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Biofertilizer Production Using Phosphate Solubilizing Bacteria Isolated from Rhizosphere Soil of Cowpea (*Vigna unguiculata*)

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Abstract **Original Research Article**

Phosphate-solubilizing bacteria (PSB) do enhance the availability of phosphorus in soil thereby promoting sustainable agriculture. This study aimed to isolate and characterize PSB from the rhizosphere soil of cowpea (Vigna unguiculata) and access its potential for biofertilizer production. Rhizosphere soils samples were collected from some farms within Sokoto metropolis. Physicochemical properties were determined using standard procedures. PSB were screened from the soil samples using Pikovskaya's agar and nutrient agar. The screened PSB were isolated and characterised biochemically using standard procedures. The potential of the screened phosphate solubilizing bacterial isolates as biofertilizer using charcoal as a base material were assessed on the growth of cowpea (*Vigna unguiculata*). Results shows slightly acidic pH ranging from 6.72 to 6.57. Temperature was recorded from 36.7 to 37.1. The cation exchange capacity (CEC) was recorded highest (23±0.021) in Arkilla Layout (ALO) and lowest (3.01±0.410) in Arkilla Gandu (AGND). Seven bacterial species were identified as *Paenibacillus dendritiformis. Pseudomonas putida, Pseudomonas aeruginosa, Bacillus megaterium, Bacillus cereus, Bacillus subtilis,* and *Bacillus licheniformis.* Paenibacillus dendritiformis exhibited the highest solubilization efficiency of (3.77±0.106) and was molecularly characterized using 16S rRNA sequencing, compared to Bacillus cereus with solubilizing efficieciency of (1.13± 0.014). The biofertilizer formulation developed and tested on cowpea plants revealed that selected PSB isolates significantly increased phosphorus availability and improved cowpea growth compared to control treatments. The tested biofertilizer on cowpea plant revealed that suspension with Paenibacillus dendritiformis had the highest growth of shoot (6.6) and number of leaves (21.50). The Isolate with highest PSI (Paenibacillus dendritiformis) was molecularly characterized as Paenibacillus dendritiformis was found to have 92.12% similarity index after running the sequence on a BLAST. These findings highlight the potential of PSB to be a good source of biofertilizers suspension with charcoal base as an eco-friendly alternative to synthetic fertilizers and organic manure.

Keywords: Biofertilizer, Phosphate-solubilizing bacteria, Cowpea, Paenibacillus dendritiformis.

INTRODUCTION

Biofertilizers are products containing living cells of different types of microorganisms which when applied to seed, plant surface or soil, colonize the rhizosphere or the interior of the plant and promotes growth by converting nutritionally important elements (nitrogen, phosphorus) from unavailable to available form through biological process such as nitrogen fixation and solubilization of rock phosphates (Kumar *et al.*, 2024). Biofertilizer and biological waste are used to replace the usage of chemical fertilizers as it does not contain any toxic substance and makes the soil enriched. The use of biofertilizer in crop cultivation will help in safeguarding the soil health and also the quality of crop products (Patel *et al.*, 2024). Biofertilizer reduce crop production costs, enhance growth and yields by increasing nitrogen availability, and promote the production of growth-promoting substances like auxins and cytokinins (Patel *et al.*, 2024). Phosphorus (P) is one of the essential elements that are necessary for plant development and growth, it makes up about 0.2% of a plant's dry weight (Patel *et al.*, 2024). It is second only to nitrogen among mineral nutrients most commonly limiting the growth of crops (Sharma *et al.*, 2024). On average, the phosphorus content of soil is about 0.05% (w/w), however, only 0.1% of this phosphorus is available for plant used (Huang *et al.*, 2024). Many species of soil fungi and bacteria are able to solubilize phosphorus in vitro and some of them can mobilize phosphorus in plants (Ramos Cabrera *et al*., 2024). Phosphate availability in the soil solution is often insufficient as most soils are poor in phosphorus, which is considered a limiting factor for plant nutrition. Its deficiency severely restricts plant growth and yields (Islam *et al*., 2024).

Phosphate solubilizing bacteria (PSB) are being used as biofertilizer since 1950s (AbouJaoudé *et al.*, 2024). Phosphate-solubilizing bacteria (PSB) play a significant role in making phosphorus available to plants by bringing about favourable changes in soil reactions and the soil micro-environment, leading to the solubilisation of inorganic phosphate sources (AbouJaoudé *et al.*, 2024). In this regard, a scientist's primary focus is to increase the yield of crop quality and health properties of foods along with organoleptic and nutritional value, which are gaining significant attention from consumers and governments (Ahmed et al., 2024). Phosphate solubilising bacteria have been shown to significantly enhance the availability of phosphorus to plants, thus improving plant growth, yield, and quality (Kaur *et al.,* 2024). They also offer the benefit of reducing the need for chemical phosphorus fertilizers, which are costly and have environmental consequences such as eutrophication and soil degradation (Kaur *et al.*, 2024).

Cowpea is an important legume crop that is widely cultivated for its high nutritional value and its ability to improve soil fertility through nitrogen fixation. Cowpea's ability to fix nitrogen is also crucial for improving the sustainability of agricultural systems, as it reduces the need for synthetic nitrogen fertilizers (Adediji *et al*., 2024). In the rhizosphere of cowpea, interactions between the plant roots, soil microorganisms, and soil particles are central to the plant's growth and the overall health of the soil ecosystem (Asghar *et al*., 2024). Cowpea is known for its ability to grow in poor, arid soils, making it an essential crop in many regions of the world, especially in sub-Saharan Africa and South Asia (Kirarei *et al*., 2024).Cowpea is an excellent source of plant-based protein, essential amino acids, fiber, vitamins, and minerals (Manole *et al*., 2024).The rhizosphere of cowpea is a rich source of beneficial microorganisms, including phosphate solubilizing bacteria, which can be harnessed for biofertilizer production (Singh *et al*.,2024).

The use of chemical fertilizer to supplement phosphorus has several environmental drawbacks, including soil degradation, and pollution (Jote, 2023). In the past centuries, farmers were eager in the usage of chemical fertilizers as it yielded great number of crops, but eventually they realized that it affects the soil fertility and it kills the beneficial microbes which enhances the growth of crops. Chemical fertilizers are costly, environmentally harmful and unsustainable. The demand for chemical-free food is rising among consumers and sparked the research for environmentally safe techniques in order to reduce the reliance on chemicals. Therefore, there is need for alternative, eco-friendly solutions that can enhance phosphorus availability in the soil. Biofertilizer, on the other hand, are made from natural ingredients and don't contain any harmful chemicals. This reduces the risk of human exposure to toxic substances and can improve overall health (Kumar *et al*., 2024).

Production of biofertilizer using locally isolated bacteria from rhizosphere soil of cowpea can be a cost-effective and sustainable alternative to chemical fertilizers. Phosphate solubilizing bacteria have been shown to play a significant role in improving soil fertility and crop yield (Kumar *et al*., 2024). These bacteria are believed to help make phosphorus more available to plants, which can improve plant health and productivity. Phosphate solubilising bacteria (PSB) have been considered important for making soluble phosphorus forms available to plants by solubilizing insoluble inorganic phosphates or mineralizing organic phosphate compounds into soluble forms (Kumar *et al*., 2024). This study was aimed to produce biofertilizer from phosphate solubilizing bacteria associated with the rhizosphere of cowpea (*Vigna unguiculata*).

MATERIALS AND METHODS

Sampling Area and Sample Collection

One kilogram (1kg) of Soil from rhizosophere of cowpea at a depth of 0-10 cm was collected from three different farms each in Arkilla Area, Sokoto, located at 13.02527^0 N latitude and 5.18771^0 E longitude, 13.03737^0 N latitude and 5.21532^0 E longitude and 13.02119^0 N latitude and 5.23399^0 E longitude respectively, using soil auger packed in a clean polyethylene bag. The Samples were aseptically packed and transported to Department of Microbiology, Postgraduate Research Laboratory for further Analysis (Reichman *et al*., 2024).

Physical and Chemical Analysis of Soil Samples

Soil has both chemical and physical properties that influence its structure,fertility and overall health, some of these properties include;

Determination of pH

Twenty (20) grams of each soil sample was weighed and put in a 100 mililiters beaker. Twenty (20) mililiters of distilled water was added to the sample. The suspension was left for 2 minutes, with occasional stirring using glass rod in order to enable it reach equilibrium. The pH of the suspension was determined using a pH meter. The determination of the pH was carried out in duplicate and the average results were recorded accordingly (Ekundayo *et al*., 2024).

Determination of Soil Particle Size

Air dried soil was sieved and 51 grams was transferred into one liter of distilled water, mixed and shaken. Fifty (50) milliliters of 5% sodium hexametaphosphate was added followed by 100 ml of distilled water. The soil suspension was stirred thoroughly using glass stirrer for 15 minutes and transferred into a cylinder containing hydrometer. Distilled water was added to the lower blue line of the cylinder. The volume changed to 1130 ml and the hydrometer was removed. The top of the cylinder was covered with hand and inverted several times until all soil was in suspension. The cylinder was placed on a flat surface and time was noted. Hydrometer was placed in the suspension and the first reading (H1) was noted immediately after 40 seconds. Subsequently, the temperature (T1) was recorded after the hydrometer was removed. The suspension was allowed to stand for 3 hours and the second reading was taken for hydrometer (H2) and the temperature (T2) (Onivefu *et al*., 2024).

Determination of Moisture Content

An empty crucible was weighed (W0) and 2 g of soil was added and weighed again (W1). Soil samples were then dried in hot air oven at 1050C for 24 hours until constant weight was achieved (W2). Both the crucible and the dried sample were weighed again (International Institute of Tropical Agriculture, 1979). The moisture content was calculated as:

% moisture = $\frac{W_{1-W_2}}{W_{1-W_0}} \times 100$

Where,
W1=Weighed soil
W2=Constant Weight
W0=Weight of the empty Crucible (Premei *et al*., 2020).

Determination of Temperature

The temperature was determined at the point of sample collection by dipping the bulb of mercury-in-glass thermometer into the soil at point of collection and recording the readings 30 seconds to one minute (Rabah and Ibrahim, 2010).

Determination of Electrical Conductivity

A 1:5 of soil and water suspension was prepared by weighing 10 g air-dry soil into a bottle. 50ml of deionized water was added and then mechanically shake at 15rpm for 1hr to dissolve soluble salts. The cell conductivity was calibrated according to the manufacturer's instructions using KCl reference solution to obtain the cell constant. The cell was rinsed thoroughly and then measured the EC of the 0.01M KCl at the same temperature as the soil suspensions. The cell was then rinsed with the soil suspension and then refilled without disturbing the settled soil. Values indicated on the cell were then recorded in ds/cm. The cell was rinsed with deionised water between solutions (Salahi *et al*., 2014).

Determination of Nitrogen Content (N)

Total nitrogen was determined by the Macro-Kjeldahl digestion method of Juo (1979). Five grams (5g) of soil sample was weighed into a 500ml Macro-Kjeldahl flask, and 20ml of distilled water was added. The content was swirled for five minutes (5mins) and allowed to stand for thirty minutes (30mins). One tablet of mercury catalyst and 10g of K_2SO_4 were added, and 30 ml of concentrated. H_2SO_4 was added through an automated pipette. The content of the flask was heated gently in the digestion stand. After cooling, 100ml of distilled water was added and transferred into another clean macro-Kjeldahl flask (750ml) and the sand residue was washed four times with 50ml of distilled water. All the washings were transferred into the same flask. Fifty millilitres (50ml) H_3BO_3 indicator solution was added into a 500ml Erlenmeyer flask, placed under the distillation apparatus's condenser and introducing 150ml of 10 NaOH. This was followed by distillation. For the condenser to remain cool (30^0C) and prevent frothing, sufficient cold water was allowed to flow through the condenser. Ammonium was determined in the distillate by treating against 0.01N standard H_2SO_4 using a 25ml burette graduated at 0.1ml intervals. The colour changed at the end point from green to pink (Juo, 1979).

Percentage nitrogen will be calculated using the formula:

%Nitrogen = $\frac{N \times 0.014 \times Vd \times 10}{A \times weight\ of\ sample} \times 100$

Where:
N = Normality of acid,
Vd = Volume of the digest,
A = Aliquot of digest (Juo, 1979).

Determination of Phosphorus Content (PO_4)

One (1) g of air-dried soil sample was weighed (passed 2 mm sieve) into a 15 ml centrifuge tube and add 7 ml of the extracting solution. It was then shake for 1 minute on a mechanical shaker and centrifuge the suspension at 2,000 rpm for 15 minutes. 2 ml of the clear supernatant was pipette into a 20 ml test tube. 5 ml distilled water and two 2 ml of ammonium molybdate `solution was added and mixed properly. 1 ml of $SnCl_2$-$2H_2O$ dilute solution was added and mixed. After 5 minutes, but not later than 20 minutes, % transmittance was measured on the electro photometer at 660 nm wave length. Using optical density, standard curve was prepared and then the exchangeable phosphorus was calculated (Okpa *et al*.,2024).

Determination of Organic Carbon

The soil sample was ground and sieved with the used of siever. One gram of soil sample was weighed in duplicates and transferred into 250ml Erlenmeyer flasks. Ten (10) milliliters of potassium dichromate solution and 20ml of concentrated sulphuric acid were added and the contents of the flasks were shaken gently until properly mixed. One hundred milliliters of distilled water was added and allowed to stand for 30 minutes. This was followed by adding 4 drops of indicator phenol red (indicator) and titrating against 0.5N ferrous sulphate solution. The percentage carbon was calculated according to the formula by IITA (1979) which is:

.% Organic Carbon = Me $K_2Cr_2O_7$ – Me $FeSO_4$ × 100
Weight (Wet soil – dry soil).
Where: Me = Mole equivalent (Abdallah and Ibrahim, 2023).

Determination of Cation Exchange Capacity (CEC)

The cation exchange capacity (CEC) was determined by extracting the cations with 1 M ammonium acetate buffered at pH 7. Thirty (30) ml of 1 M CH_3COONH_4 was added to five (5) g of soil. The suspension was shaken for 2hrs and then centrifuged (15 min, 6000 rpm). After centrifugation and filtration, the filtrate was transferred into a 100 ml flask and two other volumes of 30 ml ammonium acetate were added successively after thirty (30) min of agitation and centrifugation. The final filtrates were completed to 100 ml with ammonium acetate solution. Calcium (Ca) and magnesium (Mg) were determined by EDTA titration while potassium (K) and sodium (Na) were determined by flame photometry. Effective CEC is thus calculated by the sum of exchangeable bases (Ca, Mg, K, Na) (Gzar *et al*., 2014).

Microbiological Analysis of Soil Samples

Microbiological analysis of soil helps determine the diversity, abundance and activity of microorganisms such as bacteria, fungi and other microbes that influence soil fertility and plant health. The analysis typically involves qualitative and quantitative assessments using various laboratory techniques which involves;

Serial Dilution

A stock solution for serial dilution was made by dispensing 1 gram of soil into 100ml of distilled water, shaking thoroughly, and transferring one (1) ml into a test tube containing 9ml of sterile distilled water, subsequently making a serial dilution up to 10^{-7}. Using the spread plate method technique, 0.1 ml of the suspension from dilutions of 10^{-5}, 10^{-6}, and 10^{-7} was plated aseptically on prepared Nutrient Agar (NA) and incubated at 30°C for 24 hours. The results were determined by multiplying the number of counts with the dilution used and expressed as colony-forming units per gram (cfu/g) of soil (Premei *et al.*, 2020).

Preparation of Media

The media were prepared according to the manufacturer’s instruction.

Nutrient Agar

Twenty-eight grams (28g) of nutrient agar was dissolved into 1000ml of distilled water in a sterile conical flask. It was swirled to mix; the mixture was heated with a hotplate to dissolve thoroughly and then it was sterilized in an autoclave at 121ºC for 15mins. The media was allowed to cool at 40ºC, which was then poured into sterile Petri dishes and was allowed to solidify (Adedayo and Mohammed, 2024).

