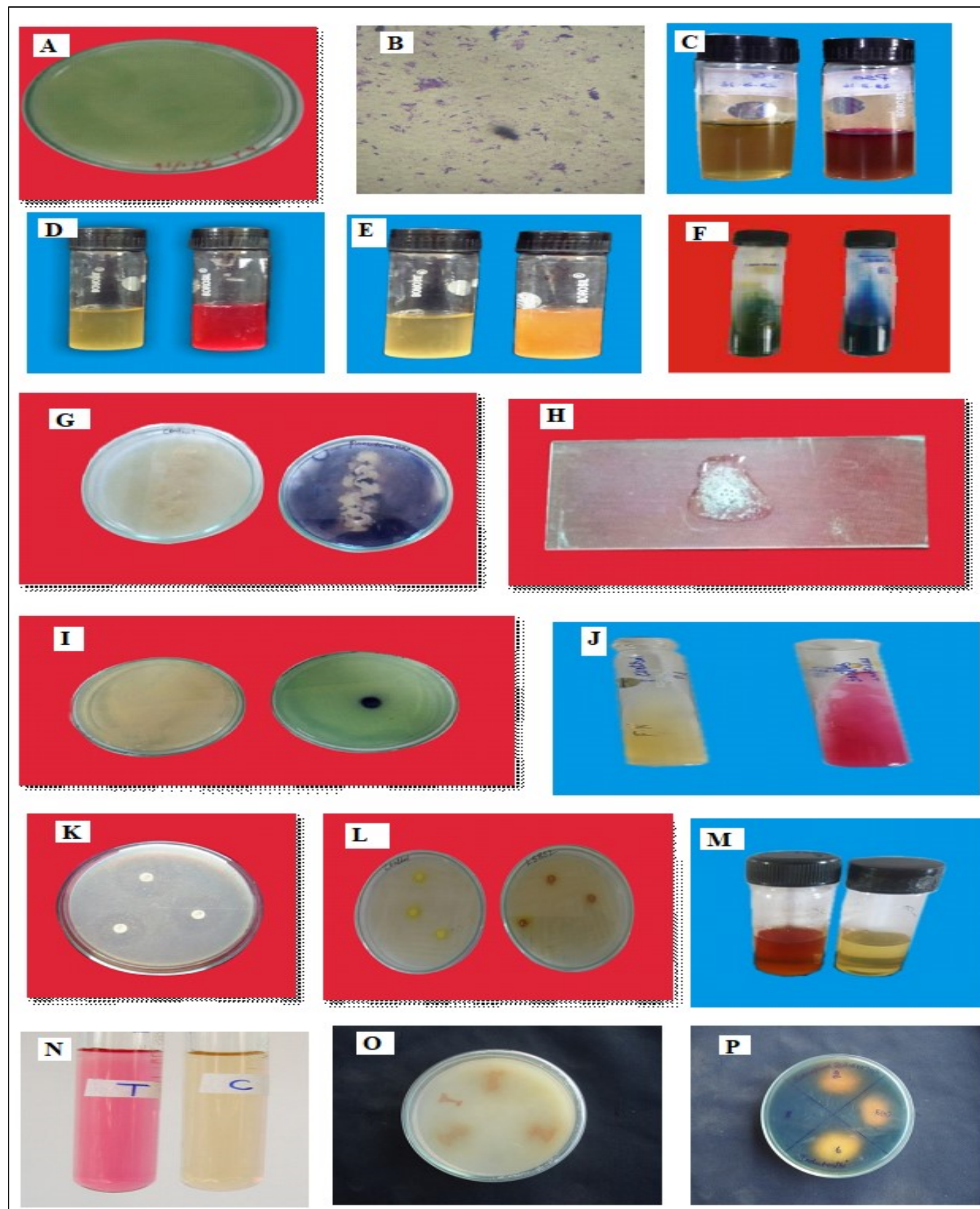

Figure 1. Biochemical characterization of LNPF1. **A.** *Pseudomonas fluorescences* **B.** Gram staining **C.** Indole production **D.** Methyl red **E.** Vogas Proskauver **F.** Citrate Utilization **G.** Starch hydrolysis **H.** Catalase test **I.** Oxidase test **J.** Urease production **K.** Antibbiotic susceptibility **L.** Hydrogen Cyanide **M.** Ammonia production **N.** Indole Acetic Acid **O.** Phosphate solubilization **P.** Siderophore production

