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Isolation and characterization of plant growth promoting rhizobacteria isolated from organically grown high yielding pole type native pea (*Pisum sativum* L.) variety *Dentami* of Sikkim, India

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ABSTRACT

Organic farming is an eco-friendly and sustainable farming practice that enhances soil fertility and helps in improving soil quality. But with the commencement of more sophisticated advances in agricultural techniques, organic farming has gradually become limited in the world. Culture-dependent plant growth-promoting bacterial isolates were isolated from the bulk and rhizospheric soil, of the native high yielding pole type organic pea (*Pisum sativum* L.) cultivar *Dentami* of Dentam, West Sikkim, India. Based on the 16S rRNA gene sequencing identification of these isolates, it was found that from the bulk soil, Actinobacteria (58%) was the dominant phyla followed by Firmicutes (28%), and Proteobacteria (14%). In the rhizospheric soil it was dominated by Proteobacteria (56%), followed by Firmicutes (33%), and Bacteriodetes (11%). A total of 40 bacterial isolates were initially screened for the plant growth-promoting (PGP) activity and out of them only four bacterial isolates i.e., *Bacillus cereus* P8, *Arthrobacter woluwensis* DP2, *Paenarthrobacter nitroguajacolicus* PP3, and *Bacillus mycoides* PP10 with accession numbers MN589697, MN559516, MN519462 and MN589696 respectively were found to possess higher PGP activity (i.e. phosphorous, potassium solubilization and nitrogen-fixing activity) as compared to the other bacteria present in the soil. Based on the indole-3-acetic acid (IAA) quantitative assay and siderophore production assay, it was found that *Bacillus cereus* (MN589697) produced the highest IAA (65.5 μg mL^{-1}) and siderophore (71%) when compared with the other isolates. The statistical correlation suggests that pH and available phosphorus were the strongest influencing factors for the distribution of Proteobacteria in the rhizospheric soil. The results indicate that these isolates can be potential plant growth promoter under the agro-climatic conditions of Sikkim, India. To the best of our knowledge the present study is the first report of its kind and showcases significant findings pertaining to the assessment of diversity, isolation and identification of plant growth-promoting rhizobacteria of organic pea grown in Sikkim.

1. Introduction

In modern agriculture, the utmost importance is the improvement and enhancement of productivity of agricultural products. Remarkable accomplishment has been achieved due to the green revolution in this field and for the farmer's life. But to achieve the goal, use of excessive chemical fertilizers and pesticides in the crop field is a major drawback to sustainable agriculture. This has deteriorated soil quality, the available mineral nutrients and also impacted the groundwater. In this adverse situation, organic farming may help to improve the situation and to reverse the decline of the ecosystem. Thus, bio-fertilizer being a mixture of growth-specific nutrients is a ray of hope for the present-day growth-oriented agriculture with sustainable crop production, while protecting and maintaining the environmental conditions.

Sikkim, a resplendent Himalayan state of India, the abode of verdurous mountains, abrupt valleys, and picturesque flora and fauna, is located between latitudes of 27 °5′ N to 20 °9′ N and longitudes of 87 °59′ E to 88 °56′ E. It is sandwiched between Nepal by Singalila range

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in the West and Bhutan in the Southeast, Chola range from Tibet in the Northeast and West-Bengal in the South and is one of the biodiversity hotspot zones of the lower eastern Himalayan region (Thakur et al., 2013; Najar et al., 2018). Sikkim is the first state of India to officially ban chemical fertilizers and pesticides in the year 2003, and subsequently gained the status of the only certified organic farming state in India in 2016 (Yadav, 2017). Generally, depletion of nutrients in the soil through leaching, volatilization, and de-nitrification are few of the setbacks related to reduced plant growth and development (Cameron et al., 2013). It is a proven fact that soil bacteriome plays a major role to supply nutrients to the crops, to stimulate the plant growth, via phyto-hormones, to inhibit/control the plant diseases and pests and to improve soil health through nutrient solubilization, and nitrogen fixation and through mineralization of pollutants (Hayat et al., 2010; Suteu et al., 2013). The sustainable crop production system, particularly organic farming sought the plant-microbe interactions in the rhizosphere for transformation, mobilization, and solubilization of nutrients from a limited nutrient pool, and subsequent uptake and represents their genetic adaptability and capability (Jacobay et al., 2017). In recent times, the use of microbial consortiums as bio-fertilizers is becoming popular as an additive to chemical fertilizers for recovering crop yield in an integrated plant nutrient management system (Bargaz et al., 2018). In this regard, the use of plant growth-promoting rhizobacteria (PGPR) has found to play a pivotal role in the development of a sustainable system in crop management. Many symbiotic and non-symbiotic bacteria (*Rhizobium* sp., *Azotobacter*sp., *Azosprillum* sp., *Klebsiella* sp., *Bacillus* sp.) are now being employed throughout the world for crop yield and diseases management (Di Benedetto et al., 2017). India is one of the most important agrarian country among the other Asian counterparts. In 2016, Sikkim, which is one of the north east states of India became a fully certified organic farming state. It is the first State in India to achieve this milestone.

Peas (*Pisum sativum* L.) are a high source of protein (28%), carbohydrates (43%), minerals such as calcium, phosphorous, vitamins, antioxidants, etc., and grow almost worldwide (Amarakoon et al., 2012; Khichi et al., 2016). For pea cultivation clay loam or silt loam type of soil having pH 6 to 7.5 is preferred (Chadha et al., 2013). In Sikkim, the pea is one of the important cash crops and is cultivated at an elevation of 914.4–2743.2 mamsl (meters above mean sea level, Subba, 2009). Peas are also cultivated at lower to mid-altitude (914.4–1524.0 mamsl), as an early winter crop whereas at higher altitudes (1828.5–2743.2 mamsl) they are cultivated chiefly as monsoon crops.

Maneybung, Begha and Utteray villages of Dentam, West Sikkim, are the major pea-producing areas in Sikkim. Dentam is considered as the origin place of local pea variety *Dentami* (Kumar et al., 2013). Three types of local pea cultivars/ genotypes (i.e. *Dentami, Local Sikkim-1* and *Local Sikkim-2*), are mostly grown in different parts of Sikkim (Kumar et al., 2013). The *Dentami* variety produces 35 pods per plant yielding 8 seeds per pod on an average. Each pod weighs 60 g approximately. Total green weight yield (t ha^{-1}) is 6.26 as compared to other varieties (*Local Sikkim-1* = 3.66; *Local Sikkim-2* = 3.47) and total dry weight yield (t ha^{-1}) is 3.33 whereas that of *Local Sikkim-1* and *Local Sikkim-2* are 1.92 and 1.75 respectively. Thus, comparing to the other studied local cultivars, *Dentami* is high yielding variety cultivar having approximately two-fold production (in weight). Each seed of this variety weighs up to 40 g (*Local Sikkim-1* = 31 g and *Local Sikkim-2* = 25 g). It has white coloured flowers whereas the other two varieties have light blue color flowers (Kumar et al., 2013).

