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Identification of plant beneficial *Bacillus* spp. for Resilient agricultural ecosystem

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ABSTRACT

The use of synthetic pesticides and chemicals to continuously increase agricultural productivity is causing severe damage to our ecosystem. Therefore, there is need to enhance our understanding about the factors which can contribute to soil processes and play key role in developing sustainable agricultural ecosystem. In this context, the bacteria from cauliflower rhizosphere were isolated and characterized for different plants beneficial attributes. The relationship of soil bacteria and its elemental composition was examined using canonical correspondence analysis. The elemental composition analysis of soil samples revealed presence of Mg, Al, Si, P, K, Ca, Fe and their oxides. In addition, the isolates were found positive for phosphorus solubilization, siderophore, chitinase and protease activity, and indole acetic acid type of growth regulator. The direct confrontation assay revealed antagonistic behavior of these isolates against *Fusarium oxysporum* and *Sclerotinia sclerotiorum*. The promising isolates were identified and affiliated to closely related species of genus *Bacillus* in phylogenetic relationship. The canonical correspondence analysis revealed distribution of elements and their relationship with the identified species in particular area. The characteristics of these isolates revealed their potential in alleviating the biotic and abiotic stresses and hence enhancing crops yield without the usage of synthetic fertilizers and pesticides. The present study is first of its kind and will open new avenues to explore microbial community structure across different farmlands soils to develop resilience agricultural ecosystem.

Introduction

The continuous change in climatic factors has negative impact on our ecosystem which severely affects the agricultural production. To counter this, agricultural practices based on natural resources and processes offers promising solution to improve the crop production without causing negative impacts to our environment (Lori et al., 2017). Soil, a source of nutrients and water for terrestrial plants, is affected by different abiotic and biotic factors such as minerals composition and their availability, microbial community, climatic factors and other activities. Moreover, soils from different areas have variations in the composition and microbial communities (Liu et al., 2015). In agricultural farming, the functions of soil are intimately linked to microbial community's dynamics (Turner et al., 2017). Therefore, the knowledge of microbial habitats and soil structure such as soil texture and their interactions are essential to understand the microbial community interaction in plant rhizosphere. Conversely, microorganisms also affect the soil structure by acting as aggregation agents (Tisdall and Oades, 1982). In parallel, soil texture, organic matter and structure form microhabitats which impact ecological and microbial dynamics (Hattori and Hattori, 1976; Stotzky, 1986; Guggenberger et al., 1999).

The interaction among soil, plants and microorganisms in rhizosphere often shapes the soil properties. Due to plethora of biological activities, microbial communities in a fertile soil are active and adaptive driving force involved in nutrients transformation and several other soil functioning (Lynch and Bragg, 1985; Emmerling et al., 2002; Garbeva et al., 2004; Rillig and Mummey, 2006; Nielsen et al., 2015). These different processes involve stem and root growth, water and nutrient uptake, respiration and rhizodeposition (Hinsinger et al., 2005). The effects of soil biochemical activities on soil microbial community have been studied on global scale for agricultural productivity (Dick, 1992; Reeve et al., 2016; Lorenz and Lal, 2016; Lori et al., 2017). The soil properties and functions evolve physicochemical and biological interactions at microscopic level (Alexander, 1964) and its estimation is often challenging (Ritz, 2011; Baveye et al. 2018). However, recent technological developments have permitted researchers to observe and

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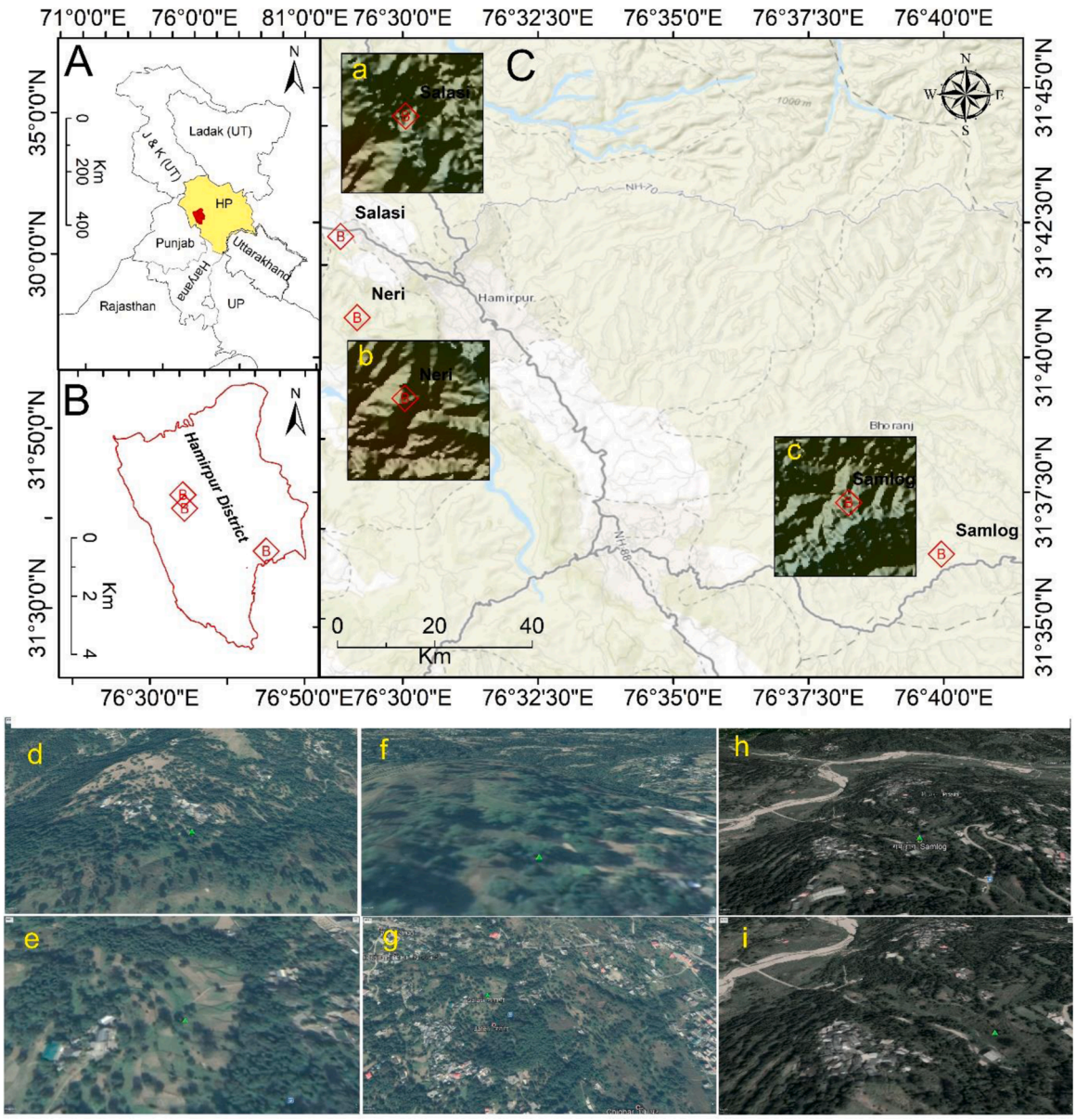

Fig 1. Mapping of agricultural soil samples collected from sites Salasi (a), Neri (b) and Samlog (c) in district Hamirpur of Himachal Pradesh, India; (d-i) different locations of sites used for soil sampling.

understand the microscopic heterogeneity of soil (Hapca et al., 2015). In this context, use of Scanning Electron Microscopy (SEM) and Energy-Dispersive X-ray spectroscopy (EDX) offers several advantages (Watteau, 2018). SEM coupled to EDS uses a beam of electron to provide information about the topography and composition of materials. SEM-EDS have been deployed for soil and various biological applications such as imaging of microorganisms which help us to understand the soils functions and the role of microorganisms in rhizosphere interface (Liu et al., 2015: Watteau, 2018).

The present study highlights the monitoring of soil-elemental composition and distribution of bacterial diversity in cauliflower rhizosphere. To the best of our knowledge, this study is first of its kind to give morpho-characteristics and elemental composition analysis of agricultural farmland soils and associated bacterial taxa.

Material and methods

Soil sample collection

Three soil samples from cauliflower rhizospheres were collected and pooled together. The sampling sites includes Neri (Lat: 31.67894, Lng: 76.48598), Samlog (Lat: 31.60591, Lng: 76.66592 and Salasi (Lat: 31.70403, Lng: 76.48089) of Himachal Pradesh, India (Fig. 1). Soil samples were air dried, ground to fine powder and then passed through sieve. Soil samples were stored at 4 °C for further experiments.

Soil profiling

The pH of soil samples was determined using standard methods in 1 N KCl extracts at 1:2.5 ratio of soil: KCl using Mettler pH meter (Mettler Toledo). Soil moisture contents were determined using formula described below:

Table 1
Elemental mapping using Energy Diffraction Spectrum (EDS) of agricultural soils.

Composition	Mass			Atom		
	Neri	Salasi	Samlog	Neri	Salasi	Samlog
O	51.713±0.379	51.94±1.498	50.89±2.84	66.69±0.444	66.63±0.703	65.67±1.972
Mg	1.4 ± 0.179	1.27	1.223±0.253	1.187±0.152	1.09	1.033±1.88
Al	8.74±0.53	9.443±0.479	10.577±0.208	6.687±0.417	7.2 ± 0.463	8.107±0.095
Si	28.79±0.544	31.36±2.401	29.09±0.719	21.15±0.382	22.873±1.32	21.463±1.009
P	0.133±0.054	0.077±0.035	0.23±0.078	0.097±0.032	0.05±0.023	0.153±0.055
K	2.477±0.128	3.373±0.127	3.32±0.459	1.303±0.069	1.803±0.067	1.763±0.27
Ca	1.297±0.593[b]	2.48±0.023[b]	0.68±0.05[a]	0.667±0.308[ab]	1.28±0.25[a]	0.353±0.023[b]
Fe	5.09±0.163[b]	6.747±0.167[a]	6.243±0.458[a]	1.88±0.064[b]	2.527±0.057[a]	2.357±0.192[a]

Values are means ± standard error. Values within rows with different superscripts are different.

$$\frac{Moisture\ content\ (w) = Initial\ weight/wet\ weight\ (W1) - Dry\ weight\ (W2)}{Dry\ weight\ of\ sample(W2)} \times 100$$

Characterization of soil samples

The air dried and fine ground soil samples were gold coated for 30 s using DII-29030SCTR Smart Coater JEOL. The SEM-EDX analysis was performed under high vacuum conditions at 20 kV with working distance 10–12 mm (JSM IT500 JEOL). EDX detector was used for area and elemental mapping using probe current between 25 and 50 μA at variable count rate (CPS) with accelerating voltage 20 kV and working distance 9.5 to 10.5 mm. The images were recorded and analyzed using automated JEOL IT 500 Software.

Isolation and purification of agriculturally important bacteria

Serial dilutions of finely grounded soil samples were prepared in autoclaved distilled water and plated on nutrient agar (Himedia). Plates were incubated at 28 °C and monitored for the growth of bacteria up to 48 h.

Screening of bacteria for antifungal activity

The purified isolates were screened for antagonistic activity using dual culture against *Fusarium oxysporum* and *Sclerotinia sclerotium* on yeast malt extract agar (Sharma et al. 2012; Sharma and Shanmugam, 2012). The plates were kept at 28 °C and observed daily for the inhibition of plant pathogen against control.

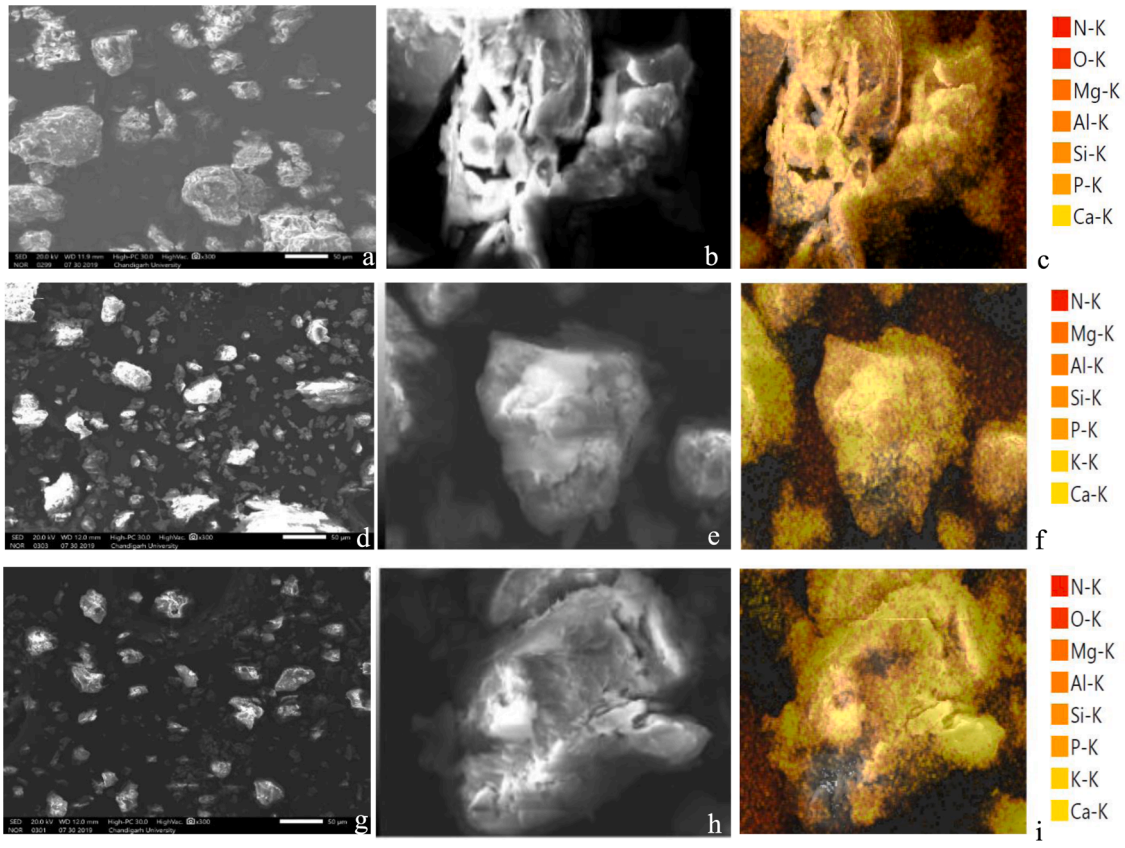

Fig 2. Morpho-characteristics of soils collected from cauliflower rhizospheres in different location of Himachal Pradesh: – particle size (a & b) and distribution pattern of elements (c) of soil collected from Neri; particle size (d & e) and distribution pattern of elements (f) of soil collected from Samlog; particle size (g & h) and distribution pattern of elements (i) of soil collected from Salasi. SEM was performed using JEOL IT500.

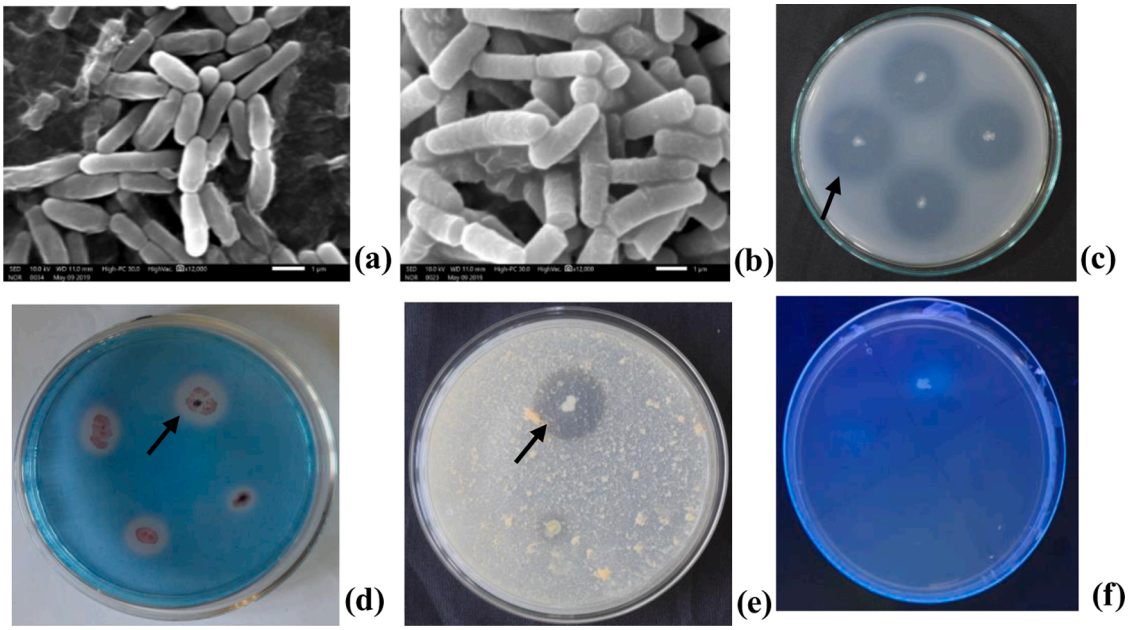

Fig 3. Isolation and characterization of bacteria for different plant beneficial attributes: (a) and (b)- morphological structure of isolate SLA 11 and SLA 8; (c)- phosphate solubilizing activity on Pikovskaya's agar; (d)-Siderophore production CAS agar; (e)- protease activity on skim milk agar; and (f)- chitinase activity using methylumbelliferyl (MUF)-labelled fluorogenic chitin analogs i.e. 4-methylumbelliferyl β-D- *N,N'*-diacetylchitobioside (MUF-diNAG) as substrate.

Chitinases and proteases production

The isolates were screened for chitinase production on agarose plate amended with methylumbelliferyl (MUF)-labelled fluorogenic chitin analogs i.e. 4-methylumbelliferyl β-D- *N,N'*-diacetylchitobioside (MUF-diNAG) (V Sharma and Salwan, 2015). Further, the isolates were spot inoculated onto skim milk agar plates and incubated at 28 °C for 3–5 days in an incubator and observed for formation of clear zone (Salwan et al., 2010).

Phosphate solubilization

Phosphate solubilization was detected by the formation of clear zones around the bacterial colonies on modified Pikovskaya (PVK) agar plates at 28 °C after 7–10 days. The quantification of phosphate solubilization was also done by using spectrophotometric method (Nautiyal 1999; Salwan et al., 2020a and b). The total soluble phosphorus was calculated and activity was expressed as release of μg phosphorus per ml compared to control.

Siderophore production

The ability of antagonistic bacteria for siderophore production was evaluated on CAS media. The active growing cultures of selected isolates were spot inoculated on CAS agar plates and incubated at 28 °C for 7–10 days (Louden et al., 2011; Sharma et al., 2018).

Indole-3-acetic acid like auxin production

The production of Indole-3-acetic acid (IAA) was performed using-tryptophan as substrate and Salkowski reagent (Loper and Schroth, 1986). The activity was determined using IAA (0 to 50 μg of IAA/ml) and expressed as μg/ml over negative control.

Characterization and identification of promising bacteria

The selected isolates were characterized based on their morphology, biochemical and molecular characterization. Morphology was studied by fixing the cultures in 1% osmium tetroxide (OsO_4) using SEM image analysis (Salwan et al., 2020b). Genomic DNA was isolated from overnight grown culture using DNA isolation kit as per manufacturer instructions (Promega). The molecular characterization was done using16S rRNA gene sequencing (Salwan et al., 2020). PCR was performed in T100 Thermal Cycler (Biorad) using initial denaturation at 98 °C for 3 min followed by 35 cycles 98 °C for 10 s, 52 °C for 40 s and 72 °C for 1.4 min and final elongation of 72 °C for 5 min. Nucleotide sequencing was done using custom sequencing (Yaazh Xenomics, Tamil Nadu, India). The nucleotide sequences were aligned together to deduce the complete sequence and analyzed for best hit in NCBI BLAST. The sequences were submitted to Genbank for accession numbers and phylogenetic analysis was performed using MEGAX (Tamura et al., 2004; Kumar et al., 2018).

Statistical analysis

The values are the mean of three replicates with standard error and analyzed based on one factor analysis in OPSTAT online statistical tool (Sheoran et al., 1998). The data was compared and considered significant based on CD value. The correlation between species distribution and soil elemental components was evaluated by applying canonical correspondence analysis using PAST software version 4.03 (https://folk.uio.no/ohammer/past/index.html).

Results

Soil characteristics

The elemental composition of soil samples based on EDS revealed the presence of O, Mg, Al, Si, P, K, Ca and Fe and amount of Fe and Ca differ significantly among all (Table 1). The association of minerals is shown in area mapping of three soil samples (Fig. 2). Further, characteristics revealed heterogeneous particles size varied from 50 to 5 μm. The average pH of these soil samples varied from 6.47 to 7.14 and average soil moisture content varied from 15.8 to 16.23%.

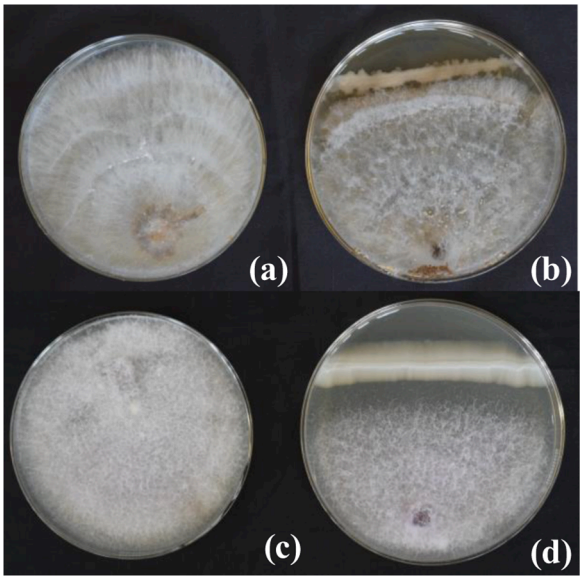

Fig. 4. Antagonistic behavior shown by plant beneficial bacteria on yeast malt extract agar: **(a)**- Control plate showing growth of *Sclerotinia sclerotiorum*; **(b)**- antagonism of bacterial isolate against *S. sclerotiorum*; **(c)**- control plate showing growth of *Fusarium oxysporum*; and **(d)** antagonism of bacterial isolate against *F. oxysporum*.