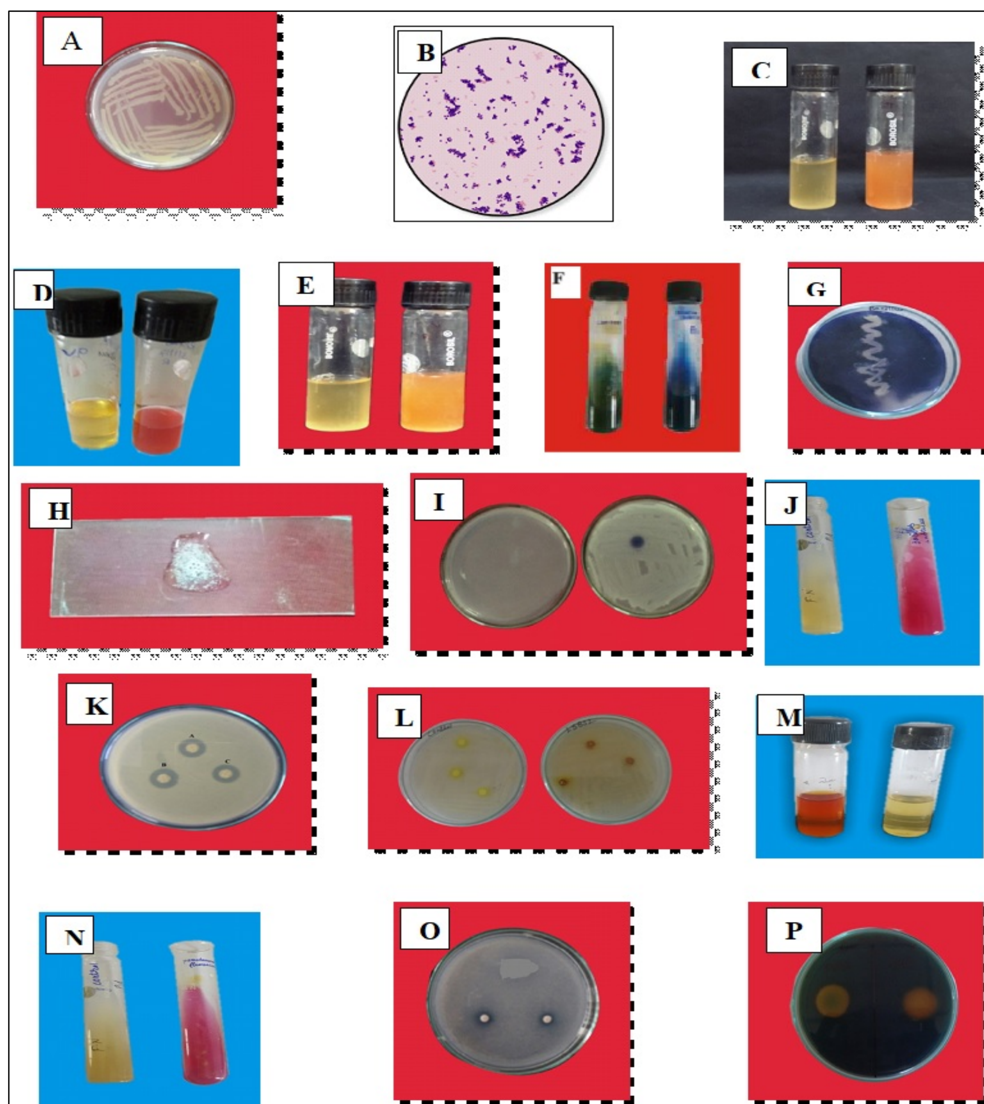

Figure 2. Biochemical characterization of LSBS2. **A.** *Bacillus subtilis* **B.** Gram staining **C.** Indole production **D.** Methyl red **E.** Vogas Proskauver **F.** Citrate Utilization **G.** Starch hydrolysis **H.** Catalase test **I.** Oxidase test **J.** Urease production **K.** Antibbiotic susceptibility **L.** Hydrogen Cyanide **M.** Ammonia production **N.** Indole Acetic Acid **O.** Phosphate solubilization **P.** Siderophore production

Methyl red test

To determine how well the bacteria were doing acid fermentation, a methyl red test was used. The test tube's bright red colour revealed LNPF1 (Figure 1b). and LSBS2 showed positive methyl red. Because acid fermentation involves extremely high acidity, methyl red was employed as a pH indicator. (Figure 2d).