Several researchers have isolated beneficial microbes from the rhizosphere of leguminous plants from different parts of the world and have reported its use as bio-fertilizers (Boraste, 2009; Bhattacharjee and Dey, 2014; Acharjee, 2017). The aim of the study was isolation and characterization of plant growth-promoting bacteria from local pea cultivar *Dentami* from Begha, Utteray and Maneybung villages of Dentam, West Sikkim, India, using culture-dependent techniques towards the development of effective bio-fertilizer for organic cultivation in the world and Sikkim in particular. This is the first-ever report on pea rhizosphere bacteria of Sikkim local pea cultivar (*Dentami*). Culture-dependent isolation of rhizosphere bacteria was done and various agronomic parameters were tested in this work.

2. Materials and methods

2.1. Sampling sites

Soil samples (rhizosphere and bulk soil) were collected in the month of December 2019, from the pea fields of Manaybung (27 °17′03.65″N, 88 °5′49.72″E; 1972 mamsl), Begha (27 °15′50.06″N, 88 °06′34.06″E; 1756 mamsl) and Utteray (27 °15′52.99″N, 88 °05′05.27″E; 2070 mamsl), villages of Dentam, West Sikkim. The mapping of the sampling sites was done by GPS MAP 78S (Garmin, India; Fig. 1).

2.2. Collection of samples

Both rhizosphere and bulk soil samples were collected from the organic agricultural fields of the above-mentioned villages. The shoots of the pea plants were manually removed and the soil which was tightly adhered to their roots (i.e. rhizosphere) separated using glass beads. The soil collected near the vicinity of the rhizosphere was accounted as the bulk soil. A total of 54 soil samples ((9 rhizosphere soil sample x 3 villages) + (9 bulk soil samples x 3 villages) = 54 samples) were collected aseptically in the sterile polypropylene bags and were immediately transported to the laboratory for the microbiological and soil physico-chemical analysis.

2.3. Physicochemical analysis of soil

For this experiment soil samples were analysed for soil organic carbon (SOC), nitrogen, phosphorus, potassium, and pH of the soil. SOC was evaluated by using the Walkley and Black method (Walkley and Black, 1965). The available nitrogen was estimated following Kjeldahl method (Subbaiah and Asija, 1956), available phosphorus by Bray's P-1 method (Bray and Kurtz, 1945), available potassium by ammonium acetate method (Hanway and Heidel, 1952) and pH of the soil samples by a digital pH meter (Mettler-Toledo, Germany).

2.4. Isolation and characterization the bacterial isolates

The plant growth-promoting bacteria from rhizosphere soil and bulk soil were isolated after serial 10-fold dilutions and pour-plate method on Nutrient Agar medium. Collected soil samples were put into a sterile glass beaker and mixed well separately. Samples were cultured in five different agar-based medium viz., Nutrient Agar (Peptone: 10 gL^{-1}; Yeast Extract: 10 gL^{-1}; NaCl: 5 gL^{-1}; Agar: 20 gL^{-1}); Pikovskay's media (Dextrose: 10 gL^{-1}; $Ca_3(PO_4)_2$: 5 gL^{-1}; $(NH_4)_2SO_4$: 0.5 gL^{-1}; NaCl: 0.2 gL^{-1}; $MgSO_4$: 0.1 gL^{-1}; KCl: 0.2 gL^{-1}; Yeast Extract: 0.5 gL^{-1}; $MnSO_4$ H_2O: 0.002 gL^{-1}; $FeSO_4$: 0.00 gL^{-1}; Agar: 20 gL^{-1}); Jensen's media (K_2HPO_4: 1.0 g L^{-1}; Sucrose: 20 gL^{-1}; $MgSO_4$: 0.5 g L^{-1}; NaCl: 0.005 g L^{-1}; $FeSO_4$: 0.10 g L^{-1}; Na_2MoO_4: 0.05 g L^{-1}; $CaCO_3$: 2.0 gL^{-1}; Agar: 20 gL^{-1}); Aleksandrow's media ($MgSO_4$: 0.5 gL^{-1}; $CaCO_3$: 0.1 gL^{-1}; $AlKO_6Si_2$: 2.0 gL^{-1}; Dextrose: 5.0 gL^{-1}; $FeCl_3$: 0.005 gL^{-1}; $Ca_3(PO_4)_2$: 2.0 gL^{-1}; Agar: 20 gL^{-1}) through the spread plate technique and was incubated at 30 °C for 48 h. Based on colony morphology and characteristics, bacterial colonies were selected, and sub-cultured through the streak plate method (Aneja, 2003). The pure bacterial isolates were preserved at - 80 °C cryo-condition in 50% (v/v) glycerol.

2.5. Molecular identification the bacterial isolates

The bacterial genomic DNA was extracted with the help of HiPurA™ kit as per the manufacturer's protocol (HiMedia, India). The bacterial 16S rRNA genes were amplified by polymerase chain reaction (PCR)

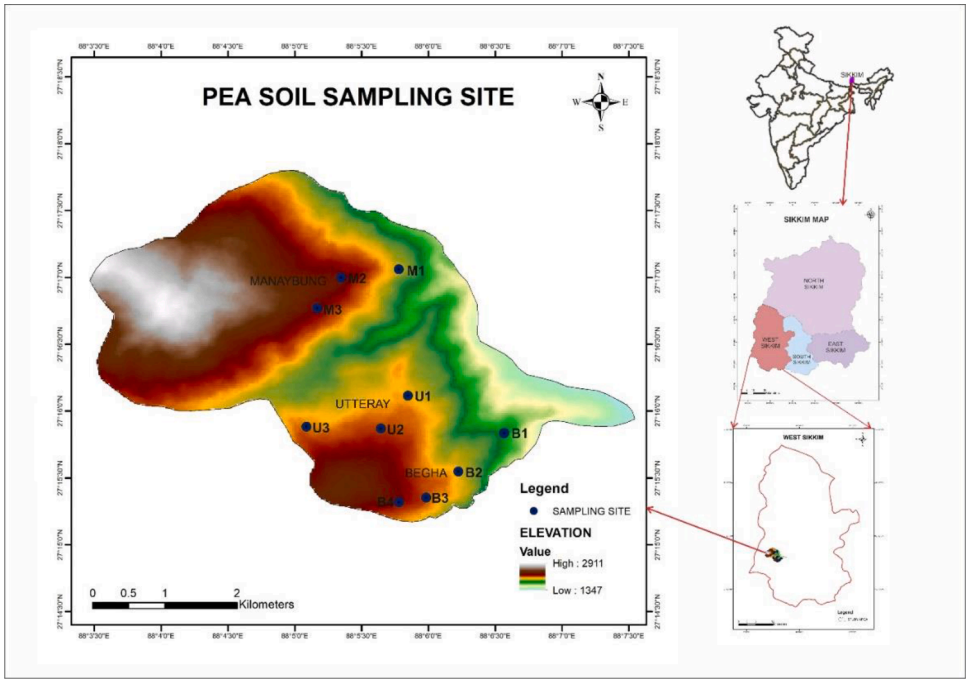

Fig. 1. Map showing Pea rhizosphere and bulk soil the sampling sites GPS coordinates of Begha (B1, B2, B3), Maneybung (M1, M2, M3); and Uttarey (U1, U2, U3), West Sikkim, India.

using two universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′), and 1492R (5′-CGGTTAC CTTGTTACGACTT-3′). PCR was carried out in a total volume of 50 μL in PCR tube using 4 μl each dNTP, 2 μL $MgCl_2$, 2 μL template DNA, 1 μL each primer (forward and reverse), 1 μL Taq DNA polymerase, and 33 μL nuclease-free water (HiMedia, India). Reactions were performed in the Mastercycler gradient (Eppendorf, India) with the following reaction conditions; 94 °C for 5 min for early denaturation stage followed by 30 cycles of 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min, and the last extension step at 72 °C for 10 min. The PCR products were purified with the HiPurA™ PCR clean-up system kit (Hi Media, India) and sequenced by Applied Biosystems ABI (3500 Genetic Analyzer, Japan) using each universal primer (Najar et al., 2018; Sherpa et al., 2018). The sequences were assembled and aligned with the aid of the Codon-Code Aligner software. The sequences were identified using the nucleotide blast tool [National Center for Biotechnology (NCBI)] and the phylogenetic tree was created by using the neighbor-joining method using MEGA v.10 software (Kumar et al., 2018; Erickson, 2010; Saitou and Nei, 1987). The identified sequences reported were submitted to the NCBI gene bank with accession numbers (Supplementary Table B (i) & (ii)).

2.6. Detection of indole acetic acid (IAA) producing bacterial isolate

The production of IAA by bacterial isolates was screened by Salkowski reagent method (Ehmann, 1977). Two sets of 2 mL supernatant of each bacterial isolate grown in Luria Bertani (LB) broth with or without tryptophan was used for the experiment. Then 1 mL Salkowski reagent (2% 0.5 $FeCl_3$ in 35% $HClO_4$ solution) was added in each broth and incubated for 30 min in dark. Production of IAA by the bacteria was confirmed by the development of the pink color of the broth. Quantification was done by spectrophotometric method, where the bacterial culture was grown in LB broth with tryptophan (100mgL^{-1}), incubated at 30±2 °C for 5 days in the dark followed by a change in color post addition of Salkowski reagent. The optical density (OD) was recorded at 530 nmusing a *uv-vis* spectrophotometer (Lambda 35, Perkin Elmer, USA) (Ehmann, 1977; Borrow et al., 1995).

2.7. Determination of phosphate, potassium solubilization and qualitative estimation of N-fixation of bacterial isolates

Phosphate and potassium solubilization ability of the bacterial isolates was initially screened on Pikovskaya's and Aleksandrow agar plates. For the qualitative estimation of phosphate and potassium solubilization, overnight grown bacterial culture, was streaked in Pikovskaya's and Aleksandrow agar plate and incubated at 30±2 °C for 5 days and the efficiency was exhibited with a clear zone formation around the colony (Katznelson and Bose, 1959; Sundara and Sinha,1963). Similarly, the qualitative estimation of N-fixation was checked using N-free Jensen's agar medium (HiMedia, Mumbai) (Kumar et al., 2017).

2.8. Quantitative and qualitative estimation of siderophores

Siderophore production was determined on Chrome Azurol S (CAS) agar plate following the method of Schwyn and Neilands (2007). The 48 h old bacterial cultures were spotted separately on CAS agar (blue dye: 10 gL^{-1}; hexadecyltrimethylammonium (HDTMA) bromide: 73mgL^{-1}. Minimal media 9 salt solution: 10 gL^{-1}, glucose: 0.2 gL^{-1}, casamino acid: 0.3 gL^{-1}, piperazine-N, N′-bis (2-ethanesulfonic acid): 3.2 gL^{-1}, and agar: 15 gL^{-1} and incubated at 28 ± 1°C for 48hr. Quantitative estimation of siderophore was done by using CAS-shuttle assay (Christina et al., 2016). Bacteria were grown on LB broth for 72hr at 28 ± 1°C with constant shaking at 150 rpm. Cell free supernatants of broth were added with an equal volume of CAS-suttler solution (0.15 mM CAS; 0.015 mM $FeCl_3$; 0.6 mM HDTMA; 0.5 mM Piperazine buffer (pH 5.6); 4.5 mM Sulfosalicylic acid) (Lau et al., 2020). The disappearance of the blue color indicates the presence of siderophores. The color change of supernatant from yellowish to orange was determined by observant at 630 nm using the spectrophotometer. The relative level of siderophore was calculated based on the following formula:

$$\%\ \text{of siderophore units} = (A_r - - A_s)/A_r \times 100$$

where, A_r= absorbance of reference CAS reagents; A_s= absorbance of the sample at 630 nm.

2.9. Preparation of bacterial inoculum

Based on the *in vitro* performance on PGP traits two promising bacterial isolates (TF20 and P8) were used for the bioassay of plant growth-promoting activity in the nursery. These isolates were grown in a 100 mL conical flask containing each 50 mL LB broth supplement with 5% sucrose and incubated in an incubated shaker (150 rpm) at 30 °C for 48 h. The concentration of bacteria in the suspension was adjusted using sterilized distilled water and the final concentration of the bacteria was maintained 2×10^9 cells mL $^{-1}$ for inoculation.

2.10. Bioassay-based evaluation of plant growth promotion

The bioassay on the effect of plant growth promotion of isolates was examined with Sikkim local pea cultivar (*Dentami*) in a pot experiment conducted under poly-house, at Department of Horticulture, 6thMile, Gangtok (25.85 °N, 93.77°E, and 1120 mamsl). In this experiment, three treatments were carried out: (1) seeds sown without any inoculation (control), and (2) seeds treated with the suspension culture of the bacterium TF20, and (3) seeds treated with the suspension culture of the bacterium P8. Initially, pea seeds were surface sterilized with a treatment of 95% ethanol for 5 min and rinsed with sterilized distilled water for four times. Then pea seeds were inoculated with two bacterial solutions (2×10^9 cells mL^{-1}) separately at room temperature for 5hr. Control seeds were also treated in the same manner with sterilized distilled water. Finally, seeds were grown in pots (20 cm diameter x 20 cm depth) filled with 3.5 kg autoclaved sterilized soil (pH 6.0). The experimental design was laid in a randomized block design (RBD). In each treatment, a block of 5 seeds planted in a pot was considered as a replicate. Three replications were conducted for all the treatments. Pots were irrigated with sterile distilled water in seven days intervals. The temperature of the poly- house was recorded everyday and it was 20–25 °C throughout the experiment.

