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ISOLATION AND CHARACTERIZATION OF *BACILLUS* SPP. FOR PLANT GROWTH PROMOTING PROPERTIES

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Abstract: A group of free-living bacteria known as plant growth-promoting rhizobacteria (PGPR) inhabit the rhizosphere and aid root development. These rhizobacteria bacteria are vital to the growth of plants and can serve as bio-fertilizer and can enhance food security through green agricultural practices. They exhibit special features which make them potential candidates as bio-fertilizer. Isolation and characterization of rhizobacteria is the first step toward their utilization as bio-fertilizers. Ten rhizobacteria from two different rice farms were isolated and characterized for plant growth promoting properties. The isolated rhizobacteria were identified morphologically, microscopically, biochemically, and molecularly. Plant's growth promoting properties of these rhizobacteria was also analyzed which includes; Indole 3-acetic acid production (IAA), phosphate solubilisation, hydrogen cyanide production (HCN), ammonia production (NH_3), and zinc solubilisation. Out of the ten isolates, three were found to have the best plant growth enhancing properties and were therefore the best candidates as bio-fertilizers. 16SrRNA study and phylogenetic analysis was performed in order to unravel the specie of these three isolates and they were identified as *Bacillus subtilis*, *Bacillus niacini*, and *Bacillus cereus* with accession numbers OM184294, OM1842295 and OM184296 respectively. These isolates have the potential to be used as bio-fertilizer, which would significantly contribute to food security.

Keywords: *Bacillus*, rhizobacteria, plant growth, Rice farm, bio-fertilizer, food security

1. Introduction

The quest to preserve ecological integrity of the soil through the use of green agricultural revolution is gaining momentum. Indiscriminate use of fertilizer, pesticides and other chemicals to enhance plant's growth occasioned by population explosion has negative consequences that affect the soil quality and microbial community surrounding the soil (Gouda et al., 2018; Molnár et al., 2020). Even in ideal situations, plants can only use 50% of the chemical fertilizer applied to them while the remaining is leached through the soil and interfere with underground and surface waters (Sharma and Singhvi, 2017). The effects of fertilizer application to the soil may not be immediately visible due to the

buffering capability of the soil; however, with time, soil degradation and disturbance of mineral elements balance eventually emerges (Sharma and Singhvi, 2017). These practices are associated with pollution of the environment, increase in soil acidity which eventually alter the pH of the soil and disruption of the ecological balance of the microbial flora in the soil. Several researches have shown the negative effects of chemical pesticides application in order to control weeds and pests (Rani et al., 2021; Sabarwal et al., 2018). This prompted a search for sustainable and less harmful green agricultural practices that can enhance plant growth while preserving the ecological integrity of the soil (Adnan et al., 2020).

Over the years, microorganisms have been linked to plant nutrient supply for agricultural development and production (Aeron et al., 2020; Péterfi and Domokos, 2018). Furthermore, the use of microorganisms to enhance plant's growth has the potential to reduce the level of pollution in the environment, raise the yields of the crops, and reduce the negative consequences of developing antibiotic resistance by pests and making the environment safer for incoming generations. Applications of plant growth-promoting rhizobacteria (PGPR) are being promoted worldwide for the expansion of sustainable agriculture. Plant growth-promoting rhizobacteria are microbes that promote beneficial effects on plant development through direct or indirect mechanisms (Rai et al., 2020). These rhizobacteria's direct mechanisms include nutrient acquisition, phytohormone synthesis, siderophore generation, and antioxidant property enhancement (Nazir et al., 2018). Through the activation of the plant immune system against phytopathogens, PGPR can indirectly accelerate plant growth (Mustafa et al., 2019). Additionally, PGPR plays crucial functions in plant physiology, which include enhancing abiotic stress responses (Rai et al., 2020).

In addition to colonizing the rhizosphere of plants, PGPR can also grow in, on, or around plant tissues and stimulate plant growth through a variety of direct (such as phosphorus solubilization, nitrogen fixation, phytohormone production, etc.) or indirect (such as reducing pathogenic infection and/or mitigating abiotic stresses) mechanisms (Prasad et al., 2019). These PGPR are the most well-known beneficial microorganisms associated with plants and the best-performing bio-inoculants, having demonstrated excellent performance under controlled settings such as phosphate solubilization, phytohormone synthesis, siderophores generation, and nitrogen fixation (Basu et al., 2021).