Vogas proskauver test

Alpha-naphthol and potassium hydroxide added to a broth in which acetyl methyl carbinol is present. LSBS2 resulted in pink colour which indicates that it was positive (Figure 2e) and absence of pink colour in LNPF1 indicates it to be negative (Figure 1e).

Citrate utilization test

The purpose of the test on citrate utilisation was to evaluate the material's potential for use as a carbon and energy source. Following incubation, the kind of the bacterial culture turned intensely blue, indicating that LNPF1 and LSBS2 were citrate positive. (Figures 1 and 2 f).

Citrate = oxaloacetate + acetate

Starch hydrolysis test

The isolated bacteria LNPF1 and LSBS2 was inoculated on starch agar plates, after the growth of bacteria it was flooded with iodine solution. The appearance of clear zones around the line positive for LNPF1 and LSBS2. (Figures 1 and 2g).

Catalase test

To determine catalase test LNPF1 and LSBS2 showed bubbles, the reasons for the bubbles formation was presence of catalase enzymes which resulted in breakdown of hydrogen peroxide (H_2O_2) into water and oxygen (Figures 1 and 2h).

$$2H_2O_2 + \text{catalase} \rightarrow 2H_2O + O_2$$

Oxidase test

Isolates of LNPF1 and LSBS2 immediately turned to dark purple which indicated oxidase positive (Figures 1 and 2i).

Urease test

To find out whether bacteria could break down urea into ammonia and carbon dioxide, urease tests were performed. After incubation the cultures in the test tubes appeared pink in colour which indicates that LNPF1 was urease positive (Figure 1j) and LSBS2 was not appeared in pink colour negative for urease test (Figure 2j).

$$(NH_2)_2\,CO + H_20 \rightarrow CO_2 + 2NH_3$$

Antibiotic susceptibility test

It has been effectively utilised to assess elite strains' competitiveness with native strains using antibiotic resistance in LNPF1, LSBS2. Chloramphenicol-25, Chloramphenicol-50, and streptomycin-25. Streptomycin-25 was the largest inhibitory zone diameter that LNPF1 displayed (Figures 1 and 2k).

HCN production (Hydrogen Cyanide)

The production of hydrogen cyanide by the isolates of LNPF1 and LSBS2 caused the filter paper to change colour from yellow to a mild brown. The filter paper's rich brown tint provided a strong indicator when glycine and $FeCl_3$ were present. (Figures 1 and 2 l).

Ammonia production

LNPF1 and LSBS2 isolates showed production of ammonia in peptone water. When 0.5ml Nessler's reagent was added in each tube containing LNPF1 and LSBS2, showed development of brown to yellow colour. Hence these isolates showed positive reaction for ammonia production (Figures 1 and 2 m).

Indole Acetic Acid

Determination of the IAA production by LNPF1 and LSBS2 was useful in its identification and acts as a helpful indicator to investigate the physiological roles or ecological significance of IAA in the group of the organism.1ml of salkowski's reagent was added with supernatant produced pink color showed the presence of IAA (Figures 1 and 2 n).

Phosphate solubilization

Phosphate solubilizing ability in pikovskaya agar showed clear zones around the colonies of LNPF1 and LSBS2 isolates. The zone of clearance which is directly proportional to the phosphate solubilizing efficiency was measured in pikovskaya agar and it ranged from 0.1 to 0.9mm. The highest phosphate solubilizing activity was shown by the isolates LNPF1 with a zone of clearance 0.5mm (Figure 1o).

Siderophore production

In CAS assay LNPF1& LSBS2 (Figure 2k) showed positive (orange colour zone) for siderophore production. The strain LNPF1 produced maximum of 10mm sized orange colour zone in CAS plate (Figure 1p). CAS plates are blue in colour due to the presence of chrome azurol S dye which is compound with ferric iron. With the presence of siderophore following reaction occurs which shows orange colour.

Molecular identification of 16S rRNA gene sequencing

Following a biochemical test and 16s r RNA sequencing analysis, the sequence showed 96.3 to 97.2% identity with the GenBank-available sequences of *Bacillus subtilis* and *Pseudomonas fluorescens.* The recently isolated LNPF1, accession number MK478897 (GenBank) (Figure 3). LSBS2 were included in the GenBank with accession number MK4832621 (Figure 4). These sequences were compared to the GenBank database's sequences of many PGPR belonging to various taxa and species within those genera.