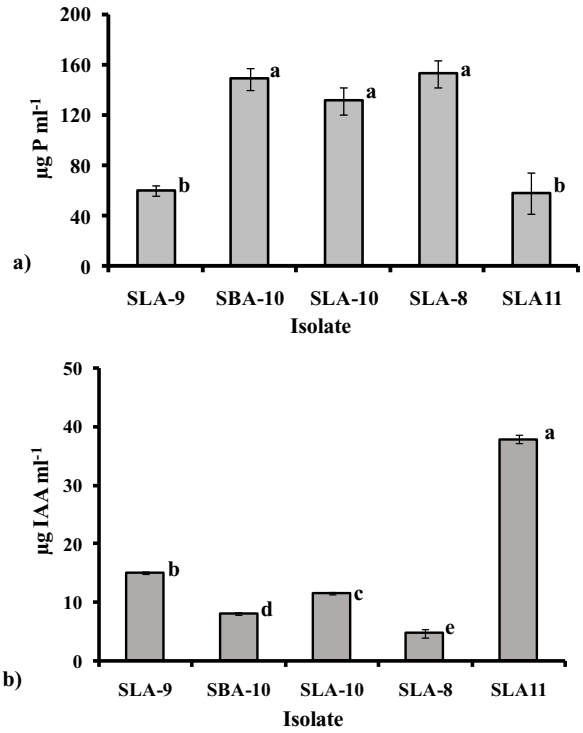

Fig. 5. Quantitative estimation of phosphate solubilization activity **(a)** and Indole acetic acid production **(b)** of isolates collected from agricultural soils of Himachal Pradesh. The values are means of three replicates with standard error. Bars with different superscript differ from each other.

Plant growth promoting attributes of selected isolates

A total of seven isolates were purified and morphological characteristics revealed variable type of growth. A typical rod shape structure of ~1 µM was observed for SBA10, SLA7, SLA8, SLA9 and SLA11 (Fig. 3a and b). The ability of selected isolates to produce phytase was observed by the formation of clear halo on Pikovskayas Agar media after 10 days at 28 °C (Fig. 3c). The siderophore production was observed by the formation of yellow color zone against bluish green background on CAS media after an incubation of 10 days at 28 °C (Fig. 3d). On the other side, extracellular protease production was observed by the formation of clear halo on skim milk agar after 3 days of incubation at 28 °C (Fig. 3e). Chitinase activity was observed as clear halo under UV light (Fig. 3f).

Table 2
Identification of plant beneficial bacterial isolates based on 16S rRNA gene in NCBI.

Isolate	Accession	Most closely related species	Identity (%)	Taxonomic designation
SLA7	MN515087	*Bacillus subtilis* IHB B 1516	99.9	*Bacillus* sp.
		Bacillus velezensis CZ-44	99.8	
		Bacillus siamensis 64X-5	99.8	
SLA8	MN515088	*Bacillus cereus* YB3	99.8	*Bacillus* sp.
		Bacillus thuringiensis NBAIR-BT25	99.7	
		Bacillus licheniformis DLSB-13	99.7	
SLA9	MN515065	*Bacillus subtilis* EB56	100	*Bacillus* sp.
		Bacillus pumilus HNS70	100	
		Bacillus altitudinis LXJ71	99.9	
		Bacillus stratosphericus CF46	99.9	
SLA11	MN515089	*Bacillus aerius* VITM4I	99.9	*Bacillus* sp.
		Bacillus licheniformis D4–10–2–2	99.7	
SBA10	MN515062	*Bacillus safensis* JCT-42	99.9	*Bacillus* sp.
		Bacillus australimaris B28A	99.8	
		Bacillus pumilus strain soapstock18B	99.8	
		Bacillus cereus UBT4	99.8	

Antagonistic activity of bacterial isolates

In direct confrontation assay, bacterial isolates restricted the growth of *Fusarium oxysporum* (Fig. 4a and b) and *Sclerotinia sclerotiorum* (Fig. 4c and d). Suppression of growth was indicated by the inhibited growth of fungal pathogen against bacterial isolates.

Quantitative estimation of phytase and IAA activity

The quantitative estimation revealed highest phosphate solubilizing activity in SLA8, SBA10 followed by SLA10 (Fig. 6a). For IAA activity, SBA-1 showed highest IAA like plant growth regulators productions followed by SLA10 (Fig.5b).

Identification of bacterial isolates

The 16S rRNA gene of bacterial isolates revealed amplified product of 1.5 kbp. Further, nucleotide sequencing and BLAST analysis showed similarity of SLA7 to *Bacillus subtilis* IHB B 1516, *Bacillus velezensis* CZ-44 and *Bacillus siamensis* 64X-5, SLA8 to *Bacillus cereus* YB3, *Bacillus thuringiensis* NBAIR-BT25 and *Bacillus licheniformis* DLSB-13, SLA9 to *Bacillus subtilis* EB56, *Bacillus pumilus* HNS70, *Bacillus altitudinis* LXJ71 and *Bacillus stratosphericus* CF46, SLA11 to *Bacillus aerius* VITM4I and *Bacillus licheniformis* D4–10–2–2 and SBA10 to *Bacillus safensis* JCT-42, *Bacillus australimaris* B28A, *Bacillus pumilus* soapstock18B and *Bacillus cereus* UBT4 (Table 2). Phylogenetic analysis showed clustering of isolates to different species of *Bacillus* lying far from each other (Fig. 6). The sequences are submitted to Genbank with accession numbers MN515062,

Fig. 6. Molecular characterization and phylogenetic analysis of selected bacterial isolates. The evolutionary history was inferred using neighbour Joining method. Bootstrap values are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Phylogenetic analysis was conducted in MEGAX.