Among the many species of bacteria that promote plant's growth, *Bacillus* spp. are the most widely distributed in the soil with several advantages over other species (Sansinenea, 2019). They shared several properties such as phosphate solubilisation, enhanced nitrogen acquisition, and siderophore production with other PGPR species. Furthermore, *Bacillus* spp. have the advantage of forming an endospore which can enable them survive high temperatures in the soil (Kaloterakis et al., 2021). They can withstand wide temperature variations, and also exhibit both the direct and indirect mechanisms of enhancing plant's growth. These and other properties make them suitable candidates for green-agricultural revolution for enhanced crop production (Saxena et al., 2020). Furthermore, the use of indigenous soil bacteria with potential plant's growth promoting properties that has already been adapted to the environment is being encouraged for easier utilization of such bacteria (Saxena et al., 2020). In this study, we aim to isolate and characterize a potential plant growth promoting *Bacillus* spp. from two

different sites; a rice farm in Kura local government area of Kano state, Nigeria, and Centre for dry land Agriculture, Bayero University, Kano, Nigeria.

2. Materials and methods

Study site

The study was conducted in the Plant Biology Department's botanical garden at Bayero University, Kano, Nigeria at coordinates 11°58′50″N, 8°28′46″E.

Collection of soil sample

Soil sample were collected from two sites; the first soil sample (Sample A) was gotten from Kura local government area, Kano State Nigeria (this soil is a rice cultivated soil). The second sample was collected from Centre for Dry Land Agriculture (CDA), Bayero University, Kano (The soil is used for cultivating rice). It was collected using hand trowel to dig up the soil to about 5cm depth; the temperature of the soil was recorded as 37˚C. 50g of the soil was collected in a zip lock transparent bag and transported to the lab for further analysis. To break up soil lumps before usage, samples were carefully mixed and run through a 0.4mm mesh sieve.

Bacteria isolation

The soil samples collected from both sites were brought to the microbiology laboratory for the isolation of the bacteria. The test tubes were arranged in a test tube rack; 9 ml of distilled water was measured and distributed in 10 different test tubes for each of the sample. Serial dilution was carried out on the soil samples. Nutrient agar was prepared according to the manufacturer's instruction and the isolates were sub-cultured into the prepared petri plates. They were then incubated at 37°C for 24 hours. Plate culture with discrete colony was used and the morphological characteristics of the colony were noted down (Garrity et al., 2005).

Microscopic examination of isolates

Glass slides were all labeled, small drop of normal saline was added on the slide. Bacteria isolates were picked with a wire loop and mixed with normal saline on the slide. After allowing it to air dry, the slide was heat fixed. After 60 seconds, a few drops of crystal violet were applied to the smear. After rinsing the slide with water, a drop of lugol's iodine was applied to the smear and left to sit for 60 seconds. After another washing with water, 95 percent ethanol was applied for 5 seconds. The slide was washed, blotted dry, and prepared for microscopic inspection (Garrity et al., 2005).

Morphological and biochemical identification

When compared to Bergey's manual, pure colonies of PGPR were identified and classified based on the findings of their Gram colouration tests, morphological traits, and biochemical characteristics (Garrity et al., 2005).

Screening of isolates for plant growth promoting properties

Phosphate solubilizing property

On a Pikovskaya's agar medium, bacterial isolates were tested for phosphate solubilization (PAM). Under usual conditions, bacterial culture was injected on PAM in the plate's center and maintained there for 5 days at 30-35 °C. Clear zones that formed surrounding the colonies demonstrated phosphate solubilizing properties. Results were displayed

as the zone's diameter in millimetres (Rai et al., 2020).

Production of indole acetic acid (IAA)

With a few minor adjustments, the technique of Mohite (2013) was used to quantify indole acetic acid (IAA). Test tubes were filled with 50 ml of nutritional broth that contained 0.1 percent DL tryptophan before being sterilized in an autoclave for 15 minutes at 121 °C. After allowing it to cool, the isolates were added to the prepared broth and maintained there for 72 hours at 28 °C.The culture was centrifuged for 10 minutes at 4°C (10,000 rpm). Orthophosphoric acid was added in two drops to the supernatant (2 ml), then, Salkowski reagent (50 ml of 35 percent perchloric acid and 1 ml of 0.5 ml $FeCl_3$ solution) was added to 4 ml of the supernatant. IAA manufacturing is indicated by the color pink. The optical density at 530 nm was captured using the spectrophotometer. IAA concentration was measured in µg/ml.

Production of NH_3

To see how bacterial isolates produced ammonia, peptone water was employed. The freshly produced culture was then added to the 10 ml of peptone water and maintained at 30 °C for 48–72 hours. 0.5 ml of Nessler's reagent was added to the mixture. Ammonia production was detected by the color changing from pale yellow to dark brown (Mohite, 2013).