Studies conducted after harvest on Arachis hypogaea (90DAI)

In the pot experiment, the inoculation of LNPF1+LSBS2 resulted in the greatest yield parameters (Figure 5), with a substantial increase in shoot length of 39.16±0.37 and root length of 31.13±0.55, respectively, as compared to uninoculated control plants. In LNPF1+LSBS2, plant fresh weight rose considerably to 12.5±0.30. In comparison to the uninoculated control plant, the dry weight also rose by 7.03±0.15, indicating a substantial increase (Table 2).

Photosynthetic pigments

When compared to control plants, the total chlorophyll content and carotenoids content in the leaves of LNPF1+LSBS2 inoculation rose considerably (0.479±0.58 mg/g and 0.573±0.01 mg/g, respectively). The increase in nitrogen accumulation was used to determine the plant nitrogen

content, and it was significantly higher in LNPF1+LSBS2 (19.33±0.64 mg N/g dry plant) than in control plants (Table 3).

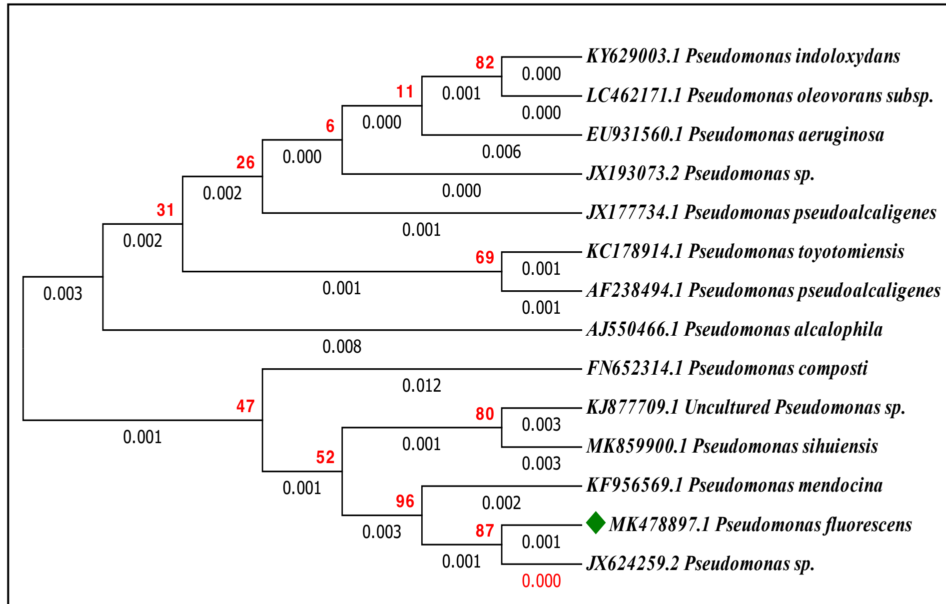

Figure 3. Phylogenetic tree on LNPF1 strains were submitted Genbank under the Accession number MK483262

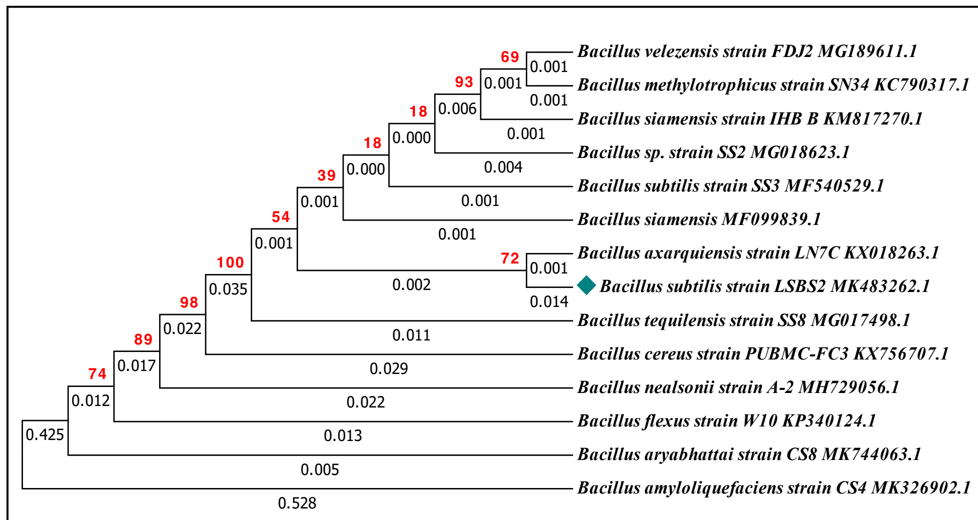

Figure 4. Phylogenetic tree on LSBS2 strains were submitted Genbank under the Accession number MK478897