Pikovskaya Agar

Pikovskaya agar medium containing the following composition in g/L: Glucose 10g/L, yeast extract 0.5g/L, ammonium sulphate 0.5g/L, magnesium sulphate 0.1g/L, calcium phosphate 5g/L, sodium chloride 0.2g/L, potassium chloride 0.2g/L, manganese sulphate 0.002g/L, ferrous sulphate 0.002g/L, agar 1.8g/L making it a total of 31.3g and distilled water 1000ml was prepared. The pH of 7.0 was used as the adjusted pH of the medium and the medium was sterilised for 1210c for 15min.The media was allowed to cool at 40ºC, which was poured into sterile Petri dishes and was allowed to solidify (Pikosvkaya,1948).

Pikovskaya Broth

This was prepared according to the manufacturer’s instructions. 16.3g of PVK broth powder was weighed and dissolved into 1000ml of distilled water in a sterile conical flask. It was swirled to mix, the mixture was heated with a hotplate to dissolve thoroughly, and then it was sterilized in an autoclave at 121ºC for 15mins. The media was allowed to cool at 40ºC (Adedayo and Mohammed, 2024).

Gram Stain

The colony of bacterial smear of different size and shape was picked with the aid of a sterilized wire loop, into a drop of sterile distilled water on a clean grease free glass slides and a smear was made, the slides were heat fixed by carefully passing them over a bursen burner flame (Amaka and Onwa, 2024). The heat fixed smear was stained with crystal violet for 60secs and it was washed off gently with water and it was drained. The smear was covered with lugol's iodine as the mordant and it was allowed for 15-30secs washed and drain. It was flooded with 0.5% counter stimulate (Safranin) for 30 seconds, and then washed using indirect stream of tap water, drained, cleaned and air dried. A drop of immersion oil was dropped on the stained smear for Microscopy. Morphological characteristics such as cell shape, arrangement and gram reaction was observed under ×100 objectives lens of the light microscope and recorded (Amaka and Onwa, 2024).

Biochemical Characterization

Biochemical characterization helps identify and classify bacteria based on their metabolic and enzymatic activities.

Catalase Test

Isolates were grown in nutrient Agar Medium for 24-48 hours at 37ºC. After incubation a thick smear of the organism was made on clean slide and a drop of 3% hydrogen peroxide was placed onto the colonies. Presence of gas bubbles indicates a positive reaction and absence of bubble indicates a negative reaction (Hadwan *et al*., 2024).

Citrate Utilizing Test

This test identifies the isolates as fast or slow growers, Simmon’s citrate agar was prepared in slants in bijou bottles as recommended by the manufacturer. Using a straight wire, the media was first streaked with the test organism and then stabbed to the butt of the bottle and was incubated at 37ºC for 24-48 hours. A blue color formation indicates a positive result while no color changes indicate a negative result (Ovuto and Tomiwa, 2024).

Starch Hydrolysis Test

A colony of the test organism was picked using a sterile wire loop, the starch plate was streaked in the form of a line across the width of the plate and incubated at 37ºC for 48hours. 2-3drops of 10% iodine solution was added directly onto the edge of the colonies. A clear zone around the line of growth indicates that the organism has hydrolyzed starch and a blue, purple or black coloration indicates a negative result (Cereda, 2024).

Oxidase Test

Isolated microorganisms were grown in nutrient agar medium for 24-48 hours at 37ºC. A filter paper was placed into a Petri dish and a few drops of dilute 1% solution of oxidase reagent (tetramethyl-phenylenediamine-dihydrochloride) which was prepared according to standard procedure was added. One large colony was picked with a loop and tapped lightly onto the wet filter paper. Formation of a blue-purple color by the cells within 30 seconds indicates a positive oxidase result (Aisyah *et al*., 2024).

Voges-Proskauer Test

Some bacteria in the fermentation of glucose, produce other products such as ethanol and 2,3-butanediol rather than large amounts of acid as does *E. coli*. A test for acethyl methyl carbinol (a precursor of 2,3-butanediol that appears in the growth medium) was performed. A pink color developing after a few minutes indicates the presence of acetyl methyl carbinol. MR-VP broth was inoculated for 48hours at 37ºC. Twelve drops of 5% solution of alpha-naphthol and 4 drops of 40% potassium hydroxide (KOH) will be added. The mixture was agitated vigorously and left to stand. Positive test results in a red or pink color (Okwelle and Amadi-Ikpa, 2024).

Indole Test

The indole test detects the ability of bacteria to produce indole from the hydrolysis of tryptophan by tryptophanase enzyme. A tryptone broth was inoculated with the bacterium. The broth was incubated. After incubation, 1 ml of Kovac's reagent was added to the broth. The color change was observed. A red color in the organic layer indicates a positive indole reaction, indicating the presence of indole after the addition of Kovac's reagent. Negative result indicates no color change or development of a yellow color (Cheesbrough, 2006).

Motility

Motility medium was inoculated with the isolate by making a fine stab with a depth 1- 2cm short of the bottom of the tube and incubate at 37°C for 24-48 hours. At the end of the period of incubation, the tube was examined. The line of inoculation would not be sharply defined and the rest of the medium would be somewhat cloudy if the organisms are motile. If the organisms are not motile, the line of inoculation will become sharply defined and the rest of the medium will remain clear (Oyeleke and Manga, 2008).

Urease Test

This test was done as described by Oyeleke and Manga (2008). A speck of each isolate was inoculated into Christensens urea agar and incubated at 37°C for 24 hours The development of a bright pink or red colour indicate a positive reaction.

Triple Sugar Ion (TSI)

Using a sterile wire loop, cultures from a solid medium was streaked on the surface of the slant and stabbed the butt twice before incubation at 37°C for 24 hours. Since TSI composite media several reactions was read from each after 24 hours incubation Gas formation was determined by the appearance of one or several bubbles in the butt Formation of hydrogen sulphide was determine by the blackening of the whole butt or streak or ring of blackening at the slant butt junction. The butt becoming yellow indicates glucose fermentation, Lactose and sucrose fermentation was also observed (Oyeleke and Manga, 2008).

Bacterial Strain Screening for Phosphate Solubilization

Following the gram staining and microscopic examination, the bacterial cultures obtained from pure culture plates and identified as phosphate-solubiliziing bacteria based on morphological features were confirmed through their ability to thrive on Pikovskaya agar media, (which is a test for phosphate-solubilizing bacteria). Using an inoculating needle, bacterial colonies were extracted from the pure culture plates and was inoculated onto a test tube containing 4ml sterile distilled water (until the sterile water turned milky in color) before inoculation on PVK agar media plates, then it was incubated at 37°C for 48hrs. A sterile syringe was used to inoculate the milky suspension of the bacterial isolates onto four Pikovskaya (PVK) agar media plates. The first plate was done by extracting 1ml of the suspension and inoculating it onto the Pikovskaya (PVK) agar media plate as a suspension, and the same goes for all the remaining 3 plates. After 48 hours of incubation, distinct halozone formations were displayed, affirming their classification as phosphate-solubilizing bacteria. These colonies were cultivated in nutrient agar media for further investigation (Zhang *et al*., 2024).

Phosphorus Solubilization Assay

Phosphorus solubilising assay is used to identify microorganisms such as bacteria and fungi that can convert insoluble forms of phosphorus into soluble forms, making it more available for plant uptake.

Determination of Phosphate Solubilisation Index (PSI)

Bacterial strains that were grown on pikosvkaya agar plates for 48hrs were brought out and zone of inhibtion was observed, the diameter of the colonies and that of the halo zone in plate assays were measured using a calliper after seven days of incubation at 37^0C in Pikosvkaya agar medium. The solubilisation index (SI) was calculated as the ratio of the total diameter to the colony diameter (Tat, 2024).