MN515087, MN515088, MN515065 and MN515089 (Table 2).

Correlation between soil elemental composition and Bacillus species

The correlation between species distribution pattern and soil elements revealed 83.4% correlation between *Bacillus* spp. and environmental variables with eigen values 0.045 and 0.009 for axis 1 and axis 2, respectively. Permutation test revealed less than 1% ($p<1$) level of significance. Elements Al, Mg, O along with Fe and K showed significant effect over *Bacillus* spp. distribution (Fig. 7). Sampling site Salasi associated with Ca and O along with Fe and K revealed distribution of closely related isolates *B. aerius, B. pumilus* and *B. subtilis* and another site Samlog associated with several elements Al, Si, P and pH and moisture content influence the distribution of closely related species belonging to *B. safensis*. The elemental composition and *Bacillus* spp. of soil collected from Neri did not reveal significant association.

Discussion

The soil and microbial community are vital source for the supply of different minerals to the plants. Therefore, understanding the structural organization of niche and residing microbes is of paramount importance. In recent times, the combination of different tools such as electron microscopy, EDX, XRD and other advance instruments are being deployed to understand the dynamics of soil compositions and biogeochemical cycles of different minerals (Williamson et al., 2017). Such integrated experimental framework associated with imaging techniques will facilitate our understanding on physico-chemical characterization and their impact on soil (Hapca et al., 2015). The present study revealed characteristic properties of rhizosphere soils from the cauliflower fields of district Hamirpur (Fig. 1) and identification of beneficial microbes with promising plant growth promoting attributes. The identified areas receive moderate amount of rainfall which causes leaching of minerals potassium, calcium, and magnesium in soil. The soil characteristics showed significant differences in the elemental composition (Table 1) and structural properties of the soils (Fig. 2). The EDS analysis revealed presence of high amount of Si^{2+} followed by Al^{3+}, Fe^{3+}, and $Mg^{2+}$and fewer amounts of Ca^{2+}, P^{+} and K^{+} and their oxides in all the soils (Table 1). Although the elemental composition varies among all soils but the amount of Ca and Fe differ significantly at $p<0.05$. Similar results covering variation in soil mineral composition have also been recorded among different soil samples (Moeskops et al., 2010). The pH profiling of selected rhizosphere soils showed the presence of neutral pH possibly due to the relatively low precipitation and buffering capacity of base-forming cations. The pH also serves as an important factor for soil profiling as metals are retained in basic pH soils while leaching and mobility in acidic pH soils (Kabata-Pendias, 2011).

Since the structure and abundance of microbial community is of vital importance for provisioning a continuous recycling of soil-based sustainable ecosystem services. Exploring and characterizing the microbial