Figure 5. Pot culture studies on *Arachis hypogaea* L.

Table 2. Post- harvest studies on *Arachis hypogaea* L. (90 DAI)

Treatment	**Shoot length cm**	**Root length cm**	**Fresh weight g**	**Dry weight g**
	Mean± SD	Mean± SD	Mean± SD	Mean± SD
Control	27.50±0.50	15.33± 0.20	6.50 ± 0.30	3.43± 0.15
LNPF1	37.33±0.7	31.30± 0.36	10.60± 0.30	5.43± 0.15
LSBS2	36.36±0.35	30.00± 0.40	9.7± 0.25	4.46±0.15
LNPF1+LSBS2	39.16±0.37	31.13± 0.55	12.5± 0.30	7.03± 0.15
F values	294.144***	259.300***	227.785***	301.179***

Note: The mean of three replicates ± the standard deviation is used to represent the data. *, **, *** signify the extent of significance LSD ($P < 0.05$) in the significant difference.

Table 3. Photosynthetic pigment and total nitrogen content on *Arachis hypogaea*

Treatment	Chlorophyll mg g^{-1}	Carotenoids mg g^{-1}	Total nitrogen content mg N/g dry plant
Control	0.046±0.01	0.019±0.00	6.7 ± 0.62
LNPF1	0.140±0.20	0.305±0.22	23.6± 1.48
LSBS2	0.112±0.05	0.231±0.17	28.15± 0.90
LNPF1+LSBS2	0.479±0.58	0.573±0.01	19.33± 0.64
F values	1.334**	7.831**	267.807 ***

Note: The mean of three replicates ± the standard deviation is used to represent the data. *, **, *** signify the extent of significance LSD ($P < 0.05$) in the significant difference.

Nodules number of Arachis hypogaea(nodule/plant)

The LNPF1+LSBS2 plants exhibited the greatest quantity of root nodules (53.00±1.00), and the fresh and dry weight of the nodules increased upon inoculation compared to the uninoculated plants (Table 4).

Table 4. Biomass growth of *Arachis hypogaea* L. (90 DAI)

Treatment	Nodules number /plant	Nodules fresh weight g	Nodules dry weight g
Control	20.00 ±2.00	0.006±0.002	0.003 ±0.000
LNPF1	48.00 ± 1.00	0.26±0.015	0.138 ±0.003
LSBS2	41.33 ± 2.08	0.17±0.015	0.092 ±0.045
LNPF1+LSBS2	53.00±1.00	0.30±0.010	0.155 ±0.002
F values	273.598***	369.140***	614.937 ***

Note: The mean of three replicates ± the standard deviation is used to represent the data. *, **, *** signify the extent of significance LSD ($P < 0.05$) in the significant difference.

Nitrogenase and Leghemoglobin content

Nodule nitrogenase activity was greater in LNPF1+LSBS2 (17.42±1.15 n moles C_2H_4 produced / h / g fresh nodules). *Arachis hypogaea* L. was found to have a high concentration in the LNPF1+LSBS2 inoculation (1.70 mg/g), which is equal to the 1.16 mg/g of uninoculated plants, according to the leghemoglobin test absorbance value of 540 nm (Table 5).

Table 5. Nitrogenase activity and leghemoglobin content of root nodules

Treatment	**Nitrogenase activity (n. moles C_2H_4 formed / h / g fresh nodules)**	**Leghaemoglobin content (mg/g)**
Control	5.43±1.15	1.16±0.57
LNPF1	12.66 ± 0.87	1.56 ± 0.57
LSBS2	9.80 ± 0.23	1.40 ± 0.10
LNPF1+LSBS2	17.42±1.15	1.70±0.10
F values	87.202***	23.792***

Note: The mean of three replicates ± the standard deviation is used to represent the data. *, **, *** signify the extent of significance LSD ($P < 0.05$) in the significant difference.