Phosphate Solubilization Index (SI) = $\frac{B}{A}$
Where;
A = Colony diameter
B = Total diameter (colony + halo zone)

Biofertilizer Production

The production of biofertilizer using phosphate solubilising bacteria involve the following major steps which are;

Preparation of Starter Cultures for Phosphate-Solubilizing Bacteria

After the screening of the PSB, bacterial strains like *Bacillus spp* and *Pseudomonas spp* from the pure culture slants were transferred to the liquid broth which was also the production media and as well as the starter culture for the growth of the cells. Production media is that media in which the number of viable bacterial cells of that particular bacterium increases because that bacterium is grown in that particular media only. Thus, in phosphate solubilising bacteria both the isolates were grown in Pikovskayas production media (Protocol followed for growth of PSB) (Ducousso-Détrez *et al*., 2024)

Thus, a 100ml of two separate conical flasks were taken and PVK media was prepared after pH adjustments to 7.0 and autoclaved at 121^0 C for 15minutes. Then inside the laminar airflow the pure cultures marked in the pure culture slants were transferred to the PVK production media conical flasks by the help of sterilized inoculating loop. Then the conical flasks were put in the rotary B.O.D shaker for 7 days. The viable cell counts in the production media or the liquid broth was found to come up to 109 Cfu/ml. Then for the mass production of PSB biofertilizer the inoculums from these starter cultures were transferred to larger flasks (Roychowdhury *et al*., 2015).

Preparation of Carrier Material for the Biofertilizer Production

Four hundred 400g of black charcoal obtained was crushed into powder using a pestle and mortar. It was sealed in a sterile polythene bag and brought into the Microbiology laboratory at Usmanu Danfodiyo University, Sokoto. The powdered charcoal was transferred into a sterile beaker for sterilization. The charcoal was sterilized following the standard sterilization method at 121^0C for 15 minutes. Sterility was maintained throughout the procedure and it was conducted within a laminar airflow environment. (Isiya, 2024).

Inoculum Preparation with Carrier Material (Mixing)

The bacterial cell cultures from storage was combined with the sterilized carrier material in separate beakers. The mixing of the carrier material and the production media was on a ratio of 2:1, which is equivalent to a ratio of 60:30 with 1 part of the production media mixed with 2 parts of the sterilized carrier material. The process was carried out manually and under aseptic conditions (Bini *et al*., 2024)

Biofertilizer Storage

Biofertilizer was produced by mixing the sterilized carrier material and production media. The produced biofertilizer was sealed in a sterile polyethylene bag and stored in a cool place for 19 hours before usage. After 19 hours, 2.5kg of the produced biofertilizer was poured in an 11cm-wide container with an open end, where sterile water was poured onto it until it finally became a bit thick suspension (Dimitrova *et al*., 2024).

Biofertilizer Testing on Crop

Green house experiment was carried out to test the effect of biofertilizer produced on the leguminious crop (*Vigna unguiculata*)

Greenhouse Experiment

A greenhouse experiment evaluated the effect of biofertilizer produced from phosphate solubilising bacteria on the growth of cowpea. The experiment was conducted in the Botanical Garden of Usmanu Danfodiyo University, Sokoto. The pots used for the experiment contained 5kg of autoclaved loamy soil (Pathak *et al*., 2017). The effectiveness of the produced biofertilizer was tested for 15 days, where, on the first day, 9 seeds of cowpea were first inserted into the biofertilizer produced for 35 seconds and then removed. The seeds were air dried for 45 seconds before planting on pots containing 5kg of loamy soil (Dimitrova *et al*., 2024).

The experiment was set up as a completely randomised design with four treatments and three replicates of each treatment, giving a total of twelve (12) pots: T1: (control; N0 –RP-PSB), T2: (PSB1: ALO 2), T3: (PSB 2: AGND 1), T4: (PSB 3: AGND 2), T5: (ASL 4). The pots were watered to 70% water holding capacity and maintained at this moisture content by watering to the constant weight every 1-2 days (Pathak *et al*., 2017). All the pots were kept in natural condition for 15 days. After completion of the trial, the plants were washed thoroughly with water, and parameters, like the length of shoots and number of leaves were recorded for comparative evaluation in different combinations (Sane and Mehta, 2015).

Characterization of the Most Effective Bacterial Suspension Using Molecular Technique

DNA Extraction

The single pure colonies of the bacterial isolates were grown in Luria-Bertani (LB) broth overnight at 28°C. Two (2) ml of the culture were centrifuged at 5000 rpm for 5 minutes, and the pellet was suspended in 200 ml of TE buffer at pH 8, containing RNase (50 ng/ml), to prevent degradation. Then, 400 µl of lysis buffer was added, followed by mixing well and incubation for 15 minutes at 37°C with intermittent shaking every 5 minutes. Immediately, chloroform and isoamyl alcohol in a ratio of 24:1 were taken and mixed by inversion. Tubes were centrifuged at 10,000 rpm for 5 minutes, and the supernatant was transferred carefully to another microcentrifuge tube. To the supernatant, 0.1 volume of 3 M sodium acetate (pH = 5.2) and 0.6 volume of isopropanol were added, mixed well by inversion, and kept on ice for 10 minutes, followed by centrifugation at 10,000 rpm for 10 minutes. The pellet was washed with 70% ethanol with gentle shaking and centrifuged at 10,000 rpm for 3 minutes. The supernatant was removed, and the pellet was air-dried. Extracted DNA was visualized using 0.8% agarose gel electrophoresis, and images were documented (Balakrishnan *et al.*, 2022).

16S rRNA gene PCR amplification

Using 16S ribosomal RNA gene-specific universal primers 27F 50-AGA GTT TGA TCC TGG CTC AG-30 and 1492R 50-GGT TAC CTT GTT ACG ACT T-30 (Sigma), the 16S rRNA gene was amplified with a 50 µl reaction mixture containing 1X reaction buffer (10 mM Tris [pH 8.3, 50 mM KCl, and 1.5 mM MgCl2), 200 µl dNTPs, 0.05 U Taq DNA polymerase enzyme (Sigma, USA), 0.5 µM of each primer, and 1 ng template DNA. The thermal cycling conditions were: 5 minutes at 94°C for initial denaturation; 31 cycles of 30 seconds at 95°C, 1 minute at 54°C, 2 minutes at 72°C, and a final extension for 5 minutes at 72°C. The amplification reaction was performed with a thermal cycler (MyCycler, Bio-Rad, USA), and the PCR amplicons (approximately 1500 bp) were resolved by electrophoresis in 1% (w/v) agarose gel to confirm the expected size of the product (Balakrishnan *et al.*, 2022).

PCR Product Purification

Isolates capable of solubilising phopshate were purified by two procedures—Ammonium sulphate precipitation method and $ZnCl_2$ precipitation method. By Ammonium sulphate precipitation method: it consisted of four steps—ammonium sulphate fractionation, chilled acetone, hexane treatment, and silica gel column chromatography. By $ZnCl_2$ precipitation method: 10 ml of the culture supernatants were concentrated by $ZnCl_2$ to a final concentration of 75 mM. The precipitated material was dissolved in 10 ml of sodium phosphate buffer (pH 6.5), extracted twice with equal volumes of diethyl ether. The pooled organic phase was evaporated to dryness, and the pellets were dissolved in 100 µl of methanol. Further purification was achieved by preparative TLC (Jiraporn and Niran, 2023).

DNA Sequencing of 16S rRNA Gene Fragment

By using the ABI DNA 3730 XL sequencing system (Applied Biosystems), the 16S rRNA purified PCR product was submitted. Sequencing of the bacterial isolate's 16S rRNA gene was carried out in both directions. The bacterial species was determined with the obtained sequence, which was searched for BLAST. The sequences were submitted to the NCBI GenBank after sequence matching percentages and accession numbers were obtained (Balakrishnan *et al.*, 2022).

Phylogenetic Analysis

The 16S rRNA gene sequence obtained in this study was aligned with the sequences published in the National Center for Biotechnology Information (NCBI). Aligned sequences were edited, and a phylogenetic tree was constructed using MEGA (version 6) software. The phylogenetic tree for the relationship among strains was constructed by the Maximum Likelihood Method with a bootstrap of 500 using the Kimura-2 parameter (Balakrishnan *et al.*, 2022)

Data Analysis

Data will be analyzed by using descriptive statistics, the samples Mean, Standard deviation, t-test Analysis of variance (ANOVA) was employed, where p=0.05 to test the level of significance (Mishra *et al*., 2019).

RESULTS

Physicochemical Parameters of the Soil Samples from the Rhizosphere of Cowpea

Table 1 presents the physicochemical parameters of soil samples from the rhizosphere of cowpea across three locations, which include Arkilla Layout (ALO), Arkilla State Lowcost (ASL), and Arkilla Gandu (AGND). The macronutrients calcium (Ca), magnesium (Mg), sodium (Na), and potassium (K) differ slightly among the samples, with ASL exhibiting the highest Ca concentration (0.39±0.014) and ALO having the highest K content (2.06±0.021). The cation exchange capacity (CEC) follows a similar trend, being highest in ALO (23±0.021) and lowest in AGND (3.01±0.410). The pH values range from 6.57 to 6.72, indicating slightly acidic to near-neutral soil conditions, which are favorable for cowpea growth. Electrical conductivity (EC) is highest in ALO (109.8±1.202), suggesting a relatively higher concentration of soluble salts compared to ASL and AGND. Phosphates (PO_4) and organic carbon (OC), crucial for soil fertility, are highest in ALO (0.155±0.007 and 0.755±0.007, respectively), decreasing across ASL and AGND. Nitrogen (N), another essential nutrient, shows a different trend, with AGND containing the highest level (0.153±0.137), significantly higher than ALO and ASL. The soil texture of the samples are predominantly sandy, with sand content exceeding 85% in all locations, while clay and silt proportions remain low. ALO and AGND have the highest sand content, above 94%, whereas ASL has a slightly lower sand percentage (85.2±5.939) with a higher clay fraction (7.15±0.212).

Mean Heterotrophic Bacterial Counts of Soil Samples

Table 2 illustrates the bacterial colony count in ALO and ASL is identical at 2.75×10^6 CFU/g ± 0.07, whereas AGND exhibits a significantly higher count of 8.3×10^6 CFU/g ± 0.14. This suggests that AGND harbors a more diverse or abundant bacterial community compared to the other two sites. The increased bacterial population in AGND could be due to better organic matter content, favorable soil conditions, or higher microbial activity in the rhizosphere.

Table 1: Physico-Chemical Parameters of the Soil Samples from the Rhizosphere of Cowpea

Parameters	ALO	ASL	AGND
Ca (cmol/kg)	0.24±0.014	0.39±0.014	0.33±0.021
Mg (cmol/kg)	1.4±0.141	1.15±0.070	1.39±0.014
Na (cmol/kg)	0.39±0.007	0.39±0.007	0.36±0.084
K (cmol/kg)	2.06±0.021	1.95±0.070	1.215±0.049
CEC (cmol/kg)	23±0.021	3.965±0.035	3.01±0.410
pH	6.57±0.007	6.67±0.042	6.72±0.014
EC (µs/cm)	109.8±1.202	81.85±2.333	66.3±666
PO_4 (mg/kg)	0.155±0.007	0.15±0.014	0.138±0.035
OC (%)	0.755±0.007	0.52±0.028	0.3±0.113
N (%)	0.065±0.004	0.05±0.007	0.153±0.137
%Sand	965±0.778	85.2±5.939	94.95±0.353
%Silt	1.6±0.141	3.15±0.212	1.4±0.141
%Clay	3.1±0.282	7.15±0.212	3.45±0.212
Temperature	36.8±1.06	36.7±0.57	37.1±0.28

Keys: ALO = Arkilla Layout, ASL = Arkilla State Lowcost, AGND = Arkilla Gandu, OC = Organic Carbon, N = Nitrogen, PO_4 = Phosphate, Ca = Calcium, Mg = Magnesium, K = Potassium, Na = Sodium, CEC = Cation Exchange Capacity, EC = Electrical Conductivity, N = Nitrogen, and % = Percentage.

Table 2: Mean Heterotrophic Bacterial Counts of Soil Samples from Arkilla layout, Arkilla State Lowcost and Arkilla Gandu

Samping Sites	Mean Bacterial Colony Count (CFU/g) ± S.D
ALO	$2.75 \times 10^6 \pm 0.07$
ASL	$2.75 \times 10^6 \pm 0.07$
AGND	$8.3 \times 10^6 \pm 0.14$

Keys: ALO = Arkilla Layout, ASL = Arkilla State Lowcost, AGND = Arkilla Gandu, and S.D = Standard Deviation.

Morphological and Biochemical Characterization of the Isolates

Table 3 shows the morphological and biochemical identification of bacteria isolated from the rhizosphere of cowpea. The Table reveals the presence of diverse bacterial species across the three sampling locations (ALO, ASL, and AGND). The majority of the isolates belong to the genus *Bacillus*, with different species identified in the ALO and ASL sites. In contrast, the AGND site shows the presence of *Pseudomonas* species. The species identified include *Bacillus megaterium*, *Bacillus cereus*, *Bacillus subtilis*, *Bacillus licheniformis*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, and *Paenibacillus dendritiformis.*

Frequency of Occurrence of the Bacterial Isolates

The frequency of occurrence of bacteria isolated from the cowpea rhizosphere reveals a dominance of *Bacillus* spp, which account for a significant proportion of the bacterial community. *Bacillus subtilis* is the most frequently isolated spp, appearing 4 out of 14 samples, representing 28.5% of the total isolates. Following closely are *Bacillus megaterium* and *Bacillus cereus*, each with 21.4% occurrence, highlighting their prevalence in the cowpea rhizosphere. *Pseudomonas putida*, *Pseudomonas aeruginosa*, *Paenibacillus dendritiformis*, and *Bacillus licheniformis* each have a lower frequency of 7.14%, appearing only once in the sample set.

Table 3: Morphological and Biochemical Characterization of the Isolates obtained from Soil Samples

Code	Shape	Spore	Gram Rn	Catalase	Oxidase	M-R	V-P	Indole	Urease	Citrate	Starch Hydrolysis	Glucose	Lactose	Sucrose	H_2S	Gas	Identified Organisms
ALO1	Rods	-	+	+	+	-	+	-	+	+	+	+	-	-	+	-	*Bacillus megaterum*
ALO2	Rods	+	+	+	-	-	+	-	-	+	+	+	-	+	+	+	*Paenibacillus dendritiformis*
ALO3	Rods	+	+	+	+	-	+	-	+	+	+	+	-	-	+	-	*Bacillus megaterium*
ALO4	Rods	+	+	+	+	-	+	+	-	-	-	+	-	+	-	-	*Bacillus subtilis*
ALO5	Rods	-	+	+	+	-	+	+	-	+	-	+	-	+	-	-	*Bacillus subtilis*
ALO6	Rods	-	+	+	+	-	+	-	+	+	+	+	-	-	+	-	*Bacillus megaterium*
ASL1	Rods	-	+	+	+	-	+	-	-	+	+	+	-	-	-	-	*Bacillus cereus*
ASL2	Rods	-	+	+	+	-	+	+	-	+	-	+	-	+	-	-	*Bacillus subtilis*
ASL3	Rods	+	+	+	+	-	+	-	-	+	+	+	-	-	-	-	*Bacillus cereus*
ASL4	Rods	+	+	+	+	-	+	-	-	+	+	+	-	-	-	-	*Bacillus cereus*
ASL5	Rods	+	+	+	-	+	+	-	-	+	+	-	-	+	-	-	*Bacillus licheniformis*
ASL6	Rods	+	+	+	+	-	+	-	-	-	-	+	-	+	-	-	*Bacillus subtilis*
AGND1	Rods	-	-	+	+	-	-	-	-	+	-	+	-	-	-	-	*Pseudomonas putida*
AGND2	Rods	-	-	+	+	-	-	-	+	+	-	+	-	-	-	-	*Pseudomonas aeroginosa*

Keys: ALO=Arkilla layout, ASL= Arkilla State Lowcost, AGND=Arkilla Gandu, M-R =Methyl Red,V-P=Voges Prauskuer.

Table 4: Frequency of Occurrence of the Bacterial Isolates from Arkilla Layout, Arkilla State Lowcost and Arkilla Gandu

Identified Isolates	Frequency of Occurrence	Percentage Frequency of Occurrence (%)
Pseudomonas putida	1	7.14
Pseudomonas aerogenosa	1	7.14
Paenibacillus dendritiformis	1	7.14
Bacillus licheniformis	1	7.14
Bacillus cereus	3	21.4
Bacillus megaterium	3	21.4
Bacillus subtilis	4	28.5
Total	14	100

The Diameter of the Colony and Halo Zone Indicating Phosphate Solubilization by the Presumed Phosphate Solubilising Bacteria

Table 5 presents the diameter of the colony and halozone indicating phosphate solubilization by the presumed phosphate solubilising bacteria. The phosphate solubilizing index (PSI) of bacteria isolated from the cowpea rhizosphere varies across the different isolates, reflecting differences in their ability to solubilize phosphate. The PSI is calculated based on the ratio of the total zone of solubilization (halozone) to the colony diameter, indicating the efficiency of each strain in breaking down insoluble phosphate into forms accessible to plants. Among the isolates, ALO2 exhibits the highest PSI (3.77±0.106), followed by ASL5 (3.00±0.141) and AGND1 (3.235±0.021). Conversely, isolates such as ASL4 (1.13±0.014), ALO4 (1.3±0.141), and ASL6 (1.3±0.282) show the lowest PSI values, indicating weaker phosphate solubilization potential. Examining the colony diameter and halozone formation further supports the PSI trends. ALO2 has a relatively small colony diameter (0.19±0.014) but a larger halozone (0.56±0.014), leading to its high PSI. In contrast, ASL4 has the largest colony diameter (255±0.077) but a relatively small halozone (0.51±0.014), resulting in a low PSI.

Effect of Biofertilizer Produced from Phosphate Solubilizing Bacteria on the Growth of Cowpea Leaves

Table 6 shows the effect of different treatments of Biofertilizer produced from phosphate-solubilizing bacteria (PSB) on the growth of cowpea leaves over a 15-day period. The control (without bacteria) shows low increase in leaf growth, starting at 0.82 cm on Day 5 and reaching 2.2 cm by Day 15. In contrast, all bacterial treatments (ALO2, ASL2, AGND1, and AGND2) show significantly higher growth rates. The treatment ALO2 consistently results in the highest leaf growth, reaching 6.6 cm on Day 15. All treatments involving PSB have greater leaf growth compared to the control, with ALO2 having the most positive effect, suggesting that phosphate-solubilizing bacteria enhance the growth of cowpea leaves more effectively than the untreated control.

Table 5 The Diameter of the Colony and Halo Zone Indicating Phosphate Solubilization by the Presumed Phosphate Solubilising Bacteria

Code	Colony Diameter	Halozone	PSI
ALO1	0.41±0.014	0.795±0.007	2.3±0.141
ALO2	0.19±0.014	0.56±0.014	3.77±0.106
ALO3	0.805±0.007	0.71±0.028	1.865±0.021
ALO4	0.49±0.014	0.215±0.021	1.3±0.141
ALO5	0.395±0.007	0.725±0.035	2.25±0.014
ALO6	1.265±0.007	0.815±0.02	1.45±0.212
ASL1	0.48±0.014	0.545±0.035	2.23±0.028
ASL2	0.525±0.007	0.26±0.014	1.35±0.212
ASL3	0.205±0.007	0.395±0.007	2.63±0.523
ASL4	255±0.077	0.51±0.014	1.13±0.014
ASL5	2.21±0.014	6.35±0.212	3.00±0.141
ASL6	0.19±0.014	0.115±0.021	1.3±0.282
AGND1	0.215±0.021	0.435±0.021	3.235±0.021
AGND2	0.545±0.035	0.78±0.028	2.25±0.212

Keys; PSI = Phosphate Solubilizing Index, ALO=Arkilla Layout, ASL=Arkilla State Lowcost, AGND=Arkilla Gandu

PSI= (Colony Diameter + Halozone Diameter) / Colony Diameter

Table 6 Effect of Biofertilizer Produced from Phosphate Solubilizing Bacteria on the Growth of Cowpea Leaves in Centimeter (cm)

Treatment	Day 5	Day 10	Day 15
CONTROL	0.82±0.29	1.0±0.15	2.2±0.45
Paenibacillus dendritiformis	1.6±0.55	4.0±0.75	6.6±0.89
Bacillus subtilis	1.4±0.55	3.4±0.55	5.8±0.84
Pseudomonas putida	1.4±0.55	3.8±0.84	6.4±0.55
Pseudomonas aeroginosa	1.4±0.55	3.6±0.89	5.8±0.84

Effect of Biofertilizer Produced from Phosphate Solubilizing Bacteria on the Shoot length of Cowpea

Table 7 shows the effect of phosphate solubilizing bacteria on the shoot length of Cowpea over 15 days, with five different treatments. The control group exhibited the lowest growth, with the shoot length increasing slowly from 117 cm on Day 5 to 159 cm on Day 15. Among the treatments, **ALO2** exhibited the highest growth, with shoot lengths reaching 21.50 cm by Day 15, far surpassing the other treatments. **AGND2** also showed substantial growth, reaching 19.60 cm on Day 15, while **AGND1** and **ASL2** showed moderate growth, with values ranging between 17.72 cm and 18.86 cm by Day 15. These results indicate that phosphate solubilizing bacteria, especially ALO2, positively influence Cowpea shoot growth, with ALO2 having the most pronounced effect.

Molecular Analysis

Gel image of Paenibacillus dendritiformis

Figure 1 shows the result of PCR product of *Paenibacillus dendritiformis* that was obtained after running the Gel electrophoresis. The anticipated band of 1500bp of 16S gene was successfully amplified as shown in the gel.The band are strong to be purified for sequencing to confirm the identity of the isolates used in the present research.

Phylogenetic Analysis of 16S rRNA of Bacteria

The evolutionary relationships between different identified species and other species are depicted in figure 2 of the phylogenetic analysis. The relationships are based on similarities and differences in the evolutionary genetic characteristics of the specie. The phylogenic relationship between the nucleotide sequences received from Inqaba Bioteh and their closest blast hit from NCBI database alighned using the clustalW program in Molecular Evolutionary Genetics Analysis (MEGA) is seen in figure 2.

Table 7: Effect of Biofertilizer Produced from Phosphate Solubilizing Bacteria on the Shoot length of Cowpea in Centimeter (cm)

Treatment	Day 5	Day 10	Day 15

CONTROL	117±0.21	122±0.19	159±0.21
Paenibacillus dendritiformis	20.83±0.23	21.29±0.25	21.50±0.29
Bacillus subtilis	17.24±0.19	17.36±0.41	17.72±0.79
Pseudomonas putida	17.52±0.79	17.98±0.99	18.86±2.43
Pseudomonas aeroginosa	18.57±0.44	19.02±0.34	19.60±0.54