Production of HCN

Glycine was supplemented with bacterial culture and streaked over nutrient agar medium. A Whatman filter paper that had previously been soaked in a particular solution (0.5 percent picric acid and 2 percent sodium carbonate (w/v) was used to cover the agar. Parafilm paper was used to seal the plates, which were then incubated at 36°C for four days. The creation of hydrogen cyanide is indicated by the development of orange or red color (Sehrawat et al., 2022).

Zinc solubilisation

Based on hollow formation on a solid basal medium containing 0.1 percent ZnO, isolated bacteria were tested for their capacity to dissolve zinc. The width of the resulting zone was measured and recorded (Hashemnejad et al., 2021).

Molecular identification of bacterial isolates

Genomic DNA isolation

Genomic DNA was isolated using Prepease genomic DNA isolation kit according to the manufacturer's instruction. Bacterial pellets from a pure bacterial culture cultured on nutrient broth were centrifuged for two minutes at 5000 rpm. The pellets were then homogenized in 100 µL of buffer, which contained 1.6 ml of 5 M sodium chloride, 5.48 g of sucrose, 1.57 g of tris, 10.16 ml of 0.5 M EDTA, and 2.5 ml of 20 percent SDS. After 30 minutes at 65 °C, 14 µL of 8 M potassium acetate (to a final concentration of 1 M), which was added, was incubated the particle was washed in 100 µL of ice-cold, 70% ethanol after the supernatant had been placed into a fresh 1.5 ml Eppendorf and 200 L of 100% ethanol had been added and vortexed. The pellet was then washed in 100 µL of ice-cold 70 percent ethanol, dried, and suspended in 100 µL of dH_20, which was then incubated at 65 °C for 10 minutes. The total concentration of DNA was then determined using nanodrop spectrophotometer.

Polymerase Chain Reaction (PCR)

KAPA Taq DNA polymerase was used to conduct the PCR procedure. The total volume of the reaction was 25 µL. The reaction mixture consists of 2 µL of each genomic DNA, 2.5 uL of 10 µM TaqA Buffer, 0.4 mM (0.85 µL) each

of the forward and reverse primers; Bact1442-F (AGAGTTGATCCTGGCTCAG) and Bact1492-R (GGTTACCTTGTTACGACTT), 1.25 mM (1.5 µL) of $MgCl_2$, 0.25 mM (0.2 µL) of dNTP The following conditions were used for the amplification: 5 min initial denaturation at 95 °C, followed by 35 cycles of 30 s at 94 °C (denaturation), 30 s at 60 °C (primer annealing), and 1 min at 72 °C for each cycle (extension). This was followed by a final extension of 10 minutes at 72^0C.

Gel electrophoresis

The 1.5 percent agarose gel used to separate the PCR products was stained with ethidium bromide. The size of the DNA fragment was measured using Ingenius' Syngene Gel Documentation System and compared to the Hyper Ladder-2K marker on a 1 percent agarose gel (CSL-AG500, Cleaver Scientific Ltd.) (IG31459). A positive result was the existence of a product with the anticipated size. The PCR product was then send for sequencing. DNA sequencing was performed according to manufacturer's instructions using the ABI Prism Big DyeTM 3730/3730XL Terminator Cycle Sequencing Ready Reaction Kit.

Alignment and phylogenetic analysis

Using BLAST at the National Center for Biotechnology Information, the 16S rRNA gene sequences of the isolated organisms were aligned and compared with the known 16S rRNA gene sequences in the Genbank database to find the closest database sequences. Using Clustal Omega for multiple sequence alignment, the isolate's 16S rRNA gene sequences were matched with sequences from the GenBank databases. The Neighbor-Joining method was used to infer the evolutionary record Molecular Evolutionary Genetics Analysis (MEGA 11.0) for Windows was used to create the phylogenetic tree. Finally, sequences were submitted to GenBank and were given an accession number.

3. Results and discussions

Plant growth-promoting rhizobacteria (PGPR) are rhizosphere bacteria that can promote plant development in response to various biotic and abiotic stressors (Aeron et al., 2020). In addition to *Rhizobium* and *Pseudomonas, Bacillus* are among the dominant species of bacteria so far isolated and proven to have plant growth promoting properties (Wang et al., 2021). In the present study, a total of 10 *Bacillus* spp. were isolated from soil in Kura local government area and CDA rice farms. Morphologically, almost all the isolates appeared to be milky white in colour and were mostly small and few large. As for shape, some were flat, irregular, and raised. All the isolates were sticky, and some are transparent while some were opaque. Microscopically, all the bacteria isolated were Gram positive, purple in colour, rod shaped and some appear singly, in chain or cluster and chained.

