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Illumina based high throughput analysis of microbial diversity of maize rhizosphere treated with nanocompounds and *Bacillus* sp.

Parul Chaudhary [a,*], Anita Sharma [a], Anuj Chaudhary [b], Priyanka Khati [c], Saurabh Gangola [a], Damini Maithani [a]

[a] Department of Microbiology, G.B. Pant University of Agriculture & Technology, Pantnagar, Uttarakhand, India
[b] School of Agriculture and Environmental Sciences, Shobhit University Gangoh, Uttar Pradesh, India
[c] Crop Production Division, Vivekananda Parvatiya Krishi Anusandhan Sansthan, Almora, Uttarakhand, India

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ABSTRACT

Soil microorganisms play a crucial role in the maintenance of the ecosystem. Their diverse enzymatic machinery facilitates the biogeochemical cycling of essential macro/micronutrients. Over the past two decades, significant amount of research has been carried out on the application of nanocompounds in agricultural practices. Some reports support the role of nanocompounds in enhancing crop productivity by providing essential nutrients to plants or by exhibiting antimicrobial activities against different phytopathogens. Meagre information is available on long term impact of agriusable nanocompounds along with plant growth promoting rhizobacteria on microbial population of an agriculture field. In this study, attempts have been made to analyse the impact of nanozeolite and nanochitosan (50 mg L^{-1}) along with a bioinoculant (*Bacillus* sp.) on the bacterial community of maize rhizosphere under field condition. Total bacterial counts, activities of soil health indicator enzymes and total microbial diversity of the experimental maize rhizosphere were assessed using Illumina based high throughput sequencing after 60 days of the experiment. Obtained results indicated higher bacterial diversity in the treated soil than the control which corresponded to increased number of Operational Taxanomic Units (OTUs). Combined treatment of bioinoculant and nanocompounds showed two fold increase in FDA (Fluorescein diacetate hydrolysis), dehydrogenase and alkaline phosphatase activity than the control. Presence of dominant bacterial genera viz. *Actinobacteria*, *Bacteroidetes*, *Acidobacteria* and *Chloroflexi* were observed in treated soil sample. Combined treatment of *Bacillus* sp. and nanocompounds had a strong influence on the composition of rhizospheric microbiota, diversity and richness. We propose that the application of nanocompounds along with a potential bioinoculant is beneficial for the survival of rhizospheric bacterial population and soil health.

1. Introduction

Soil is one of the most challenging systems for microbiologists. The extent of microbial diversity and community size of the soil must be maintained precisely to analyse soil health. Excessive use of chemical fertilizers and pesticides is detrimental to soil health and thereby quality of soil (Tilman et al., 2002). Development of new agricultural approaches for precision farming is crucially needed to boost the agricultural productivity and soil fertility while taking care of the toxicity issues equally. Nanotechnology opens up a broader scope to achieve better crop production in agricultural fields because of efficient nutrient management (Rastogi et al., 2019). Various nanocompounds have been used in agricultural field including gold NPs (AuNPs), ZnO, SiO_2 and TiO_2 (Venkatachalam et al., 2017). Nanoparticles with very special characters like small size (dimensions less than 100 nm) and large surface area show good adsorption property (Yadav, 2013). Nanosize, stability and inner structural porosity of nanocompounds result in more water retention and high cation-exchange capacity to hold nutrients (Ok et al., 2003). Likewise, uniform particle size distribution and large porosity of zeolite make it exceptionally desirable for improving soil characteristics and crop yield (Ramesh et al., 2010).

Physiochemical properties of nanozeolites are comparable to its micron-sized crystals. Decreased crystal size of nanozeolite is beneficial and broadens the application of aluminium silicate for different purposes (Mintova et al., 2016). Aminiyan et al. (2018) reported that application of nanozeolite (10 and 30%) along with plant residues

* Corresponding author.
E-mail address: parulchaudhary1423@gmail.com (P. Chaudhary).

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increased the actinomycetes population after 90 days of incubation under laboratory conditions. Karunakaran et al. (2013) observed improved growth of PGPR and total soil microbial population in maize rhizosphere under nanosilica treatment. Biomaterials like chitosan have aroused the interest of scientific community for its potential use in agriculture as they have environment friendly nature, excellent biocompatibility and bioactivity (Katiyar et al., 2015). Distinctive physicochemical properties like size, surface area and cationic nature of nanochitosan make them desirable for agricultural use (Chandra et al., 2015). Parul (2019) reported that application of nanochitosan along with *Bacillus* sp. enhanced the level of enzyme activities and improved soil health during maize cultivation. Application of different nanoparticles in agriculture is valuable but their evaluation for nanotoxicