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The growth response of rice (*Oryza sativa* L. var. FARO 44) *in vitro* after inoculation with bacterial isolates from a typical ferruginous ultisol

Musa Saheed Ibrahim[1,2*] and Beckley Ikhajiagbe[2]

Abstract

Background: Rice forms a significant portion of food consumed in most household worldwide. Rice production has been hampered by soil factors such as ferruginousity which has limited phosphorus availability; an important mineral component for the growth and yield of rice. The presence of phosphate-solubilizing bacteria (PSB) in soils has been reported to enhance phosphate availability. In view of this, the present study employed three bacteria species (BCAC2, EMBF2 and BCAF1) that were previously isolated and proved P solubilization capacities as inocula to investigate the growth response of rice germinants in an *in vitro* setup. The bacteria isolates were first identified using 16S rRNA gene sequencing and then applied as inoculum. The inolula were prepared in three concentrations (10, 7.5 and 5.0 ml) following McFarland standard. Viable rice (var. FARO 44) seeds were sown in petri dishes and then inoculated with the three inocula at the different concentrations. The setup was studied for 28 days.

Results: 16S rRNA gene sequencing identified the isolates as: isolate BCAC2= *Bacillus cereus* strain GGBSU-1, isolate BCAF1= *Proteus mirabilis* strain TL14-1 and isolate EMBF2= *Klebsiella variicola* strain AUH-KAM-9. Significant improvement in rice germination, morphology, physiology and biomass parameters in the bacteria-inoculated setups was observed compared to the control. Germination percentage after 4 days was 100 % in the inoculated rice germinants compared to 65% in the control (NiS). Similarly, inoculation with the test isolates enhanced water-use efficiency by over 40%. The rice seedlings inoculated with *Bacillus cereus* strain GGBSU-1 (BiS) showed no signs of chlorosis and necrosis throughout the study period as against those inoculated with *Proteus mirabilis* strain TL14-1 (PiS) and *Klebsiella variicola* strain AUH-KAM-9 (KiS). Significant increase in chlorophyll-a, chlorophyll-b and alpha amylase was observed in the rice seedlings inoculated with BiS as against the NiS.

Conclusion: Inoculating rice seeds with *Bacillus cereus* strain GGBSU-1, *Proteus mirabilis* strain TL14-1 and *Klebsiella variicola* strain AUH-KAM-9 in an *in vitro* media significantly improved growth parameters of the test plant. *Bacillus cereus* strain GGBSU-1 showed higher efficiency due to a more improved growth properties observed.

Keywords: Growth response, Phosphate-solubilizing bacteria, Plant growth-promoting capabilities, FARO 44

Background

Before year 2050, agricultural production must increase by 70% in order to meet up with the ever-increasing world population and food demand. However, arable lands will only increase by 6% by 2050 (Alexandratos and Bruinsma 2012). Currently, in developing countries,

*Correspondence: Musasa39id@gmail.com
[1] Department of Biological Sciences, Admiralty University of Nigeria, Ibusa, Delta State, Nigeria
Full list of author information is available at the end of the article

larger percentage of arable land is facing poor biotic and abiotic factors which have consequently affected soil fertility (Beatrice et al. 2018). Cultivation of crops, such as maize and rice, has been hampered by these soil factors including high pH, low soil nitrogen and phosphorus deficiency (Adnan et al. 2018; Joshis et al. 2007). The combination of these poor soil conditions is found in ferruginous soils (Cho and Ponnamperuma 1971). Considering the state of rice as the most consumed food in Africa (Ajala and Gana 2015; Shiferaw et al. 2011), there is a need to improve rice cultivation to meet up with the ever-increasing population (FAOSTAT 2020).

Rice (*Oryza sativa* L.) is an edible starchy cereal grain consumed by more than 50% of the world population (Maclean et al. 2002). It is the most consumed food in Africa (Ajala and Gana 2015) because it has the potentials of improving nutrition, boost food security and foster rural development (Ojo and Adebayo 2012). Rice provides more than 20% of the total calories consumed by human universally (Udemezue 2018) and 15% of the protein requirements of human beings (Kennedy et al. 2002). In Nigeria, it has become an important strategic and daily staple food crop. It is mostly cultivated in the northern States such as Kano and least cultivated in southern states such as Edo State (Daramola 2005; Obayelu 2015). Rice cultivation is highly nutrient demanding, soil nutrient depletion, loss of soil organic matter (Nye and Greenland 1961), excessive soil tillage, low soil nitrogen, phosphorus deficiency and iron toxicity, which are considered major hindrances for rice crops (Adnan et al. 2018; Joshis et al. 2007). Linking these hindrances with other reasons may be the reason why some southern states in Nigeria such as Edo is experiencing low rice production.

Ferruginous soils that cover approximately 64 million km^2 and accounted for 45.2% of the Earth's surface area have been considered as a major hindrance for rice farming (Anumalla et al. 2019). In Nigeria for example, it is predominant in some southern States such as Edo state, occupying about seven zones, including extreme north and central Benin (Doyou et al. 2017). These soils are being known for their high iron content, low bioavailable phosphate, diminished nitrogen, low organic matter and poor water retention capacity (Wang et al. 2014). The high iron in ferruginous soils forms complexes with soil insoluble phosphate, therefore limiting the bioavailable phosphorus for plants (Gyaneshwar et al. 2002). These soil properties impair plant growth and development. In a bid to improve soil fertility, modern farmers have employed the use of synthetic phosphate fertilizers that had negatively compounded the fertility problem faced by the soil and caused crop damage and food insecurity as a result of deficiency in beneficial soil microorganisms that can solubilize and mineralize the insoluble phosphate and make it bioavailable for plants (Sharma et al. 2013). Therefore, given the negative environmental and agricultural impact of synthetic phosphate fertilizers and their increasing cost, the use of beneficial soil microorganisms such as plant growth-promoting rhizobacteria (PGPR) has increased globally during the last couple of decades (Ikhajiagbe and Edokpolo 2019; Ivy et al. 2018; Balayogan and Marimuthu 2014). For example, Saneya and Muhammad (2017) observed a 75% increase in the growth of Chili when inoculated with PGPR compared to synthetic fertilizers. However, for phosphate deficient soils, there is need to employ PGP bacteria strains with capabilities to solubilize inorganic phosphates (PSB) in order to improve the availability of phosphorus for crops that are susceptible to soil phosphorus deficiency such as rice.

Wu et al. (2014), Beneduzi et al. (2013) and Mamta et al. (2010) have successfully isolated PGPR bacteria, such as *Bacillus* sp. and *Pseudomonas* sp. from different sources, which also showed PSB capabilities. These bacteria have shown different capabilities in improving growth and yield of *Stevia rebaudiana*, sugarcane and cotton. Considering the differences in PGP and PSB capabilities of different bacteria strains from different sources, a previous study by Musa and Ikhajiagbe (2020) isolated culturable bacteria from ferruginous soils comparative to control soil in order to investigate their PSB capabilities for future use in soil improvement. Six soil samples of different ferruginous levels and a control were assayed in Pikovskaya's medium at 27°C with 7.5 pH for 7days. Six distinct isolates were observed among which three species (isolate BCAC2- *Bacillus* sp., isolate EMBF2- *Klebsiella* sp. and isolate BCAF1- *Proteus* sp.) showed phosphate-solubilizing capabilities and plant growth-promoting (PGP) capacities such as IAA and siderophores production. The isolates were also observed to show tolerance to acidic and alkaline media. Therefore, the current study was conducted to investigate the possibilities of these bacteria isolates (*Bacillus* sp., *Klebsiella* sp. and *Proteus* sp.) obtained from ferruginous soils and a control to influence the growth parameters of rice plant in an *in vitro* experimental setup. The current study may improve agricultural sustainability and improve crop productivity.

Methods

Collection of microbial isolates

Three phosphate-solubilizing bacteria species (isolates BCAC2, EMBF2 and BCAF1, respectively) that were previously isolated from ferruginous ultisol and humus soil in an earlier study in Benin City by Musa and Ikhajiagbe (2020). The three bacterial isolates were isolated by using serial dilution and were subjected to biochemical test

using catalase, indole, citrate, nitrogen fixing activity and bromotyhmol blue test. The PSB screening was done by drop plate method onto a developed Pikovskaya's growth medium. The isolates were also tested for pH tolerance level with HCL following (Mondala et al. 2016). PGP capabilities of the isolates were determined by IAA and siderophores production following Gupta et al. (2012b) and Balkar (2013), respectively. The isolates were eventually streak onto petri dishes for molecular identification using 16S rRNA sequencing.

Molecular characterization of phosphate solubilizing bacteria using 16S rRNA

DNA was extracted using the protocol stated by Saitou and Nei (1987). Briefly, single colonies grown on medium were transferred to 1.5 ml of liquid medium and cultures were left to grow on a shaker for 48 h at 28 °C. After this period, cultures were centrifuged at 4600g for 5 min. The resulting pellets were resuspended in 520 µl of TE buffer (10 mMTris-HCl, 1mM EDTA, pH 8.0). 15 microliters of 20% SDS and 3 µl of Proteinase K (20 mg/ml) were then added. The mixture was incubated for 1 hour at 37 °C, then 100 µl of 5 M NaCl and 80 µL of a 10% CTAB solution in 0.7 M NaCl were added and votexed. The suspension was incubated for 10 min at 65 °C and kept in ice for 15 min. An equal volume of chloroform: isoamyl alcohol (24:1) was added, followed by incubation in ice for 5 min and centrifugation at 7200g for 20 min. The DNA was collected by centrifugation at 13000g for 10 min, washed with 500 µl of 70% ethanol, air-dried at room temperature for approximately three hours and finally dissolved in 50 µl of TE buffer. The 16S rRNA gene was amplified by using polymerase chain reaction (PCR-MJ Research PTC-100) using primers 27F 5'- AGA GTT TGA TCM TGG CTC AG-3' and - 1525R, 5′-AAGGAGGTGATCCAGCC-3′ by using BLAST programme of GeneBank database on NCBI.

Sequencing

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual, while the sequencing kit used was BigDye terminator v3.1 cycle sequencing kit. Bio-Edit software and Clustal W using MEGA 6 were used for all genetic analysis. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length = 16.34337029 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) in the number units of base substitutions per site. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018).

Preparation of inoculum

The pure phosphate solubilizing bacteria (PSB) having plant growth-promoting (PGP) traits (BCAC2, EMBF2 and BCAF1) were prepared by streaking onto agar plates and incubated at 28°C for 48 hours. Upon growth after 48 hours, the isolates were inoculated in Nutrient broth which was used to grow the isolate, and then incubated for proliferation of cells and then prepared into 0.5 McFarland Standard with Cat. No (TM50) to standardize the approximate number of bacteria in the suspension. This process was used to prepare 500 mL of each bacterial isolate and therefore having an average microbial suspension of 1.5×10^8 cfu/mL.

In vitro seed inoculation

A soil-free culture experiment was conducted using improved high-yielding rice variety (FARO 44) that was previously obtained from Center for Dryland Agriculture, Bayero University, Kano. Rice seeds were tested for viability following (AOSA 2000). Those seeds that settles at the bottom were considered viable. The viable seeds were sown onto 40 pre-sterilized Petri dishes (9 cm diameter) at the rate of 20 seeds per petri dish that was lined with Whatman filter paper. McFarland (1944) standard of the 500mL bacterial inoculum was made into three treatments with four replicates (10, 7.5 and 5.0 mL) of each bacteria. The treatments were added up to make 10 mL by using distilled water. The control was made to be 10 mL of distilled water in four replicates. The calculated inoculum volumes were introduced into the petri dishes using 5mL syringe and covered at a temperature between 25 and 26.6°C as described by Etesami et al. (2014). The three bacteria were coded as (A= *Bacillus* sp., B= *Proteus* sp. and C= *Klebsiella* sp.). The setup was monitored for 28 days using randomized blocked design and wetted with 5 mL distilled water-containing nutrient solution (NPK) every 3 days. The plant growth and yield parameters were measured and recorded.