The biochemical results of the isolates were shown in **Table 1.** Isolates from different farmlands showed varying biochemical characteristics. The result of ammonia and hydrogen cyanide production by isolated *Bacillus* sp. is presented in **Table 2**. All the isolates displayed ammonia production with the exception of SA4 and SB3 while for hydrogen cyanide production, SA2, SA5 and SB3 were found not to secrete the compound. Several parameters are used to identify plant growth promoting bacteria. Ammonia and hydrogen cyanide production are among the most important features displayed by PGPR.

Table 1. Biochemical Characteristics of the isolates

Isolate	Catalase	Oxidase	Citrate	Urease	TSI	Spore test	Indole	Starch Hydrolysis	Motility
SA1	+	-	+	-	+	+	+	-	+
SA2	+	-	+	-	+	+	+	+	+
SA3	+	-	+	-	+	+	+	-	+
SA4	-	-	+	-	+	+	+	+	+
SA5	+	-	+	-	+	+	+	+	+
SB1	+	-	+	-	+	+	+	+	+
SB2	-	-	+	-	+	+	+	-	+
SB3	-	-	+	-	+	+	+	+	+
SB4	-	-	+	-	+	+	+	+	-
SB5	+	-	+	-	+	+	+	+	+

TSI= Triple Sugar Iron Test

Table 2. Screening results for NH_3 and HCN production of the Isolates

S/N	NH_3	HCN production
SA1	+	+
SA2	+	-
SA3	+	+
SA4	-	+
SA5	+	-
SB1	+	+
SB2	+	+
SB3	-	-
SB4	+	+
SB5	+	+

PGPR indirectly support crop development by producing ammonia, which causes the soil to become alkaline, preventing the growth of some pathogenic fungus, nitrobacteria, and inhibiting the germination of some pathogenic fungal spores (Mohanty et al., 2021). Directly, ammonia production can serve as a source of nitrogen to the plant which can enhance root and shoot growth (Abdelwahed et al., 2022). HCN is toxic to animals and bacteria. The compound blocks the electron transport chain, causing the cell's energy source to be cut off, resulting in the organism's death. It also interferes with the normal function of enzymes and natural receptors, notably the action of cytochrome oxidase. This is important to plants as the action can inhibit the growth of pathogenic organism surrounding the roots of the plants (Mazumdar et al., 2020). The three isolates were found to be the most ammonia-producing bacteria, indicating their potential as PGPR. Isolated bacteria in this investigation also produced HCN, indicating that these strains are potent PGPRs.

The isolates' HCN and NH3 production result is presented in **Table 2**. For the synthesis of ammonia, SA1, SA2, SA3, SA5, SB1, SB2, SB4, and SB5 are all positive, but for the production of HCN, SA1, SA3, SA4, SB1, SB2, SB4, and SB5 are all positive.

The quantitative result of Phosphate solubilisation presented as halo-zone in diameter (mm) by the isolates is shown in **Figure 1**. Phosphates occur as insoluble complexes especially in acidic soils which dominate the tropical countries. This makes it one of the unavailable mineral required by plants (Wang et al., 2021). Majority of

phosphate solubilising bacteria are from *Pseudomonas* species and the ability of bacteria to solubilise phosphate depends upon the release of organic acid by the microorganism (Pathak et al., 2019). The hydroxyl and carboxyl groups present in the organic acids normally quench cations and convert them to soluble forms. This is extremely important to plants as the process makes phosphates available for easy absorption by the roots of the plants (Tang et al., 2020). Based on the result presented, five of the 10 isolates had a visible halo zone. SA1 has the greatest value (28mm), followed by SA3 and SB1 (25mm). Luckily enough, these isolates also appeared to produce both ammonia and hydrogen cyanide. This made them potential candidates as PGPR.

The result of Zinc solubilisation by the isolates is presented in **Figure 1**. SA1 has the highest zone diameter, Followed by SA3 and SB1. Zinc is one of the micronutrient whose deficiency is not as a result of its availability but because of low solubility as a result of complex formation (Prasad et al., 2019). The element plays a vital role as cofactor for many enzymatic reactions in both plants and animals. However, certain species of growth promoting rhizobacteria can solubilise zinc from its complex and therefore makes it available for use by the plants (Pathak et al., 2019).

Production of indole 3 acetic acid is one of the prominent features of plant growth promoting rhizosphere. PGPR uses the production of IAA to manipulate the growth of their host through cell elongation, and organ development (Kumar, Patel, Meena, and Ramteke, 2019). The result of IAA production in (µg/ml) by the isolates is presented in **Figure 2**. From the results presented, SB1 has the highest IAA value of (99.61) followed by SA1 (99.17) and then SA3 (96.56) and were selected as the candidate isolate for further studies. The ability of a plant to acquire nutrient depends on its root and shoot length, IAA production by PGPR stimulate root and shoot elongation which eventually increases the capacity of the plant to absorb more nutrient leading to rapid growth of the plant (Kumar et al., 2019).