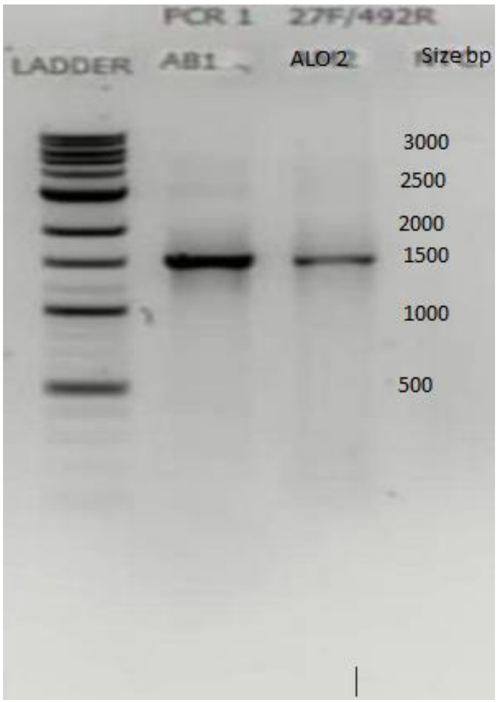

Figure 1: Gel Image of *Paenibacillus dendritiformis*

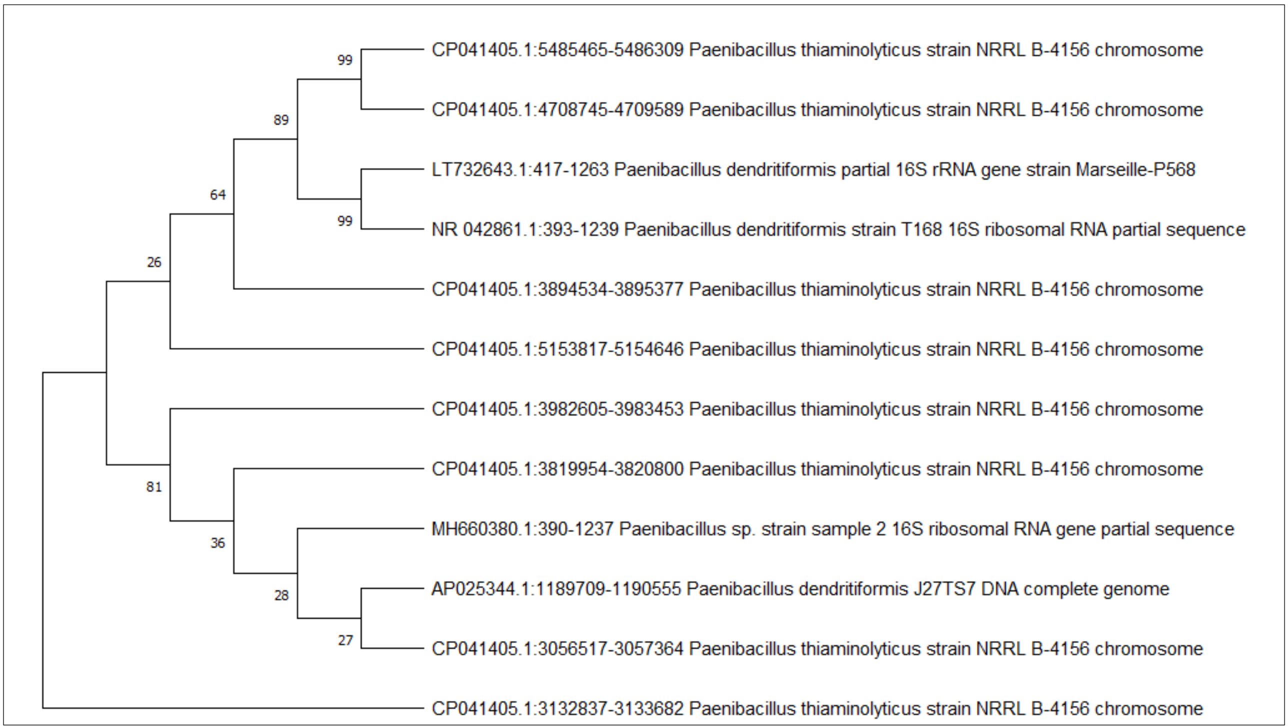

Figure 2: Phylogenetic Tree based 16S rRNA Sequence using Neighbor Joining Method

Hit Similarity of the Most Potent Isolate(s) from GenBank of NCBI

Table 8 presents hint similarity of the most potent isolate(s) from GenBank of NCBI. The bacterium *Paenibacillus dendritiformis* was found to be highly similar with *Paenibacillus dendritiformis* 92.12% percentage identity as shown in Table 8.This percentage reflects the proportion of sequence identity between the queried sequence and the reference sequence in the database, signifying a high degree of genetic homology, A higher percentage similarity, in this case, suggests that *Paenibacillus dendritiformis* is the most likely organism in the database that aligns with the queried sequence, confirming its potential in biological identity.

Table 8: The BLAST Results Showing Similarity Between the Sequence Queried and the Biological Sequences within the NCBI Database

Sample ID	Predicted Organism	Percentage Similarity	Accession Number
ALO2	*Paenibacillus dendritiformis*	92.12%	APO25341

DISCUSSION

The pH of soil samples in this present research ranges from 6.57±0.007 to 6.72±0.014 which are slightly acidic as presented in Table 1. The pH which is the measurement of the acidity and alkalinity of a substance has a scale which ranges from 0-14. A pH of 7 indicates neutrality of the substance, and pH less than 7 indicates acidity while the pH greater than 7 shows alkalinity. An irregular pH level of soil is rarely a problem. Therefore, the normal levels of soil samples should be between 6.5 and 8, but values outside this range indicate that there are severe abnormalities in the soil quality as reported by Xue *et al*., (2024). The acidity of the samples may be attributed to microbial activities as reported by Naz *et al*., (2022) who recognized microbial activities as an important cause of soil acidity. Federal Environmental Protection Agency (FEPA) permissible limit for Agricultural soil is 6-9, and the samples with pH below six (6) are revealing that the pH is not within the permissible limit set by FEPA. The pH value recorded in this research is in accordance with the findings of Zhang *et al*., (2021). The temperature of the samples in the study ranged from 36.7±0.57ºC to 37.1±0.28°C. This range shows the possibility of many bacterial species to thrive in optimum temperature which supports their growth in the plant rhizosphere. The temperature variations in this research correlates with that of Walker and Aherne (2024), which reported temperature ranges between 35°C and 45°C. The organic carbon content of the samples ranges from 0.30±0.113% to 0.755±0.007%. This is not surprising due to the soil being an agricultural soil, therefore the account for organic carbon will be much present in such sample. Also, this might be due to the increase in the deposition of nutrients containing-carbon on the soil, which may result in increased carbon content of the soil. The result of this research agrees with the findings of Maffia *et al*. (2024).

Furthermore, the nitrogen content in this study ranged from 0.05±0.007% to 0.153±0.137% indicating its low content. According to Mohammed and Dakora (2024), the rhizophere of cowpea has been reported by various studies to have low values of nitrogen and are sometimes resorted to enrichment with synthetic fertilizer. This finding conforms to that of Bashir *et al*., (2024) who studied the physicochemical characteristics of cowpea farmland. Electrical conductivity recorded ranges from 66.3±66µS/cm to 109.8±1.202µS/cm, the observed result agrees with Jang *et al*. (2022) in South Korea. Also, the phosphate (PO_4) content of the samples ranges from 0.138±0.035mg/kg to 0.155±0.007mg/kg which explained the possibility of Phosphate-solubilizing bacteria to be isolated from such habitat. This is in line with the result recorded by Çelik and Sürücü (2024).

The mean heterotrophic bacterial counts of the samples from ALO, ASL and AGND as illustrated in Table 2 were found to range from 2.75×10^6CFU/g ± 0.07 to 8.3×10^6CFU/g ± 0.14. The high microbial load recorded in this study is largely attributed to the availability of high amount of minerals, organic matter content, and favorable soil conditions in the plant rhizosphere which supports and promotes the growth of diverse microbial species. As stated by Kaur *et al*. (2024), this high bacterial count has important implications for soil fertility and cowpea growth, because beneficial bacteria in the rhizosphere play a crucial role in nitrogen fixation and organic matter decomposition. This finding is in consistence with the research conducted by Deinert *et al*. (2023).

The morphological and biochemical characterization of the isolates were presented in Table 3. According to the morphological characteristics, most bacterial species identified in this study were rod-shaped and Gram-positive, except for *Pseudomonas* species, which are Gram-negative. The seven (7) bacterial species recorded in this present research includes *Bacillus megaterium*, *Bacillus cereus*, *Bacillus subtilis*, *Bacillus licheniformis*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, and *Paenibacillus dendritiformis*, which is in conformity with the research conducted by De Mandal *et al*. (2021). The identification of *Bacillus* species in this study is noteworthy due to their cosmopolitan nature in soil environment. According to Nagaraja (2022), *Bacillus* species are Gram positive bacteria due to the availability of thick peptidoglycan on their cell wall which helps them to retain the primary stain (Crystal violet) and appear purple under the compound microscope. *Pseudomonas* species on the other hand have been linked with various studies as reported by Muindi *et al*. (2021). Their thin peptidoglycan structure disallows them to retain the primary stain after the addition of decolorizer; therefore, they are counterstained to appear pink under the microscope. As reported by Farouq *et al*. (2022), the possibility of both *Bacillus* and *Pseudomonas* species to be accounted for in this study is due to the fact that Agricultural soil, particularly the rhizophere of many plants such as cowpea can serve as a habitat for the proliferation of many microorganisms. The isolation of these species is in alignment with the research conducted by dos Santos *et al*. (2022), which highlighted the availability of *Bacillus* and *pseudomonas* species in the rhizosphere of cowpea.