Germination parameters

Germination parameters are very effective in determining the interaction of PGP bacteria and seeds. The germination test was conducted at 25°C in a dark room at the Department of Plant Biology and Biotechnology, University of Benin Postgraduate Research laboratory to determine germination parameters. Following (ISTA 2005) as:

$$\text{Germination percentage (\%)} = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds sowed}} \times 100$$

The time at which primary leaf (coleoptile) emerged was observed by monitoring the seed germination after every 6 hours. Imbibition rate was measured by weighing the rice seeds at intervals of 6 hours until an equilibrium weight was observed. The weight (g) was plotted against time (h) following Jean-Francois et al. (2018). Water use efficiency of the seed (%) was calculated following Archana et al. (2017) as:

$$\text{Water applied} - \text{Water obtained after average imbibition} \times 100.$$

The average imbibition was attained at 30 hours. Germination time was calculated as the time at which the seed stops imbibition, while the speed of germination (%) was calculated based on the time of first seed emergence (30 hours) and the average time for complete seed emergence (72 hours) following a formula developed by (Krishnaswamy and Seshu 1990) with a slight modification as:

$$\text{Speed of germination} = \frac{\text{Number of seed germinated at 30 h}}{\text{Number of seed germinated at 72 h}}$$

Morphological parameters

Morphological parameters that are related to growth and yield of rice were investigated. Fresh shoot length, fresh root length and length of the first leaf were calculated in (cm) by using a transparent ruler that was mounted on a white calibrated paper from day 4 to 28 days after sowing. To obtain the dry root length (cm), seedlings were air-dried for 24 hours and the root was measured from day 4 to 28 days after sowing. The length of internodes (cm) was measured successive nodes using a sample of 5 best seedlings from all treatments weekly, while the number of secondary roots were carefully observed and counted daily.

Physiological parameters

To analyze the inoculum effect on the growth and yield of the rice plant, physiological parameters were investigated. Total soluble sugar was estimated at week 2 and 4 by oven-drying the tallest leaf at 70°C for 24h as described by Nelson (1944) with a slight modification by Sankar and Selvaraju (2015). Growth enzyme such as alpha amylase of the seedling extract was determined at day 28 after sowing by DNS method of Lowry et al. (1951) at a pH of 7.5. Chlorophyll content index (CCI) was determined using a non-destructive method employing Apogee chlorophyll concentration meter. The CCI of the old and new leaf were measured as average of the mesocotyl, mid-seedling and top seedling at 14th, 21st and 28th days after sowing. Chlorophyll a and b contents were investigated according to methods described by Arnon (1949), Maxwell and Johnson (2000). Leaf area was calculated using an android application (Leaf-IT) following Julian et al. (2017) at 28 days after sowing. Leaf tip chlorosis and necrosis was measured as the percentage of the total number of leaves produced by plants that showed significant level of chlorosis and necrosis. This was predecided following reports from Ikhajiagbe et al. (2017).

Biomass parameters

Weight of fresh seedling (g) was measured using analytical weighing balance daily from the 4th day – 28th day after sowing. Weight of dry seedling (g) was recorded after air drying the seedling for 24 hours and measured using analytical weighing balance. Seedling vigor index was determined according to Abdul-Baki and Anderson (1973) as:

$$\text{SV} = \frac{\text{Germination percentage} \times \text{means of seedling length (Root + Shoot)}}{100}$$

Statistical analysis

Data obtained were presented in means and standard errors of four replicates. Data were analyzed following two-way analysis of variance using GENSTAT (8th edition). Where significant *p*-values were obtained, differences between means were separated using Student Newman Keuls Test (Alika 2006).

Results

Molecular characterization and phylogenetic analysis of phosphate solubilizing bacteria

The 16S rRNA gene sequencing (of 1500 bp) results showed the isolate BCAC2 was recognized to be *Bacillus cereus* strain GGBSU-1, the isolate EMBF2 was identified as *Klebsiella variicola* strain AUH-KAM-9, while the isolate BCAF1 was identified as *Proteus mirabilis* strain TL14-1. The three isolates showed 96, 66 and 100

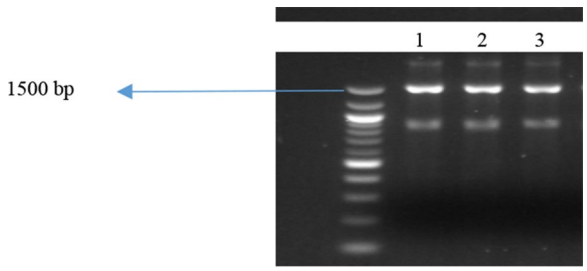

Fig. 1 PCR products of 16S rRNA (Line 1=BCAC2, Line 2= EMBF2, Line 3= BCAF1).

% resemblance with already sequenced species, respectively, as observed in the neighbor-joining dendrogram (Figs. 15, 16 and 17). The 16S rRNA gene product of all isolates in this study was equal (1.4Kb) even though the whole sequencing that includes the entire 1,500 bp region might be useful to distinguish between particular strains. The comparison bands in Fig. 1 and Table 1 showed the three isolates belongs to different taxonomic lineages.

Effect of inoculated PSBs on rice seed germination

At the first day after sowing, the 10ml BiS (A10) was observed to show the highest germination percentage (90%), while the lowest (55%) germination percentage was observed in the control. There was a significant difference (p<0.05) in the germination percentage at day one between the A10 and the 10ml PiS (B10). However, there was no significant difference between the A10 and the 10ml KiS (C10). At day 2, the A10 had complete germination (100%), while there was no increase in germination percentage at the NiS (control). At day 4, all the bacteria inoculated seeds were observed to have full germination. However, the control only had 10% increase in germination (Table 2).

The time at which seed coleoptile appears (APC) was studied (Fig. 2; Table 2). The results showed that the A10 had the first APC at 24hours, while delay was observed in PiS and KiS. The control setup had the latest APC of 48 hours. Water use efficiency (WUE) was also investigated after imbibition has been achieved at 30 hours; the control was observed to show the least WUE (38%), while the A10 showed the highest WUE (61%). A significant difference (P<0.05) was also observed between all the bacteria-inoculated seeds and the control. Seed germination time (GTM) which was calculated as the time at which the seeds stop imbibition were also monitored. The 10 ml BiS (A10), 7.5 ml BiS (A7.5), 5.0 ml BiS (A5), 10 ml PiS (B10), 10 ml KiS (C10), 7.5 ml KiS (C7.5) and 5.0 ML KiS (C5) stopped imbibition at the 30th hour after sowing, while the 7.5 ml PiS (B7.5) and 5.0 ml PiS (B5), stopped imbibition at 36th hour. The control (D10) was observed to stop imbibition at 42nd hour which showed delayed imbibition. The A10 was observed to show highest speed of germination (9%), while the lowest speed of germination was observed in the B7.5 and the control. Generally, the germination results indicated the highest positive influence of the BiS compared to PiS and KiS, while the control showed the lowest influence (1 %).

The present study investigated the rice seed imbibition rate. Five best seeds from A10, B10, C10 and the control setups were investigated for imbibition rate and presented in a graph of weight against imbibition time (Fig. 3). The A10 was observed to have the highest (0.034g) seed weight from 6 to 42 hours of the imbibition study, while the control was observed to show the lowest seed weight (0.029g). At 30 hours, imbibition was observed in the BiS, PiS and KiS, while the imbibition was delayed in the control until 36 hours after sowing. The result showed that the BiS had less time to germinate compared to PiS and KiS. However, the control took longer time to germinate.

Effect of inoculated PSBs on rice seedling morphological parameters

Significant differences (Table 3) were observed in rice shoot length between the different bacterial inoculations at varying days after sowing (DAS). The A10 showed the highest (80%) shoot length at all the reported DAS, while the control was observed to show the least shoot length (31%) at all the reported DAS. An increase in the inoculum concentration was also observed to improve the rice shoot length throughout the study. However, the C7.5 and B7.5 showed no significant difference throughout the study.

The effect of the three inoculations (*Bacillus* sp., *Proteus* sp. and *Klebsiella sp*.) on rice seedling morphology was further investigated by studying the fresh and dry root length (Table 4). The A10 was observed to show the longest root length (64%) from 4 to 28th DAS compared to the control (25%). Furthermore, the root length in KiS was observed to be significantly higher than the root length in the PiS. Meanwhile, between day 21st to 28th DAS, there was no significant different in root length between PiS, KiS and the control. The dry root length was also observed to show similar trend (Table 5).

The yield of rice seedling was determined by the distance between successive nodes. The length of internode (cm) was presented in Table 6. At 7DAS, all seedlings showed no presence of internode, while at 14DAS, the growing rice seedling in the A10 showed the highest internode length (3.1 cm) compared to the control which showed no internode. At 28 DAS, a significant increase in the internode length was observed in the growing

Table 1. Gene sequence of isolates

S/N	Isolates	Identity	Gene sequence
1	BCAC2	*Bacillus cereus* strain GGBSU-1	GGGGCATGCCTAACACATGCAAGTCGAACGGTAACAGGAAGCAGCTTGCTGCTTCGCTGACGAGTGGCGGACGGGTGAGTA ATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAATGTCGCAGGACCAAAGAG GGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTTGTTGGTGAGGTAACGGCTCACCAAGGCG ACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCA GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACT TTCAGCGGGGAGGAAGGGGATAAGGCTAATAACCTTGTTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCG TGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGT CAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTGGAGTCTTGTAGAGGGGGG TAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGGCGAAGGCGGCCCCCTGGACAAAG ACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTG GAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTAGACCGCCTGGGGAGTACGGCCGCAAGGTTAA AACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACC TGGTTTTGACATCCACAGAAGTTTNCAGAGATGNGAATGTGCCTTTGGGAACTGTGAGACAGGTGCTGCATGGCTGTCG TCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGA ACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGC TACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGT CCGGACTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTC CCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGG GCTTCCCCAT
2	EMBF2	*Klebsiella variicola* strain AUH-KAM-9	GGGGCATGCCTAACACATGCAAGTCGAACGGTAACAGGAAGCAGCTTGCTGCTTCGCTGACGAGTGGCGGACGGGTGAGTA ATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAATGTCGCAGGACCAAAGAG GGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTTGTTGGTGAGGTAACGGCTCACCAAGGCG ACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCA GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACT TTCAGCGGGGAGGAAGGGGATAAGGCTAATAACCTTGTTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCG TGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGT CAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTGGAGTCTTGTAGAGGGGGG TAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGGCGAAGGCGGCCCCCTGGACAAAG ACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTG GAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTAGACCGCCTGGGGAGTACGGCCGCAAGGTTAA AACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACC TGGTTTTGACATCCACAGAAGTTTNCAGAGATGNGAATGTGCCTTTGGGAACTGTGAGACAGGTGCTGCATGGCTGTCG TCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGA ACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGC TACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGT CCGGACTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTC CCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGG GCTTCCCCAT
3	BCAF1	*Proteus mirabilis* strain TL14-1	GGGGCGCATCTACACATGCAGTCGAGCGGTAACAGGAGAAAGCTTGCTTTCTTGCTGACGAGCGGCGGACGGGTGAGTAAT GTATGGGGATCTGCCCGATAGAGGGGGATAACTACTGGAAACGGTGGCTAATACCGCATAATGTCTACGGACCAAAGCAGG GGCTCTTCGGACCTTGCACTATCGGATGAACCCATATGGGATTAGCTAGTAGGTGGGGTAAAGGCTCACCTAGGCGACGAT CTCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGG GAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTAGGGTTGTAAAGTACTTTCAG CGGGGAGGAAGGTGATAAGGTTAATACCCTTATCAATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCA GCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCAATTAAGTCAG ATGTGAAAGCCCCGAGCTTAACTTGGGAATTGCATCTGAAACTGGTTGGCTAGAGTCTTGTAGAGGGGGGGGTAGAATTCCA TGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCA GGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTAGAGGTTGTGGT CTTGAACCGTGGCTTCTGGAGTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTG ACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGCA ATCCTTTAGAGATAGAGGAGTGCCTTCGGGAACGCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGT TGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCACGTGATGGTGGGAACTCAAAGGAGACTGCCGGT GATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCAGA TACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGAACTCATAAAGTCTGTCGTAGTCCGGATTGGAGTCTGCAACTCGA CTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCG TCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTACCACTTGGATTCAA

seedling from 5.1 cm in BiS compared to the control (1.1 cm).