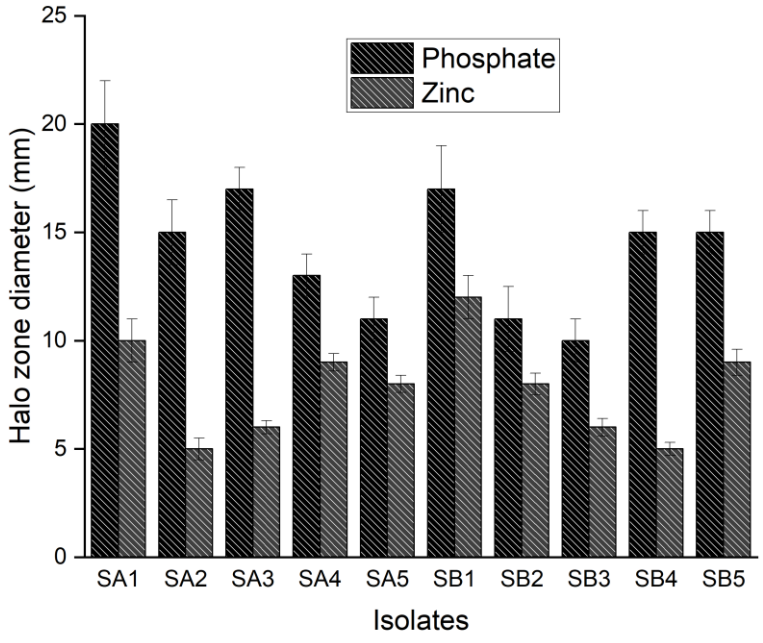

Fig. 1. Phosphate and Zinc solubilisation property of the isolates presented as halo zone diameter (mm). Values represent three independent determinations

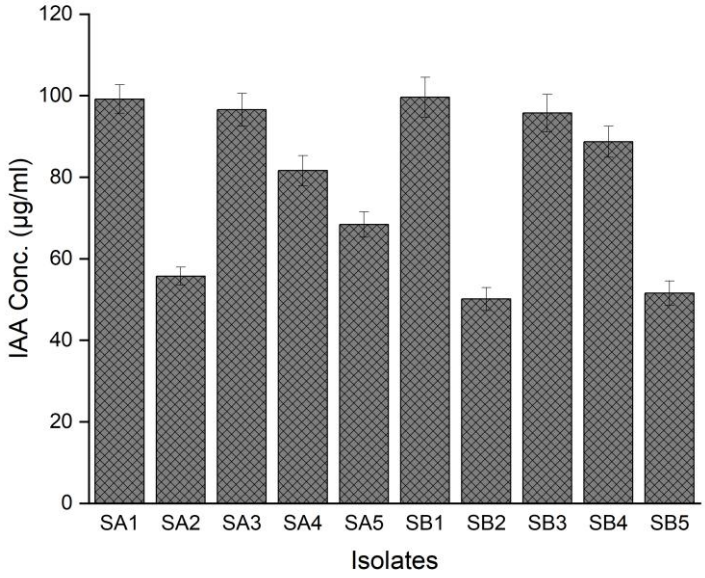

Fig. 2. Indole Acetic Acid (IAA) production by the Isolates. Values represent three independent determinations

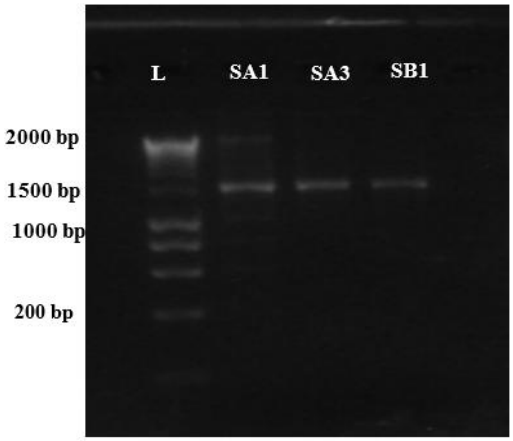

Fig. 3. Gel electrophoresis of the partial 16S rRNA of the PGPR isolates.