The percentage frequency of occurrence of the bacterial species as depicted in Table 4 showed the variation abundance of the bacterial species in the sample. From the Table, *Bacillus* species were found to account for a significant proportion of the bacterial community, which is line with de Albuquerque *et al*. (2022)'s research. This may be attributed to their ability to resist diverse environmental conditions in the soil. *Bacillus* species are known to produce spores that enable them withstand environmental harshness. Among the *Bacillus* species, *Bacillus subtilis* was the most frequently isolated species, appearing in 4 out of 14 isolates which represent 28.5% of the total bacterial isolates. Following closely were *Bacillus megaterium* and *Bacillus cereus*, each with an occurrence of 21.4%, highlighting their prevalence in the cowpea rhizosphere. Furthermore, *Bacillus licheniformis, Pseudomonas putida, Pseudomonas aeruginosa* and *Paenibacillus dendritiformis* were found to have a lower frequency of 7.14%, appearing only once in the isolates. This is in agreement with the study conducted by De Mandal *et al*. (2021). dos Santos *et al*. (2022) also supported this in research titled "Differential plant growth-promoting rhizobacteria species selection by maize, cowpea, and lima bean"

The phosphate solubilizing index (PSI) of the bacteria isolated from the rhizosphere of cowpea as presented in Table 5 showed varying index across the isolates. According to Cruz *et al*. (2022), the PSI is calculated based on the ratio of the total zone of solubilization (halozone) to the colony diameter, indicating the efficiency of each strain in breaking down insoluble phosphate into forms accessible to plants. Therefore, the diameter of both the bacterial colony and halozone were obtained in order to determine the phosphate solubilizing index of the bacterial potential to produce biofertilizer. As shown in the Table, the phosphate solubilizing index (PSI) of bacteria isolated from the cowpea rhizosphere varies significantly across the different samples, thereby reflecting differences in their ability to solubilize phosphate. ALO2 exhibited the highest PSI value of 3.77±0.106 among the isolates, followed by ASL5 (3.00±0.141) and AGND1 (3.235±0.021). Conversely, samples such as ASL4 (1.13±0.014), ALO4 (1.3±0.141), and ASL6 (1.3±0.282) show the lowest PSI values, therefore indicating weaker phosphate solubilization potential of the isolates. Examining the colony diameter and halozone formation further supports the PSI trends. ALO2 has a relatively small colony diameter (0.19±0.014) but a larger halozone (0.56±0.014), leading to its high PSI. In contrast, ASL4 has the largest colony diameter (255±0.077) but a relatively small halozone (0.51±0.014), resulting in a low PSI. This is in line with the research conducted by Janati *et al*. (2022) on isolation and characterization of phosphate solubilizing bacteria naturally colonizing legumes rhizosphere in Morocco. Similarly, Ejeagba *et al*. (2023) also support the notion in research titled "Isolation and Molecular Characterization of Phosphate-Solubilizing Bacteria from Root Nodules of Cowpea (*Vigna unguiculata*) Seeds Planted at Ota, Ogun State, Nigeria".

The effect of the different treatment of phosphate-solubilizing bacteria (PSB) on the growth of the cowpea leaves over a period of 15-day was illustrated in Table 6. As described by Omer *et al*. (2023), the effect of the biofertilizer can be determined by determining the growth of the cowpea leaves. The control sample which possess no bacterial treatment shows low increase in the growth of the leaf, starting at 0.82 cm on Day 5 and reaching 2.2 cm by Day 15. This is in alignment with that of (). In contrast to this, all bacterial treatments (ALO2, ASL2, AGND1, and AGND2) show significantly higher growth rates compared to the control sample. The most potent treatment was recorded in ALO2, having the highest leaf growth, reaching up to 6.6 cm on Day 15. Whereas ASL2 and AGND2 both possess the lowest with a length of 5.8cm, which are still many ways higher than the control, thereby showing their effectiveness on the growth of the cowpea plant. All treatments involving PSB have greater leaf growth compared to the control, with ALO2 having the most positive effect, suggesting that phosphate-solubilizing bacteria enhance the growth of cowpea leaves more effectively than the untreated control. This is in conformity with that finding of Bondok *et al*. (2024).

The effectiveness of phosphate solubilizing bacteria on the shoot length of Cowpea over 15 days, with five different treatments was presented in Table 7. From the observation, the control group exhibited the lowest growth, with the shoot length increasing slowly from 117 cm on Day 5 to 159 cm on Day 15, which is in line with that of Mohammed (2020). Among the treatments, ALO2 still exhibited the most significant growth, with shoot lengths reaching 21.50 cm after Day 15, far surpassing the other treatments. Furthermore, AGND2 also showed substantial growth, reaching up to 19.60cm on Day 15, while AGND1 and ASL2 showed moderate growth, with values ranging between 17.72 cm and 18.86 cm by Day 15. These results indicate that phosphate solubilizing bacteria, especially ALO2, positively influence Cowpea shoot growth, with ALO2 having the most pronounced effect. The shoot length shows the effectiveness of the cowpeas incorporated with the different treatment as compared with the control (untreated) sample. This finding is in agreement with the research conducted by Bondok *et al*. (2024) titled "Cowpea (*Vigna unguiculata L. Walp*) morpho-physiological and yield responses to chemical, organic, and biofertilizers at various watering levels utilizing drip irrigation system".

Molecular characterization of the bacterial isolate were conducted by firstly isolating the bacterial DNA, followed by running Polymerase Chain Reaction (PCR), and then lastly conducting 16S rDNA analysis, which showed an agarose gel indicating the amplification of the 16S target region of the isolate. According to Rana and Joshi (2023), an agarose gel electrophoresis is a laboratory technique which is used to separate and analyze DNA, RNA, or protein molecules based on their size and charges in an agarose gel matrix. The molecular identification had supported the biochemical identification of the bacterium *Paenibacillus dendritiformis* after submitting the sequence to the NCBI GenBank and 92.12% similarity was recorded with *Paenibacillus dendritiformis* with the accession number AP025341 (Table 8). The phylogenetic analysis of the obtained 16S rDNA sequence was also performed, and the phylogenetic relationship of the isolate *Paenibacillus dendritiformis* was shown with eleven (11) most closely related strains on the basis of BLAST hit as shown in Figure 2.

Conclusion

From this study, the physicochemical characteristics of the cowpea rhizopshere of the soil samples revealed that the pH of all the soil samples were slightly acidic. The average temperature ranges from 36.7±0.57ºC to 37.1±0.28°C, while phosphate (PO_4) content of the soil samples ranges from 0.138±0.035mg/kg to 0.155±0.007mg/kg. The mean heterotrophic bacterial counts of the samples ranged from 2.75×10^6CFU/g ± 0.07 to 8.3×10^6CFU/g ± 0.14. A total seven (7) bacterial species belonging to the genus of *Bacillus* and *Pseudomonas* spp. were identified biochemically with varying frequency of occurrence. They include *Bacillus megaterium, Bacillus cereus, Bacillus subtilis*, *Bacillus licheniformis*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, and *Paenibacillus dendritiformis. Bacillus subtilis* had the highest frequency of occurrence, followed by *Bacillus megaterium*, *Bacillus cereus,* while *Bacillus licheniformis*, *Pseudomonas aeruginosa*, *Pseudomonas putida,* and *Paenibacillus dendritiformis* were found to possess the lowest frequency of occurrence. Among the samples, ALO2 exhibits the highest PSI, followed by ASL5 and AGND1. While isolates such as ASL4, ALO4, and ASL6 show the lowest PSI values, indicating their weaker phosphate solubilization potential. The molecular characterization of the bacterial isolate supports the biochemical identification of the bacterium *Paenibacillus dendritiformis* after submitting the sequence to the NCBI GenBank and 92.12% similarity was recorded with *Paenibacillus dendritiformis* strain with the accession number AP025341. The phylogenetic tree of the isolate was shown with the most closely related *Paenibacillus dendritiformis* strains on the basis of BLAST.

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