Nutrient analysis

After applying the bioinoculant, the *Arachis hypogaea* L plants significantly outperformed the control plants in terms of energy (563 Kcal), protein (15.00±0.13%), carbohydrate (24.75±0.98%), fat (47.69±0.15%), fibre (15.40±0.31%), iron (7.36±0.5 mg/g), calcium (16.63±0.20 mg/g), and energy (47.69±0.15%).

Total seed oil content

Examining the overall oil content of the seeds, it can be shown that the percentage of oil content increased from 50.06±0.42% in seeds collected from plants treated with bioinoculant to 34.93±0.56% in seeds acquired from untreated plants (Table 6).

Table 6. Nutrients analysis of *Arachis hypogaea* L seed (90 DAI)

Nutrition	Control	LNPF1	LSBS2	LNPF1+ LSBS2	F values
Protein (%)	11.14 ± 0.54	13.78 ± 0.69	12.81 ± 0.82	15.00 ± 0.13	32.137***
Fat (%)	40.08 ± 0.47	46.33 ± 0.28	45.64 ± 0.08	47.69 ± 0.15	396.893***
Fibre (%)	9.21 ± 0.15	13.59 ± 0.17	12.59 ± 0.16	15.40 ± 0.31	456.511***
Carbohydrate (%)	16.39 ± 1.00	21.17 ± 0.53	21.02 ± 0.88	24.75 ± 0.98	68.230***
Energy (Kcal)	446.0 ±1.54	550.0 ± 1.00	539.0 ± 1.00	581.0 ± 1.05	161.111***
Calcium (mg/g)	11.97 ± 0.20	15.69 ± 0.55	15.14 ± 0.10	16.63 ± 0.20	122.123***
Iron (mg /g)	1.64 ± 0.0	5.52 ± 0.98	4.46 ± 0.15	7.36 ± 0.5	387.277***
Total oil content (%)	34.93 ± 0.56	42.78 ± 0.40	46.04 ±0.65	50.06 ± 0.42	452.755***

Note: The mean of three replicates ± the standard deviation is used to represent the data. *, **, *** signify the extent of significance LSD ($P < 0.05$) in the significant difference.

Discussion

After being isolated and biochemically characterised from the rhizospheric soil of *Zea mays* and *Arachis hypogaea* plants, *Pseudomonas* and *Bacillus* species were designated as LNPF1 and LSBS2, respectively, in the current study. *P. fluorescens* was previously isolated from Indian rhizosphere soil using gramme staining. As a consequence, after gram staining, the morphology of the recovered *Pseudomonas* strains showed Gram-negative, pink-colored, rod-shaped bacteria by previously described (Tripathi and Banerjee, 2011).

The same outcomes were seen when gram-negative bacteria that had been isolated demonstrated positive reactions for the enzyme's catalase, oxidase, and citrate utilisation, while amylase activity showed negative reactions for both molecular characterization (Babu *et al*., 2016; Sasirekha and Srividya, 2016; Bhojiya and Joshi, 2015). Based on *Bacillus* sp. (LSBS2) was identified as biochemical analyses, starch hydrolysis, phosphate solubilization, the synthesis of organic phosphates, and extracellular enzyme activity extracted from the plant rhizospheric area on plant growth promoting and antagonistic activity against phytopathogens (Saxena *et al*., 2020).

Positive plant growth-promoting processes included the formation of HCN, ammonium, phosphate solubilization, and IAA. (Abbamondi *et al.,* 2016) Isolates of *Bacillus* and *Pseudomonas* produced 60% and 70% of HCN and ammonium, respectively, indicating successful outcomes. Similarly, positive HCN and ammonium generation was also seen in the isolates of LNPF1 and LSBS2. Tryptophan has been found as the primary precursor for IAA pathways, and IAA production may be significant in the biosynthesis of IAA, (Shih-Feng *et al.,* 2015). It makes it possible for the microbe to solubilize the fixed form of

soil phosphate, which can raise the amount of phosphate that is available for both the bacterium and the crop (Deshwal *et al.,* 2011). Calculation the strain *Pseudomonas fluorescens's* maximum phosphate solubility potential for phosphate solubilization (Kaur *et al*., 2016).