Having analyzed plant growth promoting properties displayed by the isolates, SA1, SA3 and SB1 appeared to relatively posses the features which will enable their usage as plant growth promoting Bacillus species. These bacteria were then chosen for further analysis. Gel electrophoresis was performed in order to visualize the amplified segment of the 16sRNA which was within the range of 1500 bp **Figure 3**. The 16S rRNA gene sequences of the bacteria isolated were compared with GenBank database using Blast Server at NCBI. Blast analysis of 16S rRNA sequences of the strains SA1, SA3 and SB1 revealed homology to *Bacillus subtilis* (99%), *Bacillus niacini*(98%), *Bacillus cereus* (99%), of the existing database of National Center of Bioinformatics, respectively (NCBI). Molecular phylogenetic studies using the neighbor joining method linked the identity of the obtained bacteria sequences to *Bacillus subtilis*, *Bacillus niacin* and *Bacillus cereus* (**Fig. 4**).

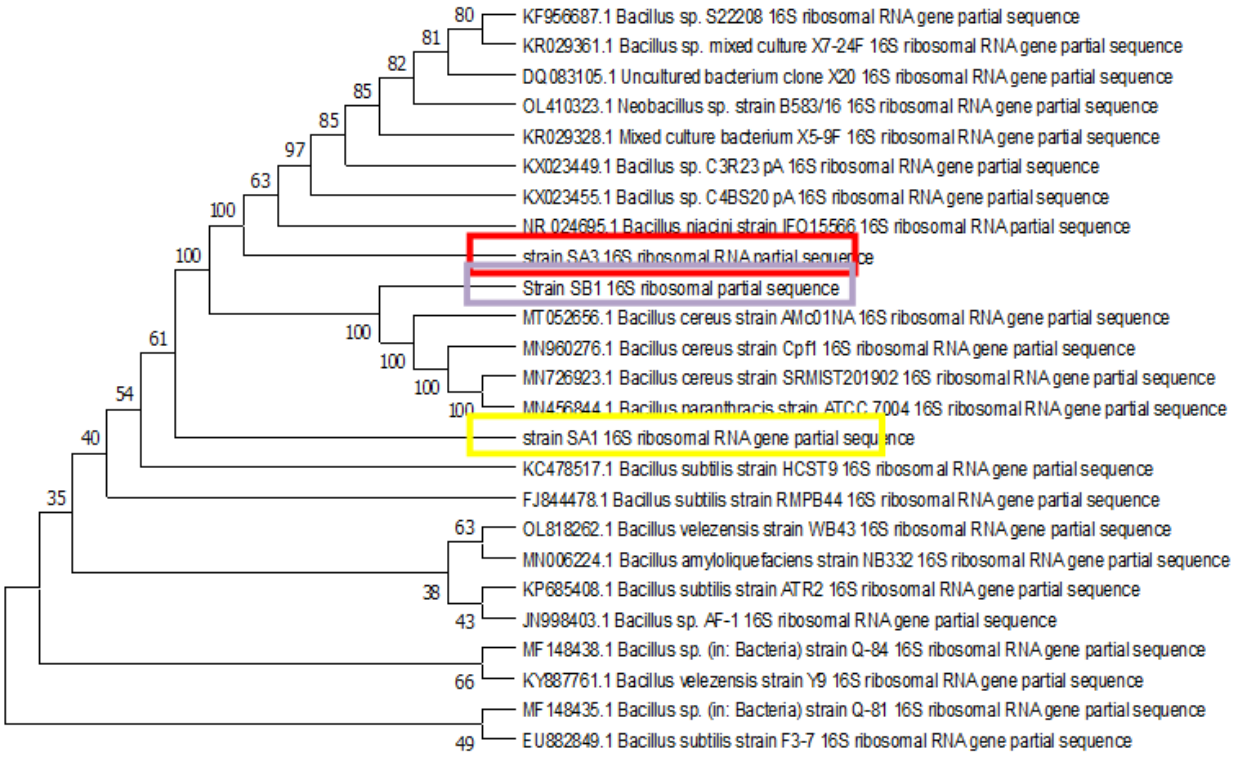

Fig. 4. Phylogenetic and Evolutionary relationships of taxa of strains. A rectangular rectangle denoted the strain's evolutionary position. Accession numbers are accompanied by the species' name. OM184294 (*Bacillus subtilis*) OM1842295 (*Bacillus niacini*) and OM184296 (*Bacillus cereus*)

Thus, these bacteria were tentatively assigned as *Bacillus subtilis strain* BUK_BCH_BTE_SA1, *Neobacillus niacini strain* BUK_BCH_BTE_SA3 and *Bacillus cereus* strain BUK_BCH_BTE_SB1 with accession numbers OM184294, OM1842295 and OM184296 respectively (**Fig. 4**). *Bacillus* spp. are the most available specie of rhizobacteria especially in tropical countries that were proven to dominate plant growth promoting specie of bacteria (Kashyap et al., 2019). Apart from promoting the growth through several mechanisms, *Bacillus* spp. were also known to reduce salinity stress in plants thereby prompting their utilization in salty environment (Kaloterakis et al., 2021).