For growth evaluation, a total of 15 randomly selected plants (5 plants in each replication in triplicate) from each treatment were uprooted after 45 days of inoculation, plant lenght (cm), fresh, dry weight of root and shoot were recorded. Dry weight was determined by placing the root and shoot samples separately into small paper bags and drying them in an oven at 80 °C for 72 h. Data were statistically analyzed using Analysis of Variance (ANOVA).

2.11. Statistical investigation

Treatment means were compared by the Least Significance Difference (LSD) using the methods of Snedecor and Cochran (1968). The principal component analysis (PCA) was employed to compare bacterial diversity and physicochemical parameters of the samples with the help of PAST software (Sherpa et al., 2018). The Shannon diversity indices and Chao 1 were computed with PAST software (Chao et al., 2006). The heatmap was exploited to investigate the comparative bacterial diversity among rhizosphere soil, bulk soil, and previously reported microbial diversity of different organic farmland soil samples through Heatmapper (Babicki et al., 2016).

3. Results and discussion

3.1. Soil physicochemical analysis

The pH of the rhizosphere soil tested ranged from 6.5 to 7.1 (i.e. slightly towards neutral) whereas that of bulk soil sample was moderately acidic (pH 5.5–6.5). The available phosphorus, potassium, and nitrogen were estimated to be 16.3 Kg ha^{-1}, 22.9 Kg ha^{-1}, and 289 Kg ha^{-1} respectively for the rhizosphere soil samples. Whereas in the case of bulk soil samples, the available phosphorus, potassium, and nitrogen was 15.4 Kg ha^{-1}, 22.5 Kg ha^{-1}, and 280 Kg ha^{-1} respectively (Table 1). The SOC value of bulk soil was higher as compared to the rhizosphere soil (Table 1). Soil plays a vital role in the agro-ecosystem for nutrient cycling, organic material returns, and xenobiotics degradation and adsorption (Doran and Michael, 2000). Microbes are the potent players causing these functions in the agro-ecosystem. Additionally, the quantity of soil microbiota helps in crop growth performance by generating plant hormones, boosting nutrient availability, and benefits plant overall quality and yield (Egamberdieva et al., 2017). In the present day, research, especially on soil microbiome and its role in improving soil health and the agricultural production system, has gained immense interest. Many studies have recognized that microbes can affect the ecosystem processes, for instance, crop yield (Deng et al., 2018), decomposition of xenobiotics (Hertmann et al., 2015), nutrient cycling, as well as protection of plants against diseases and pathogens (Cameron et al., 2014). Moreover, several studies showed that organic farming influences the composition of the soil microbial communities' (Florine et al., 2015; Ye et al., 2016; Xiong et al., 2015; Jimenez-Bueno et al., 2016). In this research,we checked the plant growth-promoting bacterial diversity of a local pea cultivar (*Dentami*) from long term organic soil of Maneybung, Begha, and Utteray villages of Dentam, West Sikkim, India.

Table 1
Physiochemical parameters of rhizosphere soil and Bulk soil form long-term organic soil of Sikkim.

A) Bulk soil Sample Name	pH	SOC (%)	Available N (Kg ha^{-1})	Available P (Kg ha^{-1})	Available K (Kg ha^{-1})
Begha (B1, B2, B3)	6.1 ± 0.1^b	0.95 ± 0.0^c	293 ± 2.0^a	14 ± 1.5^b	20.3 ± 1.5^b
Maneybung (M1, M2, M3)	6.5 ± 0.1^c	1.41 ± 0.0^a	254.3 ± 0.5^c	18.6 ± 2.8^a	28.3 ± 0.5^a
Uttarey (U1, U2, U3)	5.5 ± 0.1^a	1.0 ± 0.0^b	293 ± 1.5^b	13.3 ± 0.5^c	13.6 ± 0.5^c
SD	0.41	0.20	18.24	2.35	6.0
LSD	0.06	0.06	3.51	3.75	1.99
CV	0.51	2.87	0.60	11.10	4.49
B) **Rhizosphere soil Sample Name**	**pH**	**SOC (%)**	**Available N (Kg ha^{-1})**	**Available P (Kg ha^{-1})**	**Available K (Kg ha^{-1})**
Begha (B1, B2, B3)	7.1 ± 0.0^a	1.1 ± 0.0^a	311 ± 1.0^b	12.8 ± 0.2^b	23.0 ± 1.7^a
Maneybung (M1, M2, M3)	6.5 ± 0.0^c	1.1 ± 0.0^a	313 ± 0.5^a	15.3 ± 0.57^a	25.7 ± 1.0^a
Uttarey (U1, U2, U3)	7 ± 0.0^b	1.0 ± 0.0^a	215± 0.5^c	13.6 ± 1.5^b	14.8 ± 0.5^b
SD	0.26	0.04	45.73	1.04	4.63
LSD ($p < 0.05$)	0.108	0.065	1.48	1.47	2.65
CV	0.74	2.85	0.25	5.04	5.89

Each treatment consisted of three replications. Total number of village was three. Bulk and rhizosphere soil samples were collected from three locations of each village. Means within column followed by same letters are not significantly different from each other by F-LSD test ($p < 0.05$).
SD: Standard deviation; LSD: Least Significant Difference; CV: Coefficient of variation.

Soil is an extremely complex structure that includes organic particles in addition to thousands of living organisms such as arthropods, worms, bacteria, fungi, as well as other eukaryotic and prokaryotic organisms (Sun et al., 2017). Bacteria are considered as one of the most potent living parts of the soil ecosystem (Sun et al., 2017; Wang et al., 2016). Many of them act as decomposers while the other counterparts help for assimilation of nitrogen in soil and nutrient cycling. A higher abundance of synergistic soil bacteria improvises the soil quality and enhances plant growth by reducing the outbreak of diseases in plants (Fierer, 2017).