To verify the effect of the PSB bacteria with PGP capabilities on rice seedlings, the number of secondary roots in all the experimental setup was determined (Table 7). The results showed a significant increase ($P<0.05$) in the number of secondary root in the setup inoculated with the bacteria compared to the control for all the analyzed days. At 4DAS, no secondary root was observed in the B10, B7.5, C5 and the control, while at 6DAS, (3) secondary roots were seen in the control.

Table 2 Germination parameters of rice inoculated with PSBs and control

Samples	Germination (%) on				APC (Hrs)	WUE (%)	GTM (Hrs)	SGM (%)
	1st DAS	2nd DAS	3rd DAS	4th DAS				
A 10	90 ± 5.7^{a}	100 ± 2.3^{a}	100 ± 2.1^{a}	100 ± 2.1^{a}	24 ± 0.5^{a}	61 ± 2.1^{a}	30 ± 0.2^{a}	9 ± 0.5^{a}
A 7.5	85 ± 2.8^{a}	90 ± 2.8^{a}	95 ± 2.4^{a}	100 ± 2.4^{a}	30 ± 0.5^{b}	60 ± 5.7^{a}	30 ± 0.2^{a}	8.5 ± 0.6^{a}
A 5.0	60 ± 2.3^{b}	70 ± 2.5^{ba}	90 ± 2.6^{a}	100 ± 5.3^{a}	30±0.5cb	56 ± 0.4^{c}	30 ± 0.5^{a}	2 ± 0.5^{b}
B 10	70 ± 2.1^{ab}	75 ± 5.3^{c}	80 ± 5.1^{a}	90 ± 5.7^{a}	42 ± 0.5^{d}	51 ± 2.1^{d}	30 ± 5.3^{a}	7 ± 0.5^{a}
B 7.5	85 ± 5.7^{a}	85 ± 5.4^{ba}	85 ± 5.6^{a}	100 ± 5.7^{a}	30±0.5eb	49 ± 2.1^{d}	36 ± 0.4^{b}	1 ± 0.0^{c}
B 5.0	50 ± 2.3^{cb}	50 ± 2.4^{d}	60 ± 2.4^{b}	65 ± 5.7^{b}	48 ± 0.5^{f}	43 ± 3.0^{f}	36 ± 0.2^{b}	2.5 ± 0.2^{d}
C 10	85 ± 2.1^{a}	85 ± 2.1^{a}	100 ± 5.7^{a}	100 ± 2.8^{a}	30 ± 0.5^{gb}	53 ± 0.4^{d}	30 ± 0.4^{a}	5.6 ± 0.05^{e}
C 7.5	85 ± 3.3^{a}	90 ± 0.9^{a}	95 ± 2.8^{a}	100 ± 5.7^{a}	36 ± 0.5^{h}	51 ± 0.2^{d}	30 ± 0.4^{a}	8.5 ± 0.2^{a}
C 5.0	60 ± 2.4^{db}	60 ± 2.5^{e}	70 ± 2.8^{c}	75 ± 2.4^{c}	30 ± 0.5^{jb}	51 ± 0.3^{d}	30 ± 0.4^{a}	6 ± 0.5^{f}
D 10	55 ± 3.4^{eb}	55 ± 2.5^{f}	55 ± 5.7^{d}	65 ± 2.8^{d}	48 ± 0.5^{j}	38 ± 3.1^{j}	42 ± 0.1^{a}	1 ± 0.0^{c}

Results with similar alphabetic superscripts on same column did not differ from each other (p>0.05). Results were in mean and standard error of four replicates. DAS=Day after sowing, APC= Appearance of coleoptile, WUC= Water used efficiency, GTM= Germination time, SGM= Speed of germination. A10, A7.5 and A5.0 = 10 ml, 7.5 ml and 5.0 ml of *Bacillus* sp., respectively. B10, B7.5 and B5.0 = 10 ml, 7.5 ml and 5.0 ml *Proteus* sp. respectively. C10, C7.5 and C5.0 = 10 ml, 7.5 ml and 5.0 ml of *Klebsiella sp*., respectively. D10= 10 ml of control.

The length of first leaf in the growing rice seedling in the current experimental setup was also observed (Fig. 4). The A10 was observed to show the highest length (cm) of the first leaf at 1, 2, 3 and 4 weeks after sowing (WAS), while the control soil was observed to show the least length. A delay in first leaf emergence was also observed in the B7.5, C5 and the control until 2WAS. A significant difference was obtained between the A10 and all other bacteria inoculated setups (PiS and KiS). The significant increase in the length of the first leaf as observed in the A10 is indicating the effectiveness of the *Bacillus cereus* strain GGBSU-1 in improving yield and growth of rice.

Effect of inoculated PSBs on rice seedling physiological parameters

The effect of the bacterial inocula on the total sugar contents of rice seedling was significant at 2 and 4WAS in all the test samples (Fig. 5). At 2WAS, the content of total sugars was the highest in the A10 (18.0mg/g^{-1} fw) and the lowest (2.6 mg/g^{-1} fw) in the control. A similar trend was also observed at 4WAS. Furthermore, it was observed that the total soluble sugar of C10 at 2 and 4WAS was significantly higher than B10. However, there was no significant differences between C7.5 and B7.5.

Table 8 shows Chlorophyll-a (Chl a) and b (Chl b) contents of rice seedlings at 2 and 4WAS. For Chl a, the highest value was observed in the A10 (6.4 mg/cm^2 fw), while the least was observed in the control (2.3 mg/cm^2 fw) at 2WAS. Similar trend was also observed at 4WAS. However, there was no significant difference (P>0.05) in Chl a between A10, A7.5 and C10. Furthermore, a significant difference (P<0.05) was observed in the old and new leaf chlorophyll content index (CCI) between the BiS and the control. However, no significant difference was observed in CCI within the BiS, PiS and KiS.

During the study, necrosis and chlorosis were observed at the leaf tip of rice seedlings (Fig. 6) at 2, 3 and 4WAS and recorded in Table 8. At 2WAS, all seedlings in the control, C5, B5, B7.5 and A5 showed observable signs of different levels of chlorosis and necrosis, while the highest percentage chlorosis and necrosis (66.6–76 %) were found in the control at 3 and 4WAS. Delayed chlorosis and necrosis were observed in the A7.5, B10 and C10 treatments until 3WAS. No chlorosis were detected in A10 and C7.5 setup throughout the study.

The leaf area is one of the determinants of growth an yield of plants, in the current study, the highest leaf area (2.0 cm^2) was observed in the rice BiS, while the lowest (0.15 cm^2) was observed in the control using leaf-IT mobile application (Fig. 7). Significant difference was observed between the A10 and all other bacteria inocula (PiS and KiS).

Figure 8 indicates that BiS, PiS and KiS showed a significant improvement in seedling α-amylase at 2 and 4WAS. At 2WAS, the C10 was observed to show the highest α-amylase (15.8 U), while the control was observed to show the least (6.7 U). At 4WAS, a change in trend was observed where the A10 was observed to show the highest α-amylase (45.3 U). However, the control was still observed to show the least α-amylase (18.4 U) at 4WAS. Significant differences were also observed between the other inocula (PiS and KiS) and the control.

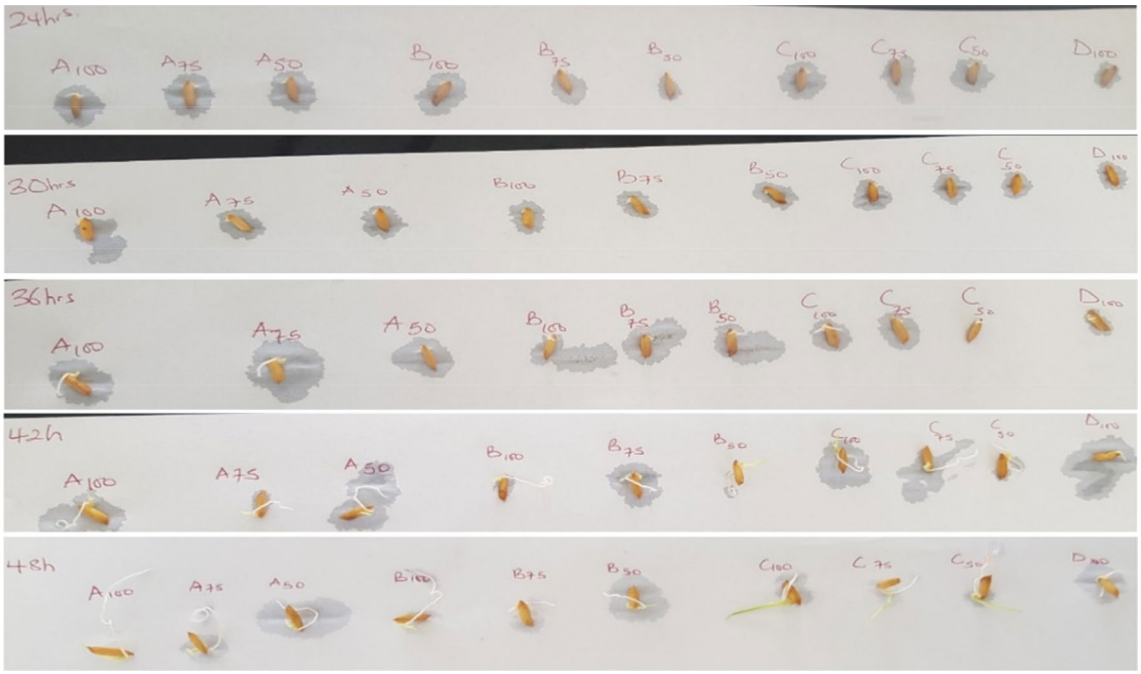

Fig. 2 Appearance of rice seed coleoptile after exposure to experimental treatments.

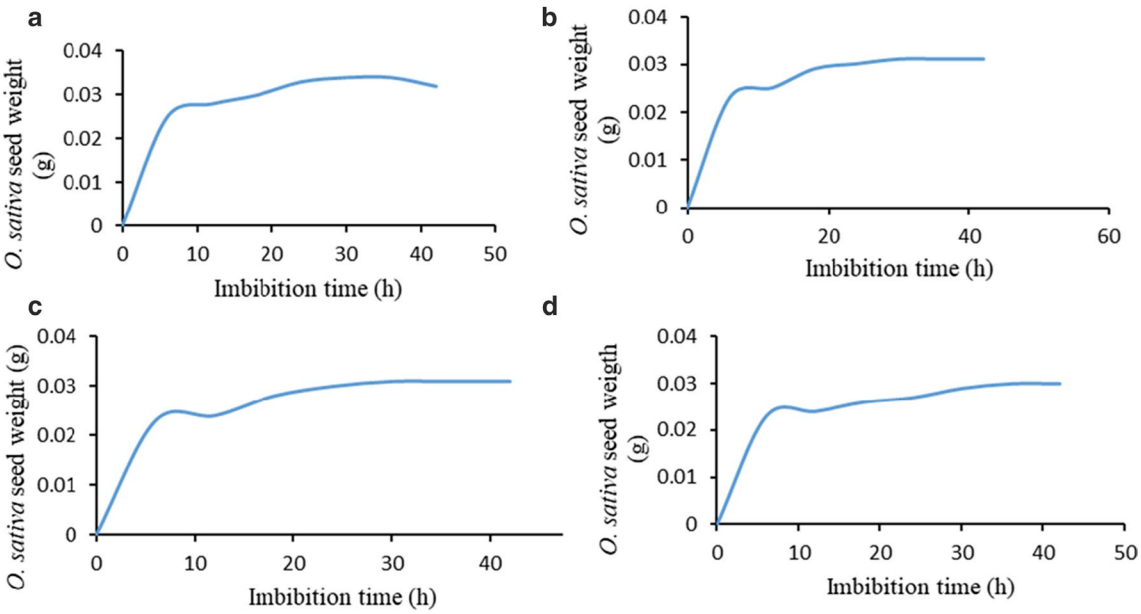

Fig. 3 Imbibition rate of rice germinants after exposure to treatments

Effect of inoculated PSBs on rice seedling biomass parameters

Table 9 shows a significant increase in the dry and fresh shoot weight in the A10 compared to the control. However, there was no significant differences between the PiS and KiS, even though the KiS was slightly higher than the PiS at all the assayed days.