P. fluorescens (LNPF1) under King's B medium and *B. subtilis* (LSBS2) under nutrient medium were the isolates from which siderophores were produced. The siderophore was successfully isolated using the CAS reagent, revealing a shift in colour from blue to orange that was consistent with previous observations of siderophore production. According to (Ines *et al.,* 2012), various zinc and magnesium concentrations also significantly influence the induction of siderophore synthesis, as seen by the creation of a yellow-green fluorescent pigment around them on Kings B medium. Additionally, the ground nut pot experiment was carried out to evaluate the siderophore-producing LNPF1 and LSBS2 isolate's bioinoculant efficacy on plant growth and other physic-chemical parameters. Many plant growth metrics showed a notable improvement in the current investigation. In the current study, the bioinoculant-treated ground nut plants exhibited a notable increase in several plant growth parameter, including plant biomass, pigment content, iron content, and oil content, as compared to the control plants.

The isolates from which siderophores were generated were *P. fluorescens* (LNPF1) in King's B medium and *B. subtilis* (LSBS2) in nutritional medium. With the use of the CAS reagent, the siderophore was effectively extracted, and the colour changed from blue to orange, matching earlier reports of siderophore formation. The formation of a yellow-green fluorescent pigment around the siderophores on Kings B medium indicates that different concentrations of zinc and magnesium also have a major effect on the stimulation of siderophore synthesis (Ines *et al.,* 2012). Furthermore, the ground nut pot experiment was conducted to assess the bioinoculant efficiency of the siderophore-producing LNPF1 and LSBS2 isolate on plant development and other physic-chemical parameters. In the current in comparison to the control plants, the bioinoculant-treated ground nut plants showed a significant increase in a number of plant growth parameters, such as plant biomass, pigment content, iron content, and oil content.

When *Arachis hypogaea* plants were co inoculated with *Bradyrhizobium* sp., they exhibited a significant increase in nodules over the course of several decades. This increase was approximately 252 nodules plant-1 when compared to nodules fresh weight, dry weight, shoot, root, plant fresh weight, dry weight, and peanut yield content, respectively (Awadalla and Abbas, 2017) According to Naik *et al.* (2020), there was an increase in the percentage of oil, protein, nitrogen, phosphate, and potassium content in seeds after *Rhizobium* and

Enterobacter treatment. Co-inoculation with phosphate-solubilizing rhizobacteria resulted in a significant increase in peanut growth potential; subsequent PGPR treatment increased nodule formation and reduced the requirement for chemical fertiliser (Awadalla and Abbas, 2017). In different research, *the P. fluorescens* strain that produces siderophores, C7, was shown to produce increased growth in *Arabidopsis thaliana* plants by mobilising enough iron uptake (Vansuyt *et al.,* 2007). Our findings also point to a favourable relationship between the rhizobacteria that promote plant development and the increased iron and oil content that we have seen. When considered as a whole, the current data revealed that the isolates of LNPF1 and LSBS2, two Plant Growth Promoting Rhizobacteria, are important for increased ground nut growth and may be used as potential bioinoculants.

In the Salem area of Tamil Nadu, India, rhizospheres of agricultural soil were found to contain rhizobacteria that promote plant development. These isolates were confirmed by 16S rRNA gene sequence analysis to be *Pseudomonas fluorescens* (LNPF1) and *Bacillus subtilis* (LSBS2) based on biochemical characteristics. The PGP activity characterisation process produced phosphate solubilisation, IAA production, HCN production, ammonium production, and siderophore production in both isolates. Additionally, a pot experiment using *Arachis hypogaea* revealed important rise in the treated plant's content of carotenoids, chlorophyll, and total nitrogen as well as in its root and shoot lengths, fresh weight, and dry weight.

The dual inoculation resulted in an increase in leghemoglobin concentration, nitrogenous activity, and biomass buildup of root nodules. When comparing the yield parameter of LNPF1+LSBS2 to uninoculated plants, the yield parameter of pod weight, number of pods per plant, total oil content, and nutrients including protein, carbohydrate, fat, calcium, energy, and iron content also increased. Thus, the strain LNPF1 (*Pseudomonas*) LSBS2 (*Bacillus)* may be used as a possible bioinoculant to promote plant development, enhance plant nutrients, improve the amount of oil that plants output, and raise the iron content of *Arachis hypogaea.*

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