3.2. Identification of bacterial isolates

A total of 24 isolates, were further subjected to 16S rRNA sequence analysis. The sequence analysis using BLAST showed that the majority of the bacterial isolates of bulk soil were Gram-positive, and rhizosphere soil was dominated by Gram negative bacteria. Based on an identity standard of a minimum of 97% for the 16S rRNA gene sequence, 14 isolates from bulk soil and 10 isolates from rhizosphere soil were identified and they were distributed within four different phyla: Proteobacteria [*Acinetobacter lwoffii* TF19 (MN559514), *Variovorax paradoxus* TF20 (MN548374), *Pseudomonas koreensis* P11 (MN519465), *Pseudomonas helmanticensis* P12 (MN58970), *Pseudomonas baetica* P16 (MN58971), and *Rhizobium etli* P18 (MN589695)], Firmicutes [*Bacillus mycoides* P10 & PP1 (MN589696 & MN589699), *Bacillus cereus* P4 & P8 (MN589698 & MN589697 respectively), *Lysinibacillus pakistanensis* DP6 (MN519468), and *Planococcus ruber* TP2 (MN519475)], Actinobacteria [Arthrobacter bambusae PP5 (MN519472), Arthrobacter woluwensis DP2 (MN559516), Micrococcus aloeverae TF17 (MN548377), and *Paenarthrobacter* nitroguajacolicus PP3 & PU11 (MN519462 & MN519465 respectively), *Paenarthrobacter nicotinovorans* UP1 (MN519461)], Bacteroidetes [Chryseobacterium oranimense P9 (MN559513). A phylogenetic tree of the identified bacteria isolates including a representative from each of the rhizosphere soil and bulk soil isolates and their closest relatives are shown in (Figs. 2& 3). Sequence analysis demonstrated that Proteobacteria (55.5%) was the main phyla in rhizosphere soil followed by Firmicutes (33.3%) and Bacteriodetes (11.1%). Similarly, Actinobacteria (57.1%) were the dominant phylum from bulk soil followed by Firmicutes (28.5%) and Proteobacteria (14.2%). Our result showed that Actinobacteria and Firmicutes were the main bacterial phyla in bulk soil. Similarly, the dominant phyla in rhizosphere soil were Proteobacteria and Firmicutes.

3.3. Screening of bacteria for plant growth-promoting traits and biochemical characteristics

The plant growth-promoting traits of the isolates were also investigated such as the production of phyto-hormone IAA, phosphate solubilization, nitrogen fixation, and siderophore production were checked (Table 2). Based on the screening and estimation of PGP traits *B. cereus* (P8), *P. koreensis* (P11), *V. paradoxus* (TF20) and *B. mycoides* (P10) showed the best performance produced 66.5 μg mL^{-1}, 61 μg mL^{-1}, 60 μg mL^{-1} and 45.1 μg mL^{-1} IAA respectively as compared to other test strains (Table 2). Similarly, screening of bacterial siderophore by CAS agar plate method and Chrome Azurol Sulphonate (CAS) assay method showed that the *B. cereus* was the highest siderophore producer followed by *V. paradoxus* as compared to other isolates. The phosphate solubilization studies based on Pikovsky's agar plate method showed that *A. woluwensis* (DP2), *P. nicotinovorans* (UP1), *B. cereus* (P8), and *V. paradoxus* (TF20) showed significantly higher zone of inhibition on agar plate than other isolates. Further, out of the selected isolates *B. mycoides* PP1, *B. cereus* P4, *B. mycoides* P10 and *P. koreensis* P11 showed potential nitrogen fixation ability.

The relative percentage of bacteria was higher in the bulk soil as compared to that of the rhizosphere soil. Actinobacteria and Firmicutes were the key phyla in bulk soil in our study. Similarly, the dominant phyla in rhizosphere soil were Proteobacteria and Firmicutes. Our findings were supported by the results of García-Salamanca and coworkers (Garcia-Salamanca et al., 2013), that reported Proteobacteria as the dominant phyla in rhizosphere soil and Actinobacteria as the predominant phyla in the bulk soil (Yang et al., 2017). Heat- Map analysis showed that most of the bacterial isolates of rhizosphere soil from organic farmlands of Sikkim belonged to Proteobacteria which was similar to that of Lithuania and Shangai organic farmlands (Fig. 4).

3.4. Correlation of physicochemical parameters and bacterial diversity of rhizosphere soil and bulk soil

The principal component analysis was employed to understand the correlation between physico-chemical parameters and bacterial diversity at the phylum level of the rhizosphere soil and bulk soil. The top two phyla from both the samples and physicochemical parameters such as pH, soil organic carbon, available phosphorus, potassium, and nitrogen were studied as shown in (Fig. 5). The PCA revealed that the community composition was differently correlated to various physicochemical parameters. Firmicutes of the bulk soil were positively correlated to SOC, and available potassium, whereas from the rhizosphere soil

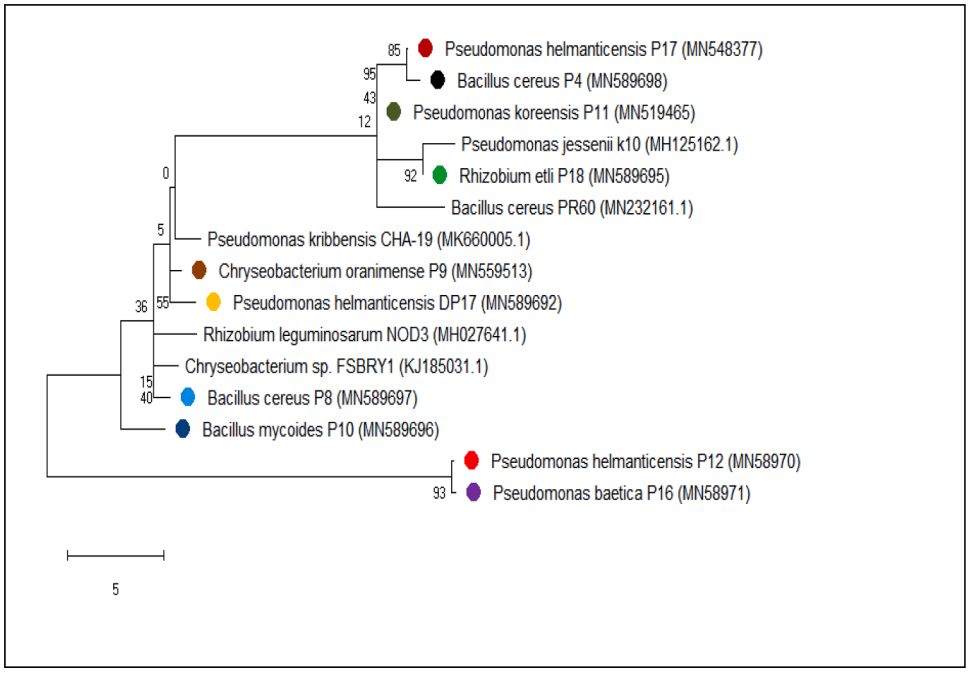

Fig 2. The evolutionary history of rhizosphere soil isolates was inferred using the Neighbor-Joining method. The evolutionary distance was calculated via the Kimura 2-parameter model method. Evolutionary analyses were conducted in MEGA v.10.