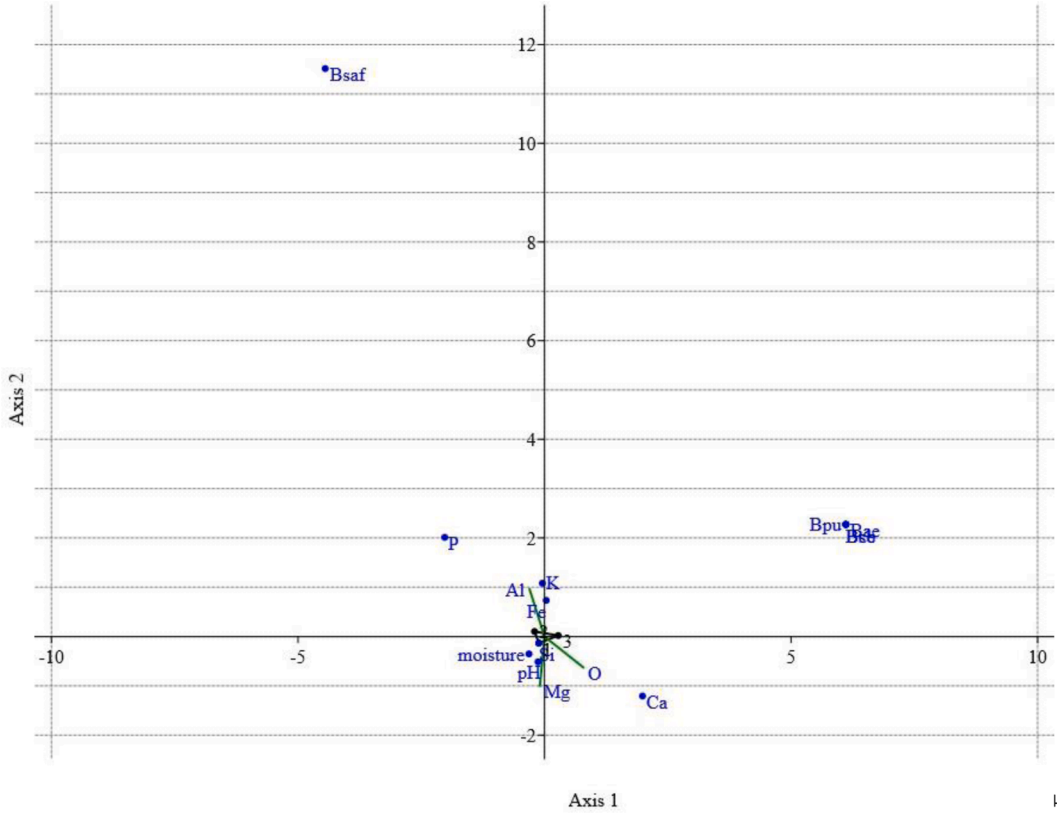

Fig. 7. A plot showing the relationship of identified species with soil elemental components among different sampling sites using canonical component analysis. Abbreviations- bae represents SLA11, bce as SLA8, bpu as SLA9, bsaf SBA10 and bsu SLA7. 1, 2 and 3 in triplot indicates sampling sites Neri, Samlog and Salasi, respectively.

community structure, abundance and their activity can be a good indicator of soil quality and health. The prevalence of microbes is found to exert positive effects on agricultural system at global scale (Lori et al., 2017). The bacterial population may be associated with differences in soil composition, climatic factors and as a function of agricultural practices adopted by the farmers. The present study revealed diverse species belonging to genus *Bacillus* according to their phylogenetic relationship (Fig. 6) and the isolates showing identity with the reference strains of two or more species were not assigned to any species in accordance with the current taxonomic practices (Table 2). The morphological characteristics revealed a typical rod-shaped structure with variation (Fig. 3a and b). These strains have shown plant growth promoting attributes including phosphate solubilization (Fig. 3c and 5a), siderphores (Fig. 3d), and chitinase and protease activity (Fig. 3e). These strains were also reported to produce indole acetic acid-like growth hormone (Fig. 5b). Moreover, these strains were effective in inhibiting the growth of phytopathogens *Fusarium oxysporum* (Fig. 4a and b) and *Sclerotinia sclerotiorum* (Fig. 4c and d). Such studies provide evidences for their utilization in agricultural practices and advocate the positive effect conferred by plant beneficial communities and farming practices in soil health (Lori et al., 2017).

The relationship between environmental variables and identified *Bacillus* spp. revealed the importance of soil elemental composition and physico-chemical parameters on bacterial distribution (Fig. 7). The multivariate component based canonical component analysis explained 83.4% correlation between *Bacillus* spp. and environmental variables. Elements Al, Mg, and their oxides along with Fe and K showed significant effect over *Bacillus* spp. distribution (Fig. 7). The isolate identified as *B. safensis* is distributed in area with high amounts of phosphorus level, hence more efficient in phosphate solubilization. Similarly, isolates closely related to *B. aerius, B. cereus, B. pumilus* and *B. subtilis* are distributed in areas with high calcium levels (Table 1). Besides this, the ability of the isolates to solubilize minerals such as Fe through siderophore production can help to reduce the extensive usage of fertilizers in soil containing higher amount of minerals. Various species of bacteria such as *B. megaterium* produce siderophores to provide Fe in soluble form to the plants during deficiency (Robin et al., 2009). Since the availability of minerals like P and K to plants is being decreased due to imbalanced use of fertilizers, soil erosion, leaching and intensive cropping (Zörb et al., 2014). Similarly, various *B. mucilaginosus, B. edaphicus, B. circulans* and *Paenibacillus* spp. have been reported for potassium solubilization (Friedrich et al., 1991) and *B. circulans, Paenibacillus* and *Bacillus* spp. for phosphorus availability to the plants (Etesam et al., 2017; Alori et al., 2017).

The present study is first of its kind to provide a view of soil mineral composition and their associated plant beneficial bacteria in agricultural soils. Further, studies on role of microbes in mineral recycling and pathogen suppression will help in developing a sustainable solution to counter biotic and abiotic stresses.

Conclusion

The soil composition, microbial community structure and abundance are crucial as integrated components of sustainable agricultural farmland in order to provide continuous recycling of soil-based ecosystem services. Our efforts to underpin the soil minerals and the plant beneficial bacteria with plethora of beneficial activities to counter biotic and abiotic stresses will help in enhancing the agricultural productivity and counter climatic factors in efficient ways.

Ethics approval (include appropriate approvals or waivers)

Not applicable

Consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

Not applicable

Code availability (software application or custom code)

Not applicable

Authors' contributions

Richa Salwan and Vivek Sharma were involved in the execution of experimental work, MS writing and editing. Raj Saini provided soil samples. AS assisted in conducting experiments and Dr. Manish Pandey contributed in preparing geographic map.

Declaration of Competing Interest

The authors declared no conflict of interests.

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