Table 3 Effect of PSB inoculum on in vitro rice fresh shoot length

Inoculum	Fresh shoot length (cm) on					
	4DAS	5DAS	6DAS	2WAS	3WAS	4WAS
A/10	2.6 ± 0.07^{a}	4.4 ± 0.22^{a}	5.3 ± 0.36^{a}	8.5 ± 0.02^{a}	11.9 ± 0.64^{a}	15.4 ± 0.35^{a}
A/7.5	1.9 ± 0.07^{b}	3.8 ± 0.14^{a}	5.3 ± 0.21^{a}	7.5 ± 0.38^{b}	10.5 ± 0.26^{b}	13.2 ± 0.36^{b}
A/5	1.4 ± 0.06^{c}	3.7 ± 0.25^{a}	4.5 ± 0.08^{b}	7.1 ± 0.35^{b}	9.5 ± 0.00^{c}	11.2 ± 0.23^{c}
B/10	1.3 ± 0.07^{c}	2.8 ± 0.28^{b}	3.2 ± 0.11^{c}	5.7 ± 0.04^{c}	6.8 ± 0.25^{d}	8.4 ± 0.13^{d}
B/7.5	1.2 ± 0.03^{c}	3.2 ± 0.10^{c}	3.3 ± 0.06^{c}	5.0 ± 0.09^{c}	6.2 ± 0.37^{d}	7.0 ± 0.43^{e}
B/5	1.4 ± 0.16^{c}	3.2 ± 0.02^{ac}	3.3 ± 0.63^{c}	4.5 ± 0.07^{d}	5.3 ± 0.11^{e}	6.0 ± 0.18^{f}
C/10	1.9 ± 0.18^{b}	3.4 ± 0.21^{ac}	3.4 ± 0.08^{c}	6.0 ± 0.26^{e}	7.2 ± 0.15^{f}	8.9 ± 0.59^{d}
C/7.5	1.6 ± 0.05^{c}	3.7 ± 0.26^{a}	3.2 ± 0.31^{c}	5.0 ± 0.56^{c}	6.8 ± 0.34^{d}	7.6 ± 0.08^{e}
C/5	1.1 ± 0.03^{c}	3.2 ± 0.08^{ac}	3.4 ± 0.21^{c}	4.7 ± 0.10^{d}	6.2 ± 0.16^{d}	7.9 ± 0.25^{ed}
D/10	1.1 ± 0.03^{j}	2.2 ± 0.06^{cd}	2.3 ± 0.20^{d}	3.7 ± 30.03^{f}	4.7 ± 0.20^{g}	4.9 ± 0.37^{g}

DAS= Days after sowing, WAS= Weeks after sowing. Results with similar alphabetic superscripts on same column did not differ from each other (p>0.05). A10, A7.5 and A5.0 = 10 ml, 7.5 ml and 5.0 ml of *Bacillus* sp., respectively. B10, B7.5 and B5.0 = 10 ml, 7.5 ml and 5.0 ml *Proteus* sp., respectively. C10, C7.5 and C5.0 = 10 ml, 7.5 ml and 5.0 ml of *Klebsiella sp*., respectively. D10= 10 ml of control

Table 4 Effect of PSB inoculum on in vitro rice fresh root

Inoculum	Fresh root length (cm) on					
	4DAS	5DAS	6DAS	2WAS	3WAS	4WAS
A/10	3.3 ± 0.36^{a}	5.0 ± 0.08^{a}	5.2 ± 0.10^{a}	7.0 ± 0.10^{a}	8.8 ± 0.24^{a}	9.3 ± 0.01^{a}
A/7.5	2.8 ± 0.21^{b}	4.2 ± 0.15^{b}	4.6 ± 0.30^{b}	5.2 ± 0.06^{b}	6.8 ± 0.38^{b}	7.1 ± 0.04^{b}
A/5	2.2 ± 0.88^{c}	4.3 ± 0.49^{b}	4.6 ± 0.26^{b}	5.1 ± 0.30^{b}	6.8 ± 0.37^{b}	7.0 ± 0.23^{b}
B/10	1.2 ± 0.11^{d}	3.3 ± 0.05^{c}	3.3 ± 0.06^{c}	3.7 ± 0.13^{c}	4.9 ± 0.44^{c}	5.6 ± 0.11^{c}
B/7.5	1.1 ± 0.06^{d}	2.6 ± 0.24^{d}	3.1 ± 0.07^{c}	3.2 ± 0.16^{d}	4.4 ± 0.15^{c}	5.1 ± 0.23^{c}
B/5	1.7 ± 0.63^{e}	3.2 ± 0.06^{c}	3.3 ± 0.07^{c}	3.6 ± 0.03^{c}	4.1 ± 0.07^{d}	5.1 ± 0.03^{c}
C/10	3.2 ± 0.08^{e}	4.5 ± 0.09^{b}	4.7 ± 0.12^{b}	4.9 ± 0.11^{e}	5.3 ± 0.33^{e}	5.9 ± 0.06^{c}
C/7.5	2.6 ± 0.31^{b}	3.3 ± 0.12^{c}	3.5 ± 0.14^{c}	4.4 ± 0.34^{f}	5.3 ± 0.32^{e}	5.6 ± 0.05^{c}
C/5	1.7 ± 0.21^{e}	3.5 ± 0.28^{c}	3.7 ± 0.38^{c}	4.6 ± 0.15^{e}	5.4 ± 0.30^{e}	5.5 ± 0.07^{c}
D/10	0.6 ± 0.20^{f}	1.7 ± 0.27^{e}	2.0 ± 0.25^{d}	3.1 ± 0.03^{d}	3.8 ± 0.23^{f}	4.0 ± 0.09^{d}

DAS= Days after sowing, WAS= Weeks after sowing. Results with similar alphabetic superscripts on same column did not differ from each other (p>0.05). A10, A7.5 and A5.0 = 10 ml, 7.5 ml and 5.0 ml of *Bacillus* sp., respectively. B10, B7.5 and B5.0 = 10 ml, 7.5 ml and 5.0 ml *Proteus* sp., respectively. C10, C7.5 and C5.0 = 10 ml, 7.5 ml and 5.0 ml of *Klebsiella sp*., respectively. D10= 10 ml of control.

Seedling vigor index

The maximum seedling vigor index (22.23) was observed in the A10, while the least (4.89) was observed in the control (Fig. 9). There was significant differences (P<0.05) in the seed vigor index between all the bacterial inocula and the control. However, no significant difference were observed between the A5, C7.5 and B7.5. These results clearly showed the beneficial influence of the growth-promoting bacteria on rice seedling health and yield.

Discussion

The 16S rRNA results showed *Bacillus cereus* strain GGBSU-1, strain TL14-1, and *Klebsiella variicola* strain AUH-KAM-9 as the thr*Proteus mirabilis*ee identified PGP bacteria with proven PSB capabilities. Numerous *Bacillus* strains express plant growth-promoting (PGP) activities and they have been used to improve growth of crops. For example, Beneduzi et al. (2008) had isolated the plant growth-promoting strain SVPR30 identified by 16S rRNA gene sequence as *Bacillus* spp. and had been used to promote plant growth through *in vivo* experiments. Behera et al. (2014) reported *Bacillus* and *Proteus* strains as the most powerful and abundant PGP bacteria and improved the growth of rice species. *Klebsiella* sp. has also been frequently found to be associated with hospital infections (Podschun and Ullmann 1998); however, the pathogenicity of the *K. variicola* isolates from plants has not been determined (Rosenblueth et al. 2004), but *K. variicola* DX120E have been documented by Bashan et al. (2013) to promote sugarcane growth.

Table 5 Effect of PSB inoculum on in vitro dry root of rice.

Inoculum	Dry root length (cm) on					
	4DAS	5DAS	6DAS	2WAS	3WAS	4WAS
A/10	2.0 ± 0.11^{a}	3.2 ± 0.46^{a}	4.1 ± 0.15^{a}	4.8 ± 0.10^{a}	6.8 ± 0.52^{a}	7.3 ± 0.03^{a}
A/7.5	1.1 ± 0.08^{b}	2.6 ± 0.15^{b}	3.3 ± 0.16^{b}	4.1 ± 0.18^{b}	5.7 ± 0.20^{b}	6.1 ± 0.04^{b}
A/5	1.2 ± 0.17^{b}	2.3 ± 0.12^{c}	3.4 ± 0.03^{b}	3.9 ± 0.36^{b}	5.4 ± 0.04^{c}	5.9 ± 0.11^{c}
B/10	0.5 ± 0.16^{c}	1.3 ± 0.05^{d}	2.4 ± 0.03^{c}	3.3 ± 0.17^{c}	4.5 ± 0.11^{d}	5.0 ± 0.32^{d}
B/7.5	0.7 ± 0.00^{c}	1.0 ± 0.06^{e}	2.1 ± 0.07^{d}	2.8 ± 0.26^{d}	4.4 ± 0.40^{d}	4.8 ± 0.43^{e}
B/5	0.2 ± 0.05^{d}	1.2 ± 0.06^{de}	2.4 ± 0.06^{c}	3.2 ± 0.18^{c}	4.0 ± 0.25^{e}	4.3 ± 0.11^{f}
C/10	1.2 ± 0.65^{b}	2.0 ± 0.31^{f}	2.8 ± 0.23^{e}	3.5 ± 0.05^{cb}	6.0 ± 0.32^{f}	7.0 ± 0.09^{a}
C/7.5	2.3 ± 0.00^{a}	2.7 ± 0.79^{b}	3.1 ± 0.14^{b}	3.5 ± 0.05^{cb}	5.2 ± 0.07^{cg}	5.6 ± 0.78^{c}
C/5	0.2 ± 0.00^{d}	1.0 ± 0.06^{e}	1.8 ± 0.32^{f}	2.9 ± 0.33^{d}	5.0 ± 0.35^{g}	5.5 ± 0.54^{c}
D/10	0.2 ± 0.15^{d}	1.1 ± 0.09^{d}	1.4 ± 0.90^{g}	2.1 ± 0.08^{e}	4.1 ± 0.05^{e}	4.6 ± 0.32^{e}

DAS = Days after sowing, WAS = Weeks after sowing. Results with similar alphabetic superscripts on same column did not differ from each other ($p>0.05$). A10, A7.5 and A5.0 = 10 ml, 7.5 ml and 5.0 ml of *Bacillus* sp., respectively. B10, B7.5 and B5.0 = 10 ml, 7.5 ml and 5.0 ml *Proteus* sp., respectively. C10, C7.5 and C5.0 = 10 ml, 7.5 ml and 5.0 ml of *Klebsiella sp*., respectively. D10= 10 ml of control.