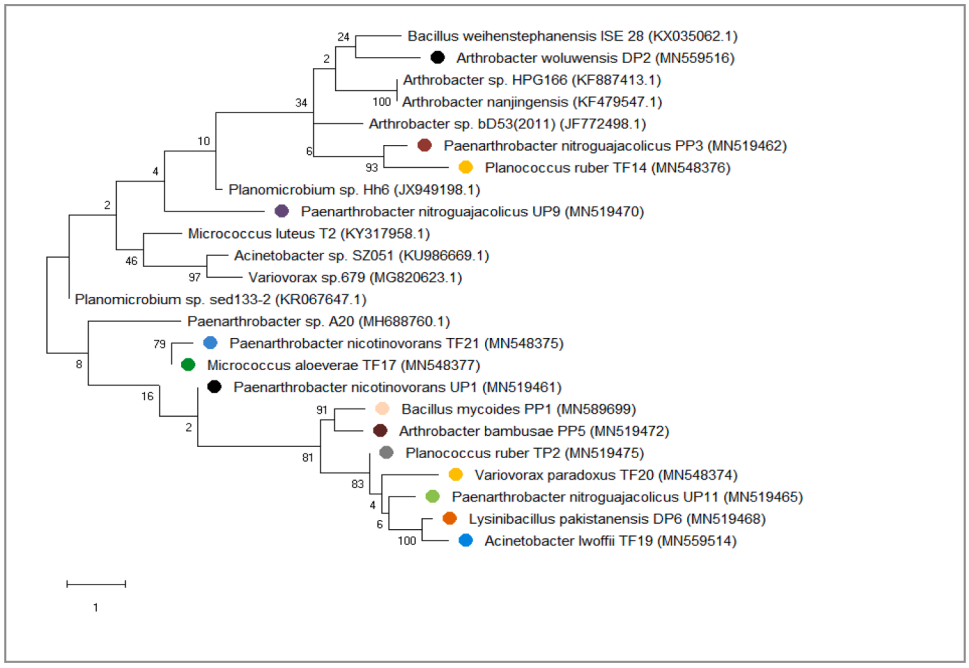

Fig. 3. The evolutionary history of bulk soil isolates was inferred using the Neighbor-Joining method. The evolutionary distance was calculated via the Kimura 2-parameter model method. Evolutionary analyses were conducted in MEGA v.10.

Table 2
Plate assay and biochemical estimation of selected bacterial isolates.

Strain	IAA (μg mL^{-1})	P-solubilization activity	K-solubilization activity	N-fixing activity	% of Siderophore production
PP1	45.1	-	+	+	49
DP2	10	+	+	-	42
UP1	07	+	+	-	17
P8	66.5	+	+	-	71
TF20	60	+	+	-	60
UP11	51	-	+	-	00
DP6	55	+	+	-	00
TF19	15.5	+	+	-	35
P17	10	+	-	-	30
P4	09	+	+	-	40
P11	61	+	+	+	23
P18	55	+	-	+	17
P9	45	-	+	-	35
DP17	25	+	+	-	31
P10	20	+	+	-	41
P12	22	+	-	-	31
P16	15.5	+	-	-	24
PP3	35	+	+	+	0
TF14	15	+	+	-	31
UP9	30	+	+	+	26
TF21	10	+	+	-	27
TF17	16	+	+	-	34
PP5	09	+	+	-	36
TP2	14	+	-	-	32

"+" indicates positive, "-" indicates negative

Proteobacteria exhibited a high correlation to pH and available phosphorus. However, nitrogen was negatively correlated with Firmicutes and Proteobacteria of the rhizosphere soil as well as bulk soil (Table 3).

The recent data showed that the Proteobacteria, Acinetobacteria, Acidobacteria, Firmicutes and Bacteriodetes were more prevalent in near-neutral pH (Zhang et al., 2017). The chemical composition of soil mainly the quantity of phosphorus is also an important feature for the microbial population (Liu et al., 2013) but is still ambiguous the relationship between the amount of phosphorous and microbial diversity. There were no major changes observed in the microbial diversity at the genus level with varying level of phosphorus in the rhizosphere soil and bulk soil (16.3 Kg ha^{-1} vs 15.1 Kg ha^{-1}). Whereas significant difference among the dominance of Proteobacteria in the rhizosphere soil was detected, and it was two times populated than the bulk soil. The predominance of Proteobacteria in the rhizosphere soil might be due to their rapid growth rates and, this nutrient-rich environment was suitable for Proteobacteria, or certain classes within this phylum (Johnston-Monje et al., 2016). Gram-negative bacteria found were mostly responsible for nitrogen fixation and polycyclic aromatic hydrocarbon degradation (Yang et al., 2017). The prevalence of Actinobacteria from bulk soil might play a critical role in the decomposition and humus formation process (Buée et al., 2009).

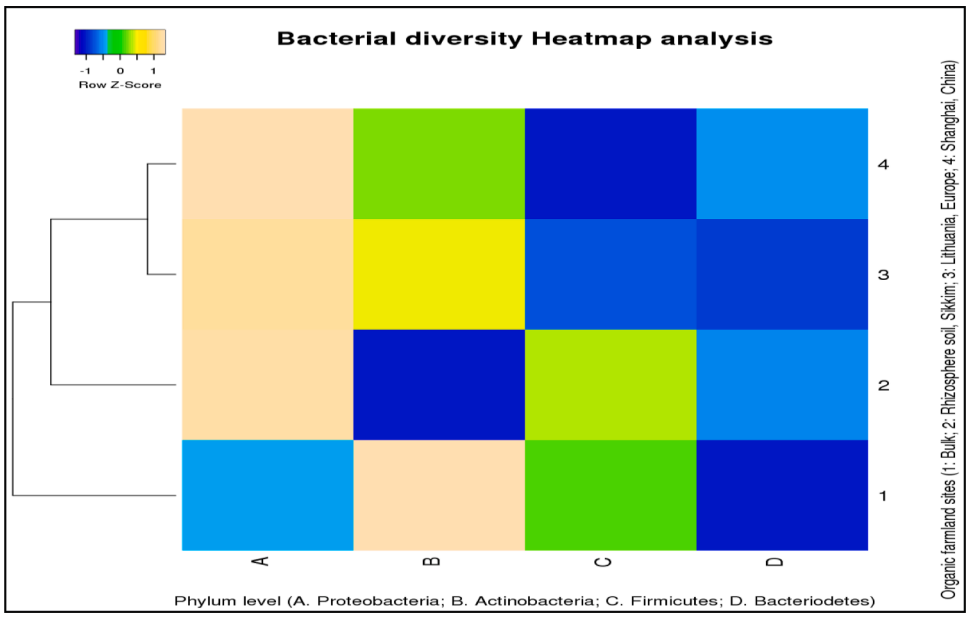

Fig 4. Heat-map analysis of top phylum among four organic farm land soil samples from different parts of the World [1 =bulk soil (Sikkim); 2 = rhizosphere soil (Sikkim); 3 =Lithuania organic soil, Europe; 4 = Shangai organic soil, China].