Conclusions

In conclusion, ten bacterial strains were isolated from rhizosphere of two rice farms and screened for their plant growth promoting properties. Out of these ten, three isolates SB1, SB3 and SA proved to be the best from the results of the screening test. They were identified as *Bacillus subtilis*, *Bacillus niacini* and *Bacillus cereus* using 16SrRNA gene-based sequence. These three isolates have proven to be PGPRs. PGPRs are well known for their ability in plant growth promotion. Therefore, they could be used to enhance green agricultural revolution in crop production.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or

financial relationships that could be construed as a potential conflict of interest.

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References

1. Abdelwahed S, Saadouli I, Kouidhi S, Masmoudi AS, Cherif A, Mnif W, Mosbah, A (2022). A new pioneer colorimetric micro-plate method for the estimation of ammonia production by plant growth promoting rhizobacteria (PGPR). Main Group Chemistry 21(1):55-68. DOI: 10.3233/MGC-210077.
2. Adnan N, Nordin SM, Anwar A (2020) Transition pathways for Malaysian paddy farmers to sustainable agricultural practices: An integrated exhibiting tactics to adopt Green fertilizer. Land use policy 90:104255. https://doi.org/10.1016/j.landusepol.2019.104255.
3. Aeron A, Khare E, Jha CK, Meena VS, Aziz SMA, Islam MT, Rajashekara H (2020) Revisiting the plant growth-promoting rhizobacteria: lessons from the past and objectives for the future. Archives of microbiology 202(4):665-676. doi:10.1007/s00203-019-01779-w.
4. Basu A, Prasad P, Das SN, Kalam S, Sayyed R, Reddy M, El Enshasy H (2021) Plant growth promoting rhizobacteria (PGPR) as green bioinoculants: recent developments, constraints, and prospects. Sustainability 13(3):1140. https://doi.org/10.3390/su13031140.
5. Garrity GM, Brenner DJ, Krieg N, Staley J, Manual BS (2005) Systematic bacteriology. The Proteobacteria, Part C: The Alpha-, Beta-, Delta-, and Epsiloproteobacteria, Bergey's Manual Trust, Department of Microbiology and Molecular Genetics. Springer USA P2.
6. Gouda S, Kerry RG, Das G, Paramithiotis S, Shin HS, Patra JK (2018) Revitalization of plant growth promoting rhizobacteria for sustainable development in agriculture. Microbiological research 206:131-140. https://doi.org/10.1016/j.micres.2017.08.016.
7. Hashemnejad F, Barin M, Khezri M, Ghoosta Y, Hammer EC (2021) Isolation and identification of insoluble zinc-solubilising bacteria and evaluation of their ability to solubilise various zinc minerals. Journal of soil science and plant nutrition 21(3): 2501-2509. 10.1007/s42729-021-00540-x.
8. Kaloterakis N, van Delden SH, Hartley S, De Deyn GB (2021) Silicon application and plant growth promoting rhizobacteria consisting of six pure Bacillus species alleviate salinity stress in cucumber (*Cucumis sativus* L). Scientia Horticulturae 288: 110383. https://doi.org/10.1016/j.scienta.2021.110383.
9. Kashyap B K, Solanki MK, Pandey AK, Prabha, , Kumar P, Kumari B (2019) Bacillus as plant growth promoting rhizobacteria (PGPR): a promising green agriculture technology Plant health under biotic stress. Springer, pp. 219-236. DOI: 10.1007/978-981-13-6040-4_11.
10. Kumar A, Patel J, Meena VS, Ramteke P (2019) Plant growth-promoting rhizobacteria: strategies to improve abiotic stresses under sustainable agriculture. Journal of Plant Nutrition 42 (11-12):1402-1415. https://doi.org/10.1080/01904167.2019.1616757.