Germination parameters are very effective in determining the interaction of PGP bacteria and seeds. In the present study, the *Bacillus cereus* strain GGBSU-1 inoculated seed showed the highest germination percentage in comparison with the *Proteus mirabilis* strain TL14-1, *Klebsiella variicola* strain AUH-KAM-9 and the control which showed delayed germination. This indicated a positive impact of the *Bacillus cereus* strain GGBSU-1 inocula on the rice seed germination. This positive impact had led to a fast appearance of coleoptile at 24 hours in the *Bacillus cereus* strain GGBSU-1 inocula compared to the control which observed delayed until 48 hours. Certain bacteria species such as *Bacillus subtilis* have been shown to significantly enhance germination parameters of mustard family and several other plant species (Wu et al. 2016). The water use efficiency of rice seeds, germination time and speed of germination were also influenced by the *Bacillus cereus* strain GGBSU-1, *Proteus mirabilis* strain TL14-1 and *Klebsiella variicola* strain AUH-KAM-9 inoculation. This is likely as a result of the ability of these bacteria to change the seed microbiome, thereby improving germination parameters as against the control. This is consistent with the work of Gupta et al. (2012a) who observed that treatment of plants with certain PSB bacteria consequently improved germination of maize seeds.

Table 6 Effect of PSB inoculum on in vitro rice length of internode

Inoculum	Length of internode (cm)			
	1WAS	2WAS	3WAS	4WAS
A/10	Not present	3.1 ± 0.26^{a}	4.3 ± 0.25^{a}	5.1 ± 0.11^{a}
A/7.5	Not present	2.0 ± 0.26^{b}	3.2 ± 0.33^{b}	4.1 ± 0.56^{b}
A/5	Not present	0.8 ± 0.26^{c}	2.7 ± 0.22^{c}	3.3 ± 0.11^{c}
B/10	Not present	0.3 ± 0.23^{c}	1.1 ± 0.12^{d}	2.2 ± 0.22^{d}
B/7.5	Not present	0.0 ± 0.03^{c}	1.2 ± 0.22^{d}	1.9 ± 0.34^{e}
B/5	Not present	Not present	0.5 ± 0.11^{e}	0.8 ± 0.54^{f}
C/10	Not present	1.6 ± 0.23^{b}	3.4 ± 0.43^{b}	3.7 ± 0.11^{c}
C/7.5	Not present	1.5 ± 0.12^{b}	2.9 ± 0.13^{c}	3.2 ± 0.44^{c}
C/5	Not present	0.7 ± 0.31^{c}	2.2 ± 0.54^{c}	2.6 ± 0.77^{d}
D/10	Not present	Not present	0.3 ± 0.56^{e}	1.1 ± 0.32^{g}

DAS = Days after sowing, WAS = Weeks after sowing. Results with similar alphabetic superscripts on same column did not differ from each other ($p>0.05$). A10, A7.5 and A5.0 = 10 ml, 7.5 ml and 5.0 ml of *Bacillus* sp., respectively. B10, B7.5 and B5.0 = 10 ml, 7.5 ml and 5.0 ml *Proteus* sp., respectively. C10, C7.5 and C5.0 = 10 ml, 7.5 ml and 5.0 ml of *Klebsiella sp*., respectively. D10= 10 ml of control.

Seed germination could be divided into three phases (Bewley 1997). As reported, phase II of rice seed germination is the stage between 20 and 50h after sowing (Yang et al. 2007). In order to determine the influence of the PSBs on rice seed germination, the present study observed a faster imbibition rate in the *Bacillus cereus* strain GGBSU-1-inoculated seed compared to the control. This clearly showed the positive influence of the *Bacillus cereus* strain GGBSU-1 and its ability to influence the seed microbiome, leading to rapid cell division and early activation of enzymes such as amylase that are liable for solubilizing spare food material and deliver energy and other fundamental food material for the germinating embryo (Renu 2018).

Morphological parameters are related to plant physical and external properties. In the present study, all the bacterial inoculations were observed to improve rice seedling morphological characters such as shoot length, fresh and dry root length, internode length and length of the first leaf. However, the *Bacillus cereus* strain

Table 7 Influence inoculum on number of secondary roots

Inoculants	Number of secondary roots						
	4DAS	5DAS	6DAS	7DAS	2WAS	3WAS	4WAS
A/10	4DAS	5 ± 0.47^{a}	9 ± 0.47^{a}	11 ± 0.47^{a}	15 ± 0.47^{a}	20 ± 0.47^{a}	27 ± 0.47^{a}
A/7.5	3 ± 0.47^{a}	4 ± 0.47^{a}	8 ± 0.47^{a}	9 ± 0.47^{b}	12 ± 0.47^{b}	14 ± 0.47^{b}	16 ± 0.47^{b}
A/5	1 ± 0.47^{b}	4 ± 0.47^{a}	7 ± 0.47^{b}	8 ± 0.47^{b}	11 ± 0.47^{b}	14 ± 0.47^{b}	19 ± 0.47^{c}
B/10	Not present	2 ± 0.47^{b}	7 ± 0.47^{b}	9 ± 0.47^{b}	9 ± 0.47^{c}	13 ± 0.47^{b}	16 ± 0.47^{b}
B/7.5	Not present	2 ± 0.47^{b}	7 ± 0.47^{b}	9 ± 0.47^{b}	9 ± 0.47^{c}	11 ± 0.47^{c}	15 ± 0.47^{b}
B/5	1 ± 0.47^{b}	1 ± 0.47^{d}	3 ± 0.47^{c}	5 ± 0.47^{c}	6 ± 0.47^{d}	9 ± 0.47^{d}	13 ± 0.47^{d}
C/10	1 ± 0.47^{b}	2 ± 0.47^{b}	7 ± 0.47^{b}	9 ± 0.47^{b}	10 ± 0.47^{d}	13 ± 0.47^{b}	17 ± 0.47^{b}
C/7.5	3 ± 0.47^{a}	1 ± 0.47^{d}	6 ± 0.47^{b}	6 ± 0.47^{c}	8 ± 0.47^{c}	13 ± 0.47^{b}	15 ± 0.47^{b}
C/5	Not present	3 ± 0.47^{a}	4 ± 0.47^{c}	6 ± 0.47^{c}	6 ± 0.47^{d}	8 ± 0.47^{d}	11 ± 0.47^{e}
D/10	Not present	Not present	3 ± 0.47^{c}	3 ± 0.47^{d}	5 ± 0.47^{d}	7 ± 0.47^{de}	9 ± 0.47^{f}

DAS = Days after sowing, WAS = Weeks after sowing. Results with similar alphabetic superscripts on same column did not differ from each other ($p>0.05$). A10, A7.5 and A5.0 = 10 ml, 7.5 ml and 5.0 ml of *Bacillus* sp., respectively. B10, B7.5 and B5.0 = 10 ml, 7.5 ml and 5.0 ml *Proteus* sp., respectively. C10, C7.5 and C5.0 = 10 ml, 7.5 ml and 5.0 ml of *Klebsiella sp*., respectively. D10= 10 ml of control.

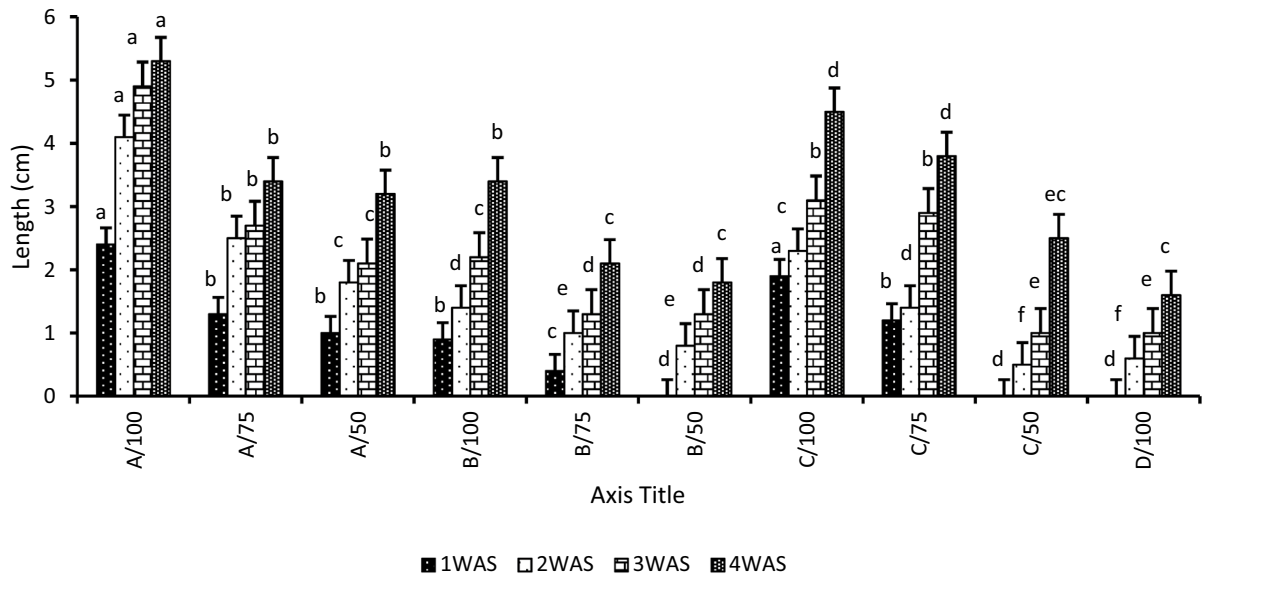

Fig. 4 Length of first leaf (WAS—weeks after sowing). Results with similar alphabets on same bars did not differ from each other ($p>0.05$)

GGBSU-1-inoculated seed was observed to show the highest improvement than *Proteus* sp. and *Klebsiella sp*. inoculations. This indicates the ability of the *Bacillus cereus* strain GGBSU-1 to improve growth properties of rice. This may be as a result of the ability of the bacteria to produce some metabolites such as IAA and siderophores that facilitate the plant growth. According to Dey et al. (2004), IAA secreted by rhizobacteria may effectively improve root growth by stimulating plant cell elongation or division, resulting in greater root surface area, which enables the plant to access more nutrients from soils. The present study indicated that the inoculation of *Bacillus cereus* strain GGBSU-1, *Proteus mirabilis* strain TL14(1) and *Klebsiella varicola* strain AUH-KAM-9 influence the morphological parameters of rice seedlings at different levels.

The significant increase in rice seedling secondary root observed in all the PSB bacteria-inoculated setup at all the assayed days indicates growth-promoting capabilities of the isolates. Some earlier reports have

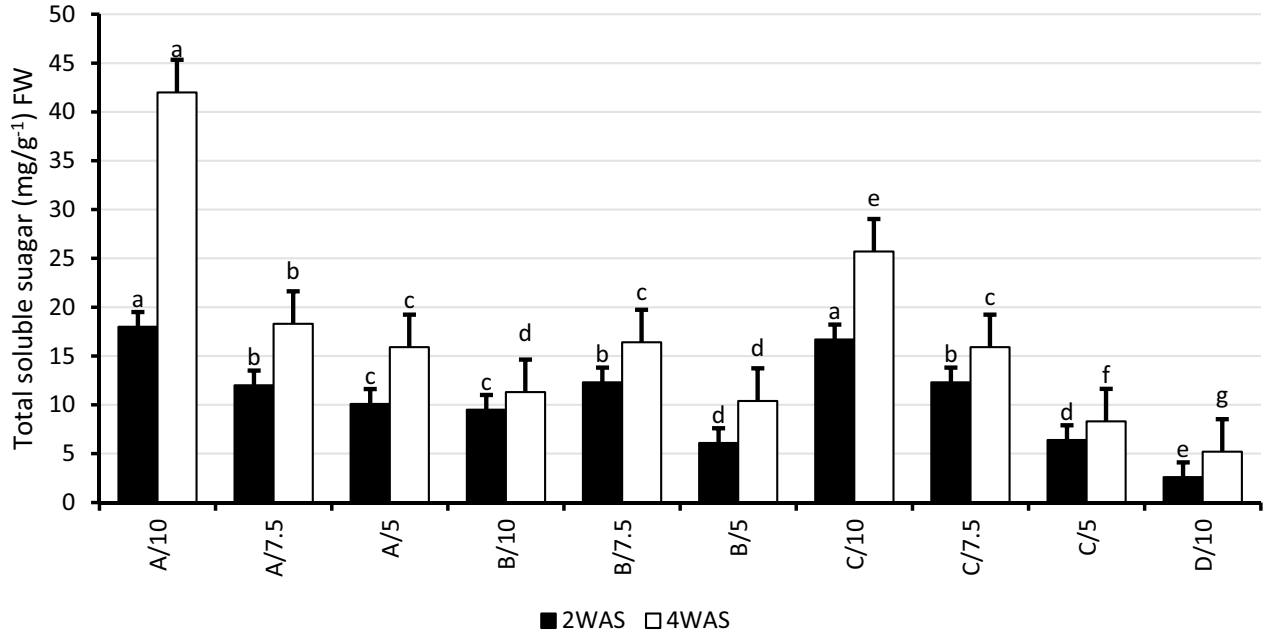

Fig. 5 Total soluble sugar contents of rice seedlings at 2 and 4 weeks after sowing. Results with similar alphabets on same bars did not differ from each other ($p>0.05$).

Table 8 Effect of PSB inoculum on in vitro rice physiological parameters

Inoculum	Chlorophyll-a (mg/cm^2) FW on		Chlorophyll-b (mg/cm^2) FW on		OL CCI (µmol/m^2) on			NL CCI (µmol/m^2) on			Leaf tip % Necrosis and Chlorosis on			LLA (cm^2) on
	2WAS	4WAS	2WAS	4WAS	2WAS	3WAS	4WAS	2WAS	3WAS	4WAS	2WAS	3WAS	4WAS	4WAS
A/10	6.4	8.9	1.9	4.6	1.2	1.3	1.5	1.1	1.4	1.7	ND	ND	ND	2.0
A/7.5	6.1	8.1	1.6	3.2	1.1	1.1	1.3	0.9	1.0	1.3	ND	33.3	25	0.6
A/5	4.5	6.5	0.6	1.0	1.1	1.2	1.3	1.1	1.2	1.4	50	66.6	50	0.6
B/10	4.0	7.2	2.4	3.0	1.1	1.1	1.2	1.2	1.3	1.5	ND	33.3	50	0.4
B/7.5	4.3	6.5	1.7	2.3	1.2	1.3	1.4	0.6	0.9	1.2	50	33.3	25	0.3
B/5	4.1	5.1	0.8	1.2	1.1	1.2	1.3	0.4	0.7	1.0	50	33.3	50	0.4
C/10	6.1	8.0	2.5	4.3	1.3	1.4	1.5	1.1	1.3	1.5	ND	33.3	25	0.6
C/7.5	5.3	7.8	1.2	3.0	1.3	1.3	1.4	1.0	1.2	1.4	ND	ND	ND	0.3
C/5	4.0	6.5	0.8	1.0	1.0	1.1	1.2	0.6	0.9	1.3	100	66.6	50	0.3
D/10	2.3	4.1	0.3	0.7	0.6	0.8	1.0	0.3	0.7	1.0	100	66.6	76	0.15

identified certain bacteria as effective in improving plant root length, thereby improving the uptake of water and nutrient which consequently increase seedling growth and yield morphology (Walker et al. 2012) as observed in the present study. This is consistent with the work of (Zamioudis et al. 2013) who observed that under the influence of certain beneficial bacteria, the root number and structure of Arabidopsis seedlings were improved.

Plant productivity can be accessed using total sugars. The increase in total soluble sugar content observed in rice seedlings at 2 and 4WAS indicated the important role of the inoculum in improving rice seedling sugar content, which consequently plays an important role in plant metabolism, growth and hormone signaling (Rolland et al. 2002). Seedlings with high sugar contents may signify improved growth and yield. According to Lucas et al. (2014), different bacterial strains modify different

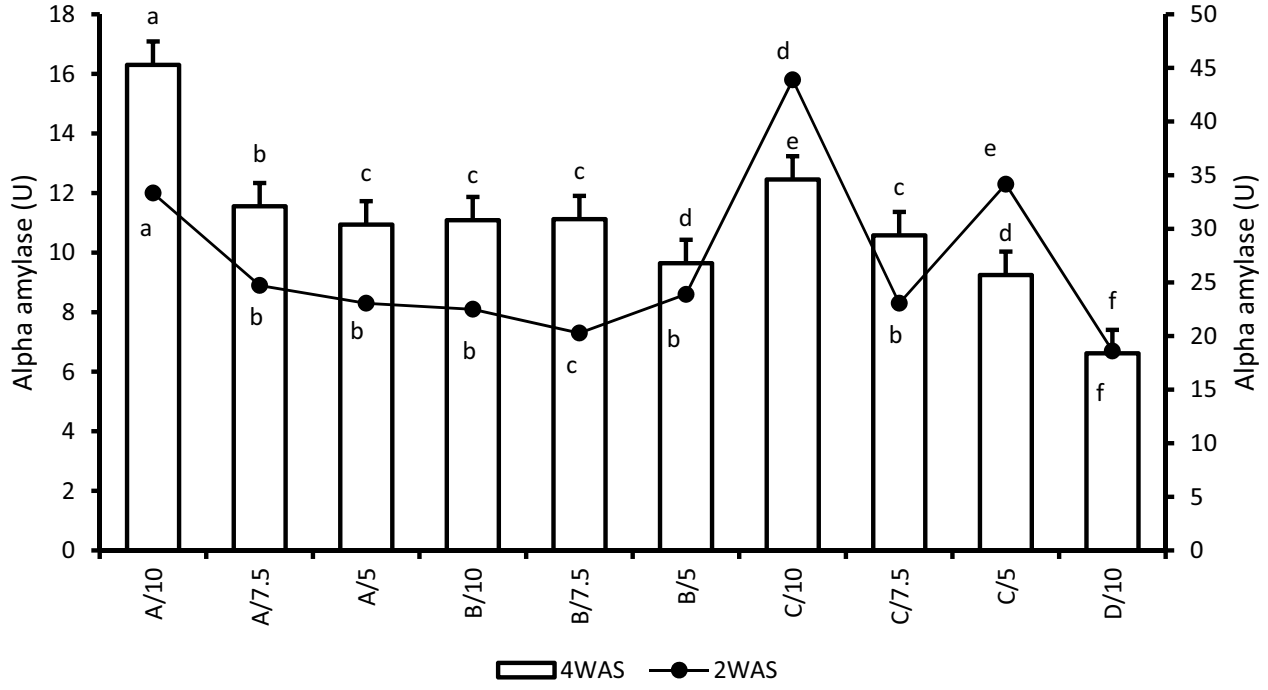

Fig. 6 Leaf tip necrosis and chlorosis

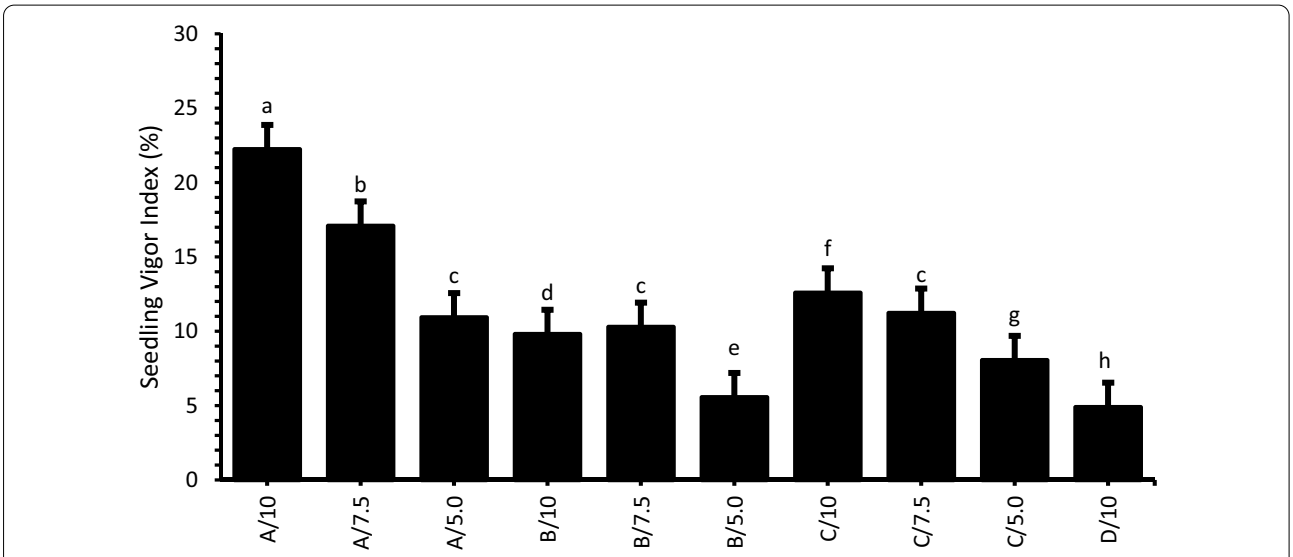

Fig. 7 Leaf area measurement using leaf-IT

innate plant mechanism such as total soluble sugar at different levels. Garcia-Cristobal et al. (2015) also reported the ability of a *Chryseobacterium* strain to enhance total sugar level in pathogen-stressed rice plants.

The increase in chlorophyll levels is considered to be a parameter indicating increase in photosynthetic capacity and consequently increase plant yield and vigor (Bashan et al. 2006). The increase in Chlorophyll-a (Chl a) and b (Chl b) contents and chlorophyll index content (CCI) of rice seedlings at 2 and 4WAS was significant in influencing the bacteria. Bacterial strains can improve plant photosynthetic rate that benefit plant growth (Chaves et al. 2003). Since lack of chlorophyll, damaged roots and hormonal imbalances are known to result in chlorosis

Fig. 8 Alpha amylase at week 2 and 4. Results with similar alphabets on same bars (or line graph) did not differ from each other ($p>0.05$).

and eventual necrosis of seedlings, the lack of chlorosis and necrosis observed in the *Bacillus cereus* strain GGBSU-1 and *Salmonella enterica sub. arizonae* strain DSM9386 inocula indicate the morphological influence of the bacteria in rice seedling. This may be attributed to that the inoculum improved the seedling hormone levels in the *Bacillus cereus* strain GGBSU-1 and *Salmonella enterica sub. arizonae* strain DSM9386-inoculated seeds. The increase in leaf area in the bacteria-inoculated seeds as against the control can be linked to the beneficial influence of the bacteria on seedling enzymes. According to Ehrlich (1990), the major role of bacteria in seed is to stimulate plant growth through production of phytohormones.

The improved levels of α-amylase at 2 and 4WAS in the PSB inoculated seeds may likely be as a result of the improved photosynthetic capacity and enzymes metabolism caused by the PGP nature of the isolates. Since α-amylase has been shown to be very important in initiating starch degradation in cereal grains (Beck and Ziegler 1989), higher α-amylase showed higher starch degradation and higher energy for further development and metabolic activities.

The improved fresh and dry shoot weight observed in the inoculum-induced rice seedling showed the plant growth-promoting capabilities of the isolates. The higher shoot weight may consequently signify improved growth and yield of the test plant. This result is consistent with the work of Sarma and Saikia (2014) who suggested that *Bacillus lentimorbus* and *Pseudomonas aeruginosa* strains enhanced seedling height and shoot weight of *Vigna radiata* plants under drought stress.

Significantly higher levels of seed vigor are requisite in high seed health and improved seedling yield. The effect of the bacteria inocula on rice seedling vigor showed the beneficial influence of the growth-promoting bacteria on rice seedling health and yield. The present result agreed with the work of Yadav et al. (2016), who investigated

Table 9 Fresh and dry weights of rice shoot

Inoculum	Weight of fresh shoot (g^{-1}) on						Weight of dry shoot (g^{-1}) on					
	4DAS	5DAS	6DAS	2WAS	3WAS	4WAS	4DAS	5DAS	6DAS	2WAS	3WAS	4WAS
A/10	0.43 ± 0.23^{a}	0.72 ± 0.11^{a}	0.97 ± 0.12^{a}	1.67 ± 0.43^{a}	2.31 ± 0.44^{a}	3.01 ± 0.12^{a}	0.20 ± 0.11^{a}	0.32 ± 0.55^{a}	0.53 ± 0.43^{a}	0.69 ± 0.23^{a}	0.72 ± 0.42^{a}	0.89 ± 0.66^{a}
A/7.5	0.33 ± 1.20^{b}	0.45 ± 0.32^{b}	0.48 ± 0.23^{b}	0.62 ± 0.77^{b}	1.00 ± 0.12^{b}	2.12 ± 0.33^{b}	0.20 ± 0.12^{a}	0.22 ± 0.67^{b}	0.28 ± 0.43^{b}	0.31 ± 0.23^{b}	0.33 ± 0.41^{b}	0.39 ± 0.56^{b}
A/5.0	0.30 ± 0.34^{b}	0.37 ± 0.22^{c}	0.42 ± 0.45^{c}	0.67 ± 0.92^{b}	0.92 ± 0.01^{c}	1.02 ± 0.33^{c}	0.20 ± 0.12^{a}	0.23 ± 0.65^{b}	0.28 ± 0.65^{b}	0.30 ± 0.34^{b}	0.43 ± 0.23^{c}	0.44 ± 0.45^{c}
B/10	0.36 ± 0.22^{cb}	0.42 ± 0.23^{b}	0.47 ± 0.11^{b}	0.62 ± 0.22^{b}	0.91 ± 0.01^{c}	1.11 ± 0.34^{d}	0.20 ± 0.45^{a}	0.22 ± 0.65^{b}	0.24 ± 0.67^{b}	0.28 ± 0.65^{b}	0.34 ± 0.34^{b}	0.39 ± 0.32^{b}
B/7.5	0.30 ± 0.37^{b}	0.32 ± 0.23^{d}	0.39 ± 0.12^{d}	0.57 ± 0.11^{c}	0.87 ± 0.87^{d}	1.01 ± 0.44^{c}	0.18 ± 0.43^{a}	0.20 ± 0.45^{b}	0.21 ± 0.78^{c}	0.24 ± 0.34^{c}	0.29 ± 0.54^{d}	0.34 ± 0.34^{b}
B/5.0	0.29 ± 0.44^{b}	0.33 ± 0.23^{d}	0.39 ± 0.11^{d}	0.52 ± 0.21^{d}	0.80 ± 0.09^{d}	1.02 ± 0.65^{c}	0.12 ± 0.22^{b}	0.19 ± 0.42^{b}	0.21 ± 0.43^{c}	0.23 ± 0.34^{c}	0.30 ± 0.54^{b}	0.33 ± 0.33^{b}
C/10	0.39 ± 0.44^{d}	0.48 ± 0.34^{b}	0.46 ± 0.11^{b}	0.73 ± 0.11^{e}	1.12 ± 0.76^{e}	1.98 ± 0.76^{b}	0.23 ± 0.22^{a}	0.25 ± 0.43^{c}	0.29 ± 0.23^{b}	0.57 ± 0.33^{d}	0.63 ± 0.64^{e}	0.71 ± 0.32^{d}
C/7.5	0.31 ± 0.46^{b}	0.38 ± 0.12^{c}	0.42 ± 0.11^{c}	0.56 ± 0.11^{d}	0.76 ± 0.44^{d}	0.98 ± 0.55^{e}	0.20 ± 0.12^{a}	0.22 ± 0.23^{b}	0.24 ± 0.56^{b}	0.29 ± 0.43^{b}	0.33 ± 0.64^{b}	0.37 ± 0.12^{b}
C/5.0	0.29 ± 0.42^{b}	0.32 ± 0.23^{d}	0.35 ± 0.21^{e}	0.42 ± 0.12^{f}	0.64 ± 0.35^{f}	0.77 ± 0.64^{f}	0.16 ± 0.34^{c}	0.17 ± 0.12^{b}	0.19 ± 0.76^{c}	0.24 ± 0.64^{c}	0.27 ± 0.23^{d}	0.29 ± 0.12^{e}
D/10	0.27 ± 0.42^{e}	0.27 ± 0.68^{e}	0.28 ± 0.11^{f}	0.39 ± 0.11^{g}	0.60 ± 0.23^{f}	0.72 ± 0.23^{f}	0.11 ± 0.45^{d}	0.11 ± 0.12^{d}	0.12 ± 0.65^{d}	0.21 ± 0.64^{c}	0.26 ± 0.42^{d}	0.28 ± 0.23^{e}

WAS= Weeks after sowing, DAS= Days after sowing. Results with similar alphabetic superscripts on same column did not differ from each other ($p>0.05$). A10, A7.5 and A5.0 = 10 ml, 7.5 ml and 5.0 ml of *Bacillus* sp., respectively. B10, B7.5 and B5.0 = 10 ml, 7.5 ml and 5.0 ml *Proteus* sp., respectively. C10, C7.5 and C5.0 = 10 ml, 7.5 ml and 5.0 ml of *Klebsiella sp.*, respectively. D10= 10 ml of control.

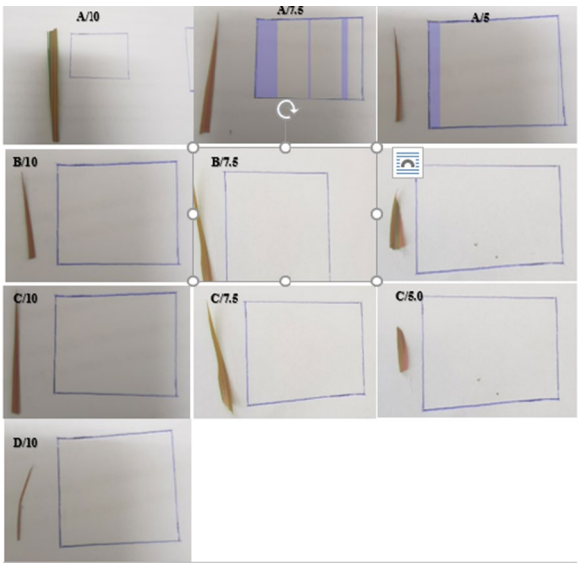

Fig. 9 Seedling vigor index. Results with similar alphabets on same bars did not differ from each other (p>0.05).

the influence of endophytic fungi on plant growth and seed vigor of green gram seeds. Furthermore, Singh et al. (2010) has also reported improved seed germination and seedling vigor upon inoculation with plant growth-promoting rhizobacteria on lentil seeds.

Conclusion

The present study investigated the application of three distinct phosphate solubilizing bacteria with plant growth-promoting capabilities that were previously isolated from ferruginous soils and humus soil as a trial to investigate the growth and yield response of rice seeds. Results of this study showed that bacteria-inoculated rice had significant growth and yield properties compared to the un-inoculated setup. Also, the concentration of the inoculum was observed to be directly proportional to the improving growth and yield properties. The three bacteria showed different levels of growth and yield properties on rice seedling. The setup inoculated with *Bacillus cereus* strain GGBSU-1 (A) was observed to show the highest yield effect on the rice seeds, followed by *Klebsiella variicola* strain AUH-KAM-9 (C), while *Proteus mirabilis* strain TL14-1 (B) had the least rice growth parameters. Since these bacteria proved effective in improving growth and yield parameters of rice in an *in vitro* setup, further studies should be conducted to trail the *in vivo* effectiveness of these bacteria in phosphorus-deficient soils such as ferruginous soils. This would provide a sustainable way of enhancing crop production and toxic metals remediation.

Appendix

See Figs. 10, 11, 12, 13, 14, 15, 16, and 17.

Fig. 10 Showing *In vitro* germination of rice after inoculation with the three PSBs at 2DAS. DAS= Days after sowing, A= *Bacillus* sp., B= *Proteus* sp., C= *Klebsiella sp*., D= Control.

Fig. 11 Showing a sample of *In vitro* growth of rice after inoculation with PSB at 3DAS. DAS= Days after sowing.

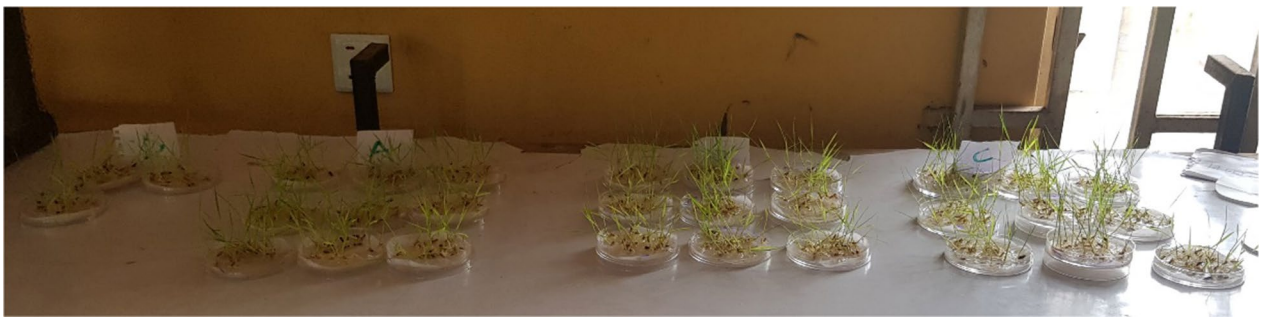

Fig. 12 *In vitro* growth of rice after inoculation with the three PSBs at 5DAS. DAS= Days after sowing, A= *Bacillus* sp., B= *Proteus* sp., C= *Klebsiella sp*., D= Control.

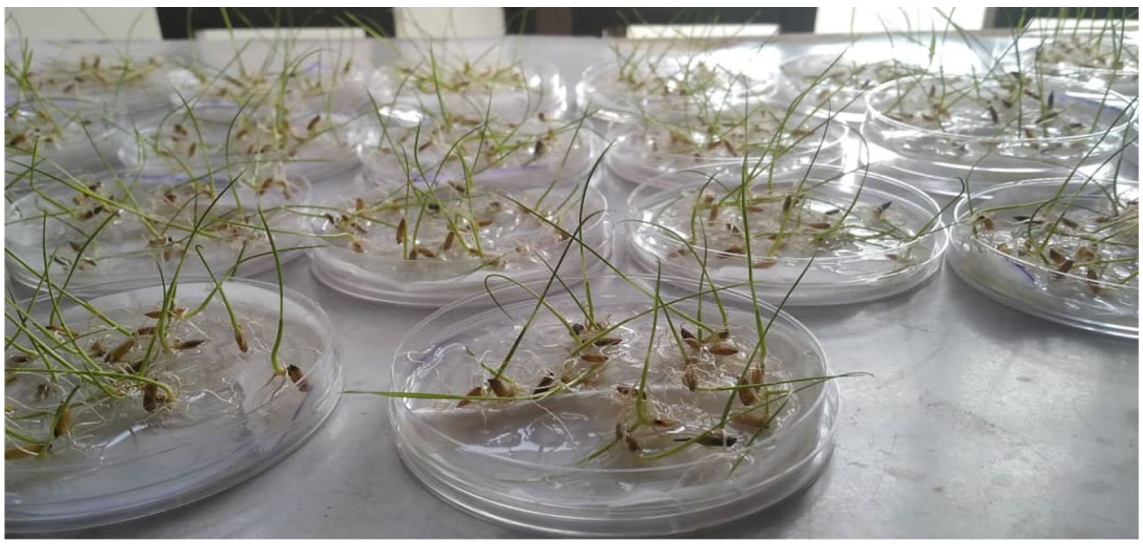

Fig. 13 *In vitro* growth of rice after inoculation with the three PSBs at 6DAS. DAS= Days after sowing.

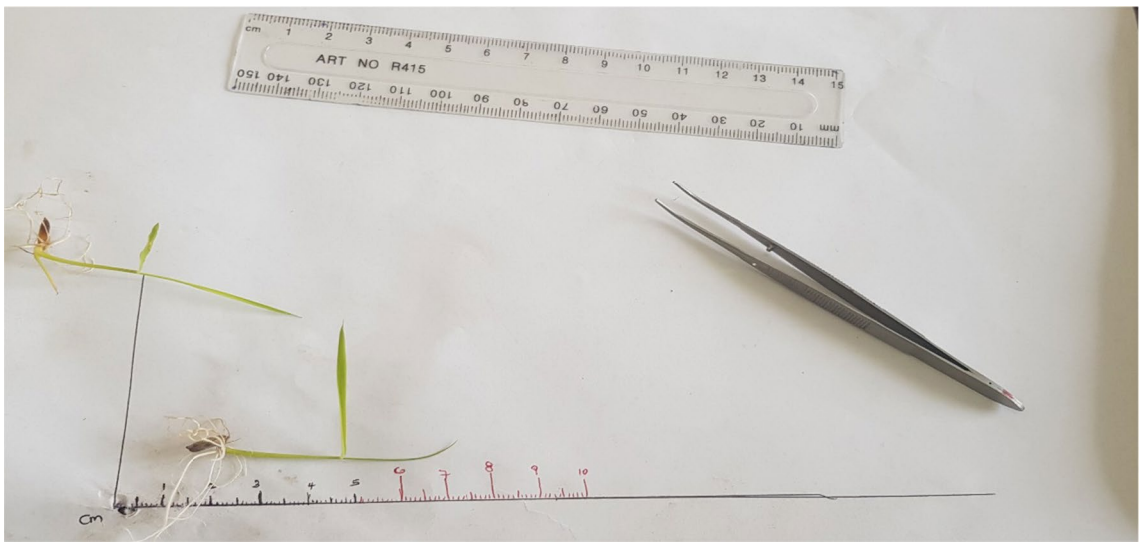

Fig. 14 Measurement of germinating seedling using a transparent ruler on a calibrated white paper.

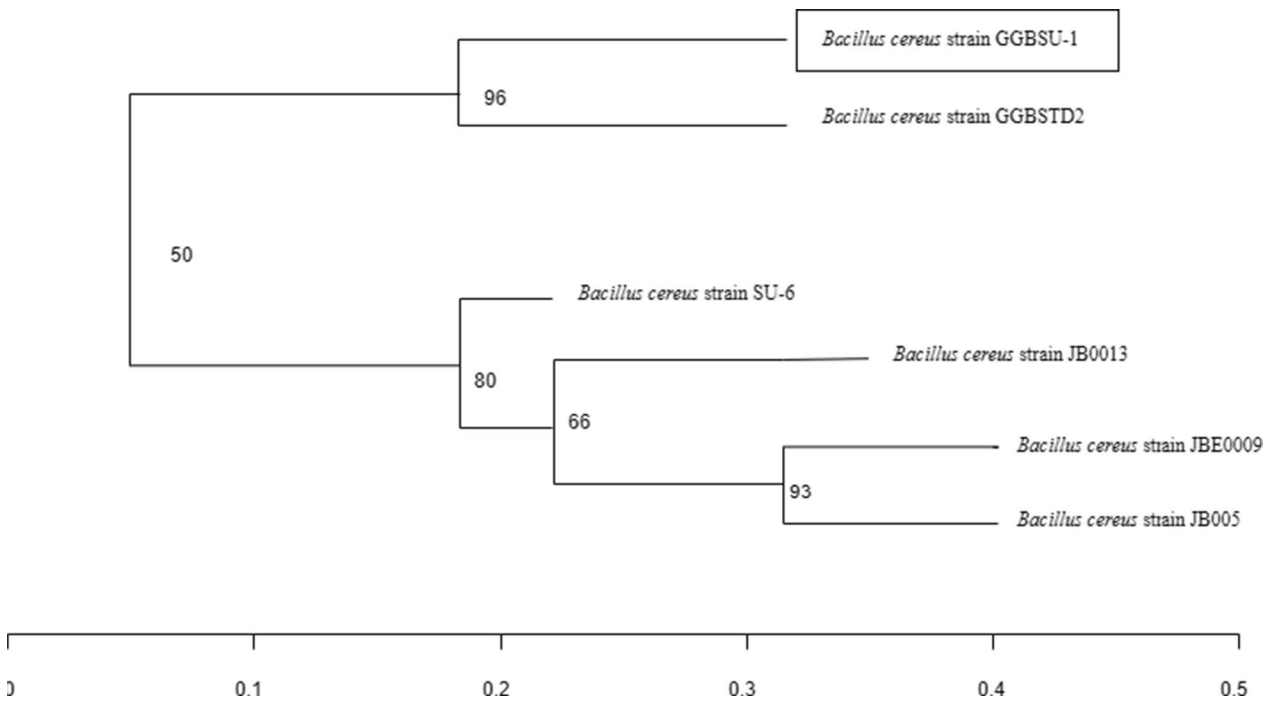

Fig. 15 Phylogenetic tree showing the relationships between the phosphate-solubilizing bacteria (PSB) isolates in this study and their closest phylogenetic relatives based on 16S rRNA gene sequencing. Isolate with rectangle was identified in this study.

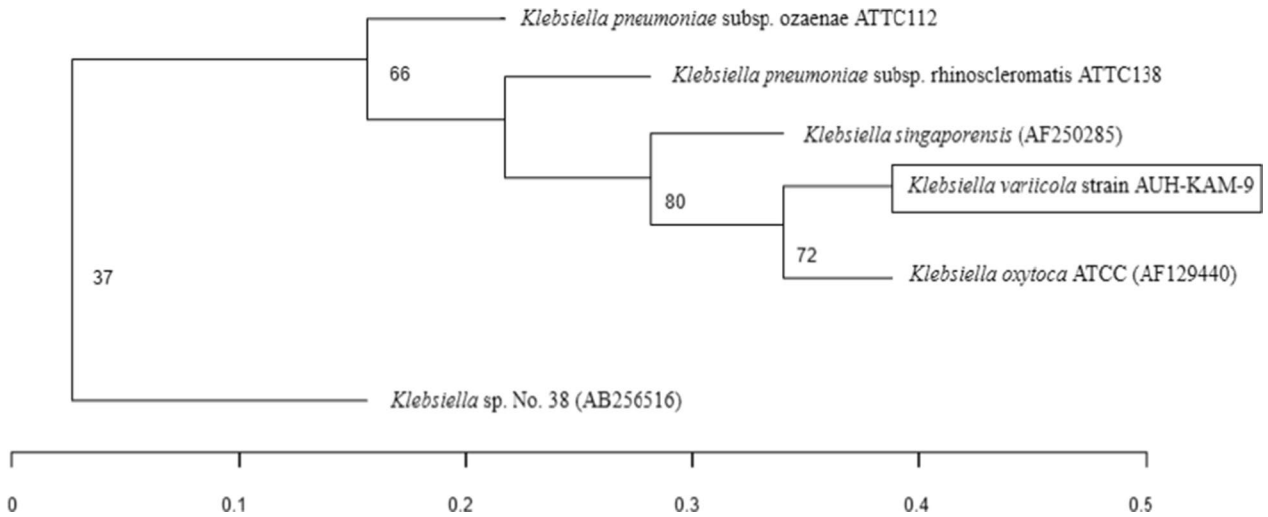

Fig. 16 Phylogenetic tree showing the relationships between the phosphate-solubilizing bacteria (PSB) isolates in this study and their closest phylogenetic relatives based on 16S rRNA gene sequencing. Isolate with rectangle was identified in this study.

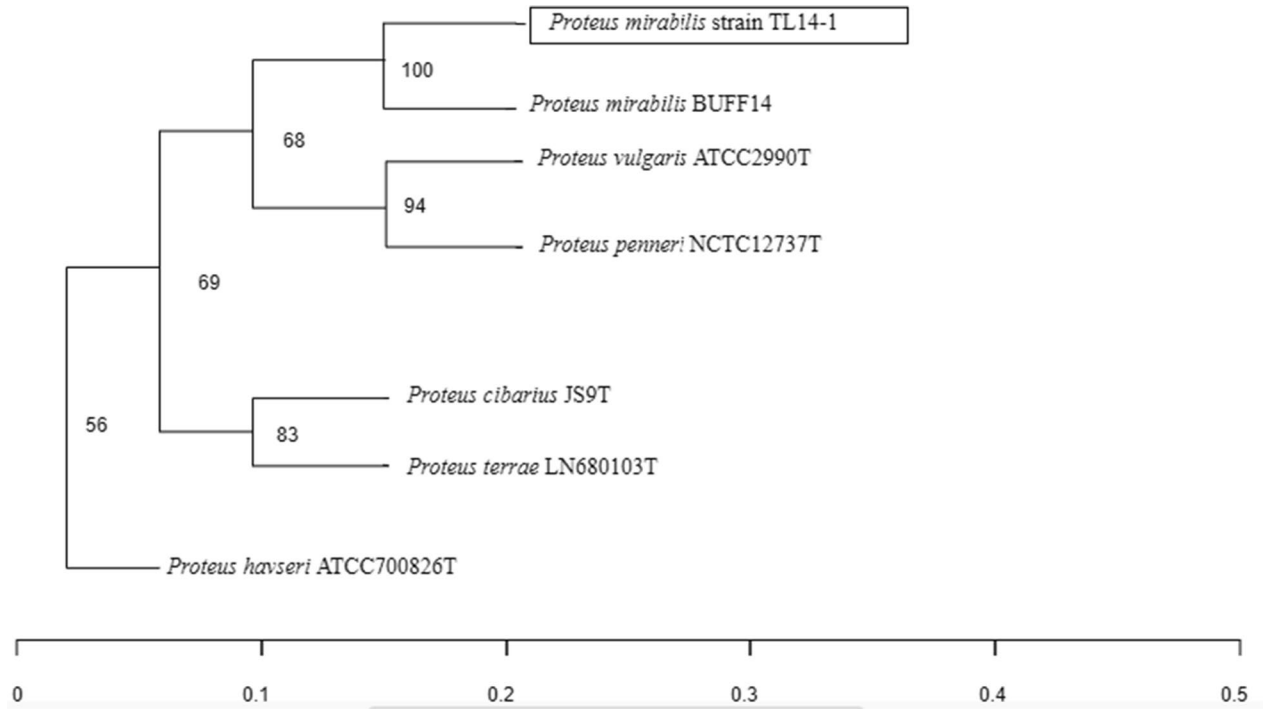

Fig. 17 Phylogenetic tree showing the relationships between the phosphate-solubilizing bacteria (PSB) isolates in this study and their closest phylogenetic relatives based on 16S rRNA gene sequencing. Isolate with rectangle was identified in this study.

Abbreviations
NiS: Non-inoculated seed; BiS: *Bacillus* sp. inoculated seed; PiS: *Proteus* sp. inoculated seed; KiS: *Klebsiella* sp. inoculated seed; PGPR: Plant growth-promoting rhizobacteria; PSB: Phosphate-solubilizing bacteria; WAS: Week after sowing; DAS: Day after sowing; OL: Old leaf; CCI: Chlorophyll content index; NL: New leaf; LLA: Largest leaf area; FW: Fresh weight; APC: Appearance of coleoptile; WUE: Water use efficiency; GTM: Germination time; SGM: Speed of germination; SV: Seed vigor; Chl a: Chlorophyll-a; Chl b: Chlorophyll-b.

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Authors' contributions
MSI and BI designed the study, MSI carried out the research under the supervision of BI. MSI carried out the statistical analysis and interpretation of data. MSI wrote the first draft. BI edited the final draft of the manuscripts. Both authors read and approved the final manuscript.

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Availability of data and materials
The datasets generated and/or analyzed during the current study are included in this published manuscript.

Declarations

Ethics approval and consent to participate
Not applicable

Consent for publication
Consent is given for publication of this manuscript when accepted.

Competing interests
The authors declare no conflicts of interests.

Author details
[1] Department of Biological Sciences, Admiralty University of Nigeria, Ibusa, Delta State, Nigeria. [2] Department of Plant Biology and Biotechnology, University of Benin, Benin City, Edo State, Nigeria.

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