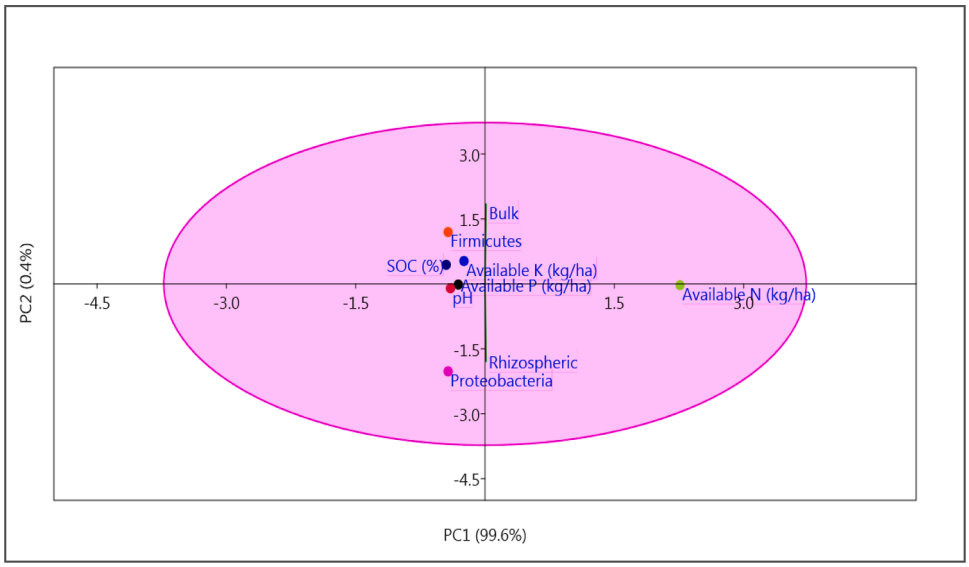

Fig 5. Principle component analysis (PCA) of bacterial diversity at the phylum level and physicochemical parameters of the rhizosphere soil and bulk soil of Sikkim.

3.4.1. Assessment of microbial diversity

The microbial community structure of organic farmland reported from different parts of the world was evaluated with our studies using a heat-map plot using the Bray Curtis dissimilarity method. Phylum level diversity comparison showed that the community structure of rhizosphere soil positively correlated with the Lithuania organic farmland, Europe (Armalyate et al., 2019), and Shanghai organic farmland, China (Liao et al., 2018). This result indicates a possible role of organic farming soil in shaping microbial diversity.

3.4.2. Diversity index and rarefaction curve analysis

Diversity indices for instance Fisher Alpha, Shannon H, and Chao1 were estimated by using the PAST software. The results showed that the bulk soil is more diverse than the rhizosphere soil. The Shannon index was 0.76 and 0.54 for bulk and rhizosphere soil respectively (Fig. 6a). The rarefaction curve permits the computation of species richness in a sample. The curve is a plot of the total number of species annotated as a function of the number of sequences sample (Sherpa et al., 2019). The rarefaction curve analysis also supported that the bulk soil samples were higher species richness than the rhizosphere soil (Fig. 6b).

Earlier studies confirmed the dynamics of soil physico-chemical parameters in altering the soil microbial diversity (Thomson et al., 2015). The soil nutrient contents as well as pH, chiefly in terms of C, N, and P availability, are very crucial factors (Ramirez et al., 2012) that significantly affects the microbial abundance in soil (Zhong et al., 2010). When the soil pH was lower than 6.5, the pH was believed to be the major factor that affected the microbial diversity, structure, and activity, and higher microbial richness and diversity were detected at a near to neutral pH (Preem et al., 2012; Bergkemper et al., 2016). In contrast, Lauber et al. (2009), have suggested that there is a pessimistic relationship between soil bacterial diversity and soil pH. The study also revealed that their soil pH and microbial diversity were negatively

Table 3
Physiochemical parameters of *Variovorax paradoxus* TF20 and *Bacillus cereus* P8 inoculated pea soil.

A) *Variovorax paradoxus* (TF20) **inoculated soil**				
pH	SOC (%)	Available N (Kg ha^{-1})	Available P (Kg ha^{-1})	Available K (Kg ha^{-1})
7.2 ± 0.0[a]	1.5 ± 0.0[a]	311 ± 1.0[a]	18.3 ± 0.5[a]	28 ± 0.5[a]
B) ***Bacillus cereus*** **P8 inoculated soil**				
pH	SOC (%)	Available N (Kg ha^{-1})	Available P (Kg ha^{-1})	Available K (Kg ha^{-1})
7.0 ± 0.1[b]	1.3 ± 0.0[b]	288 ± 1.0[b]	15.3 ± 0.5[b]	24 ± 0.5[b]
C) Control soil				
pH	SOC (%)	Available N (Kg ha^{-1})	Available P (Kg ha^{-1})	Available K (Kg ha^{-1})
6.5 ± 0.0[c]	1.2 ± 0.0[b]	270 ± 0.5[c]	15.0 ± 0.5[b]	23 ± 1.0[b]
LSD (*P = 0.05*)	0.127	2.02	0.66	1.67

Means within column followed by same letters are not significantly different from each other by F-LSD test ($p < 0.05$).

correlated, which is analogous with the study by Deng (Deng et al., 2018). Previous studies have recommended that soil microbial diversity was mostly affected by SOC (Siles and Margesin, 2016). In our study, Firmicutes from bulk soil positively correlated with the SOC and available potassium, which was inconsistent (Siles and Margesin, 2016; Ren et al., 2018).

3.5. Evaluation of plant growth promoting potential of bacteria in pea plant

Plant growth promotion of these isolates was demonstrated through a plant-based bioassay under poly-house condition. The bacterial inoculations were effective in improving the overall growth performance of pea plants (cultivar *Dentami*). The use of these bacterial isolates as inoculant resulted in a statistically significant increment in root and shoot biomass of pea plants after 45 days of growth (Table 4).