11. Mazumdar D, Saha SP, Ghosh S (2020) Isolation, screening and application of a potent PGPR for enhancing growth of Chickpea as affected by nitrogen level. International Journal of Vegetable Science 26(4):333-350. https://doi.org/10.1080/19315260.2019.1632401.
12. Mohanty P, Singh P K, Chakraborty D, Mishra S, Pattnaik R (2021) Insight into the role of PGPR in sustainable agriculture and environment. Frontiers in Sustainable Food Systems 5:667150. https://doi.org/10.3389/fsufs.2021.667150.
13. Mohite B. (2013) Isolation and characterization of indole acetic acid (IAA) producing bacteria from rhizospheric soil and its effect on plant growth. Journal of soil science and plant nutrition 13(3):638-649. http://dx.doi.org/10.4067/S0718-95162013005000051.
14. Molnár K, Nyárádi II, Bíró-Janka B, Simó I, Bálint J, Domokos E. (2020) Preliminary Study of the Effect of Chemical and Organic Fertilizers on a Semi-Natural Grassland in Vlăhiţa, Harghita Mountains, Romania. Acta Biologica Marisiensis 3(2):56-65. DOI: 10.2478/abmj-2020-0011.
15. Mustafa S, Kabir S, Shabbir U, Batool R (2019) Plant growth promoting rhizobacteria in sustainable agriculture: from theoretical to pragmatic approach. Symbiosis 78(2):115-123. doi:10.1007/s13199-019-00602-w.
16. Nazir N, Kamili AN, Shah D (2018) Mechanism of plant growth promoting rhizobacteria (PGPR) in Enhancing plant growth: A review. Int. J. Manag. Technol. Eng 8:709-721.
17. Nicolopoulou-Stamati P, Maipas S, Kotampasi C, Stamatis P, and Hens L (2016) Chemical pesticides and human health: the urgent need for a new concept in agriculture. Frontiers in public health 4:148. https://doi.org/10.3389/fpubh.2016.00148.
18. Pathak D, Lone R, Khan S, Koul K (2019) Isolation, screening and molecular characterization of free-living bacteria of potato (*Solanum tuberosum* L.) and their interplay impact on growth and production of potato plant under mycorrhizal association. Scientia Horticulturae 252: 388-397. https://doi.org/10.1016/j.scienta.2019.02.072.
19. Péterfi O, Domokos E (2018) Mutualistic and Endophytic Microorganisms of: Description, Role and Use. Acta Biologica Marisiensis 1(2):5-21. DOI:https://doi.org/10.2478/abmj-2018-0009.
20. Prasad M, Srinivasan R, Chaudhary M, Choudhary M, Jat LK (2019) Plant growth promoting rhizobacteria (PGPR) for sustainable agriculture: perspectives and challenges. PGPR amelioration in sustainable agriculture 2:129-157. https://doi.org/10.1016/B978-0-12-815879-1.00007-0.
21. Rai PK, Singh M, Anand K, Saurabh S, Kaur T, Kour D, Kumar M (2020) Role and potential applications of plant growth-promoting rhizobacteria for sustainable agriculture New and Future Developments in Microbial Biotechnology and Bioengineering Elsevier, pp. 49-60. https://doi.org/10.1016/B978-0-12-820526-6.00004-X.
22. Rani L, Thapa K, Kanojia N, Sharma N, Singh S, Grewal AS, and Kaushal J. (2021) An extensive review on the consequences of chemical pesticides on human health and environment. Journal of Cleaner Production 283:124657. https://doi.org/10.1016/j.jclepro.2020.124657.

23. Sabarwal A, Kumar K, and Singh, RP (2018) Hazardous effects of chemical pesticides on human health–Cancer and other associated disorders. Environmental toxicology and pharmacology 63:103-114. https://doi.org/10.1016/j.etap.2018.08.018.
24. Sansinenea E (2019) Bacillus spp. as plant growth-promoting bacteria: Secondary metabolites of plant growth promoting rhizomicroorganisms. Springer pp225-237. DOI: 10.1007/978-981-13-5862-3_11.
25. Saxena A, Kumar M, Chakdar H, Anuroopa N, Bagyaraj D.(2020) *Bacillus* species in soil as a natural resource for plant health and nutrition. Journal of applied microbiology 128(6): 1583-1594. https://doi.org/10.1111/jam.14506.
26. Sehrawat A, Sindhu SS, Glick BR (2022) Hydrogen cyanide production by soil bacteria: Biological control of pests and promotion of plant growth in sustainable agriculture. Pedosphere 32(1): 15-38. https://doi.org/10.1016/S1002-0160(21)60058-9.
27. Sharma N, and Singhvi, R (2017) Effects of chemical fertilizers and pesticides on human health and environment: a review. International journal of agriculture, environment and biotechnology 10(6):675-680. 10.5958/2230-732X.2017.00083.3.
28. Tang A, Haruna AO, Majid NMA, and Jalloh MB (2020) Potential PGPR properties of cellulolytic, nitrogen-fixing, phosphate-solubilizing bacteria in rehabilitated tropical forest soil. Microorganisms 8(3):442. https://doi.org/10.3390/microorganisms8030442.
29. Wang Y, Peng S, Hua Q, Qiu C. Wu P, Liu, X, Lin X (2021) The long-term effects of using phosphate-solubilizing bacteria and photosynthetic bacteria as biofertilizers on peanut yield and soil bacteria community. Frontiers in Microbiology 3:12-18. 10.3389/fmicb.2021.693535.