Application of *V. paradoxus* TF20 and *B. cereus* P8 in pea plant demonstrated better growth with an average plant length increment were 75% higher (average plant length 28.06 ± 0.20 cm) and 31.81% higher (average plant lenght 21.13±0.70 cm) respectively to that of control plants (average plant lenght 16.03 ± 0.15 cm). Likewise, plant fresh weight increment was also recorded 78.57% higher (average plant fresh weight 7.5 ± 0.10 g) for both the bacterial inoculations compared to control plants (average plant fresh weight 4.2 ± 0.25 g). In case of plant dry weight it was 146% higher (average plant dry weight 5.12 ± 0.0 g) and 47.59% higher (average plant dry weight 3.07 ± 0.0 g) respectively in inoculated plants compared to control plants (average plant dry weight 2.08 ± 0.0 g). For root analysis, fresh weight of root increments were 24.53% (average root fresh weight 1.32 ± 0.13 g) and 31% (average root fresh weight 1.39 ± 0.18 g) respectively in inoculated plants compared to control plants (average root fresh weight 1.06 ± 0.13 g) and when analysed root dry weight it was 77.77% more (average root dry weight 0.32 ± 0.0 g) and 72.22% more (average root dry weight 0.31 ± 0.0 g) in inoculated plants respectively when compared with control plants (average root dry weight 0.18 ± 0.0 g) (Fig. 7, Table 4). The present results are correlated with earlier studies (Di Benedetto et al. 2019), who has described the plant-growth-promoting potential of *Bacillus* spp. Results of this experiment are correlated with earlier studies as demonstrated by Di Benedetto et al. (2019), who have defined the plant-growth-promoting potential of *Bacillus* spp. (Panneerselvam et al. (2019) showed that *B. luciferensis* K2 and *B. amyloliquefaciens* K12 were higher IAA producer (97.1 μg mL^{-1} and 49.9 μg mL^{-1}) which is consistent with our result of *B. cereus* P8 and *V. paradoxus* TF20 (66.5 μg mL^{-1} and 60 μg mL^{-1}). In general, the application of *V. paradoxus* TF20 inoculum either or *B. cereus* P8 inoculum significantly increased plant growth parameters as compared to the un-inoculated control plant. Numerous studies have also proved that *B. aryabhattai, B. megaterium, B. polymaxa, B. cereus*, and *V. paradoxus* possessed plant growth-promoting attributes and enhance plant growth and increase yield in several agricultural and horticultural corps (Lau et al., 2020; Park et al., 2017; Dodd

Table 4
Pea bioassay on growth promotion by inoculation with *Variovorax paradoxus* TF20 and *Bacillus cereus* P8.

Isolates	Plant length (cm)	Plant fresh weight (g)	Plant dry weight(g)	Root fresh weight (g)	Root dry weight (g)
TF20	28.06 ± 0.20[a]	7.5 ± 0.10[a]	5.12 ± 0.0[a]	1.32 ± 0.13[ab]	0.32 ± 0.0[a]
P8	21.13 ± 0.70[b]	6.26±0.25[b]	3.07 ± 0.0[b]	1.39 ± 0.18[a]	0.31 ± 0.0[a]
Control	16.03 ± 0.15[c]	4.2 ± 0.25[c]	2.26 ± 0.4[c]	1.06 ± 0.13[b]	0.18 ± 0.0[a]
LSD (*p = 0.05*)	0.975	0.489	0.603	0.336	0.043

Response was recorded on the basis of 15 plants per treatment.
Data on the average fresh plant weight, dry plant weight, total length, fresh root weight, dry root weight were recorded after a total of 45 days.
Means within column followed by same letters are not significantly different from each other by F-LSD test ($p < 0.05$).

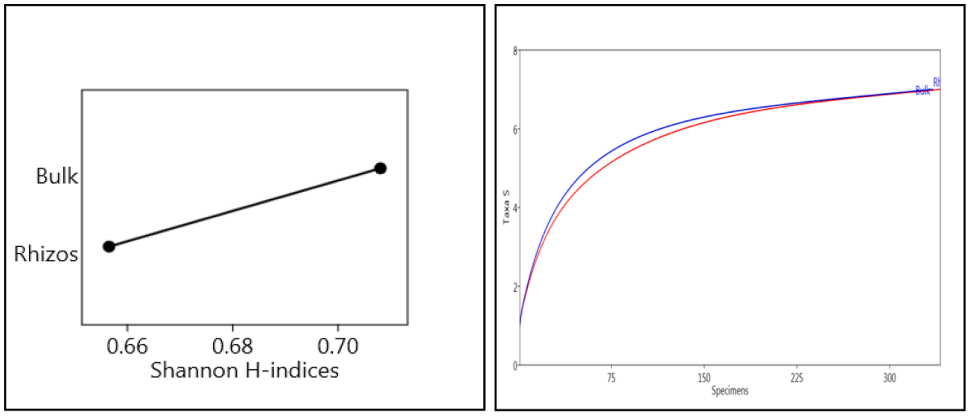

Fig 6. Representation of the abundance of bacterial diversity in both the soil sample with bulk soil having higher Shannon H-index than the rhizosphere soil and rarefaction curve, red curve show species richness in the rhizosphere soil whereas blue line represents bulk soil.

Fig. 7. Growth performance of pea plants (cultivar *Dentami*) inoculated with *Variovorax paradoxus* TF20 and *Bacillus cereus* P8 in a pot experiment. (a- inoculated with *Variovorax paradoxus*TF20, b- inoculated with *Bacillus cereus* P8 and c-control). **(A)** Plants grown in pots. **(B & C)** Plantlets taken out from soil. **(D)** Root system of plants.

et al., 2009). These microbial inoculums could be potential candidates for bio-intensive nutrient management in organic farming systems.

4. Conclusion

The present work is the first study on the plant growth-promoting bacteria isolated from rhizosphere soil and bulk soil of local pea cultivar *Dentami* of Sikkim, India. Phosphate solubilization and siderophore producing bacteria belonging to *Bacillus* and *Arthrobacter* were the most predominant colonizer in the local pea cultivar *Dentami. B. cereus* P8, *B. mycoides* P10, L. *pakistanensis* DP6, *P. nitroguajacolicus* UP11, *A. woluwensis* DP2, *A. bambusae* PP5 and *V. paradoxus* TF20 are being reported for the first time from the pea rhizosphere soil of Sikkim. Major variation exists in the rhizosphere and bulk soil on bacterial diversity suggesting the hypothesis that physiochemical parameters are the influencing factors for the distribution of Proteobacteria. Therefore, the results suggest that Proteobacteria is more dominant in rhizosphere soil than bulk soil. *V. paradoxus* TF20 and *B. cereus* P8 showed the best plant growth-promoting and biocontrol traits, such as phosphorous and potassium solubilization, nitrogen-fixing activity, and siderophore production. Plant-based bioassay under greenhouse conditions also showed very impressive results on growth performance by inoculating with *V. paradoxus* TF20 and *B. cereus* P8 as compared to the control plant. Bacterial isolates *B. cereus* P8 and *B. mycoides* PP1 were found to be the highest IAA producer (66.5 μg ml^{-1} and 45.1 μg ml^{-1} respectively). Sikkim is the first state in India to practice organic agriculture farming; hence, such studies on soil microbiology are of immense significance.

CRediT authorship contribution statement

Mingma Thundu Sherpa: Conceptualization, Investigation, Methodology, Writing – original draft. **Niladri Bag:** Supervision, Writing – review & editing. **Sayak Das:** Writing – review & editing, Visualization. **Paolenmang Haokip:** . **Laxuman Sharma:** Writing – review & editing, Visualization, Supervision.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2021.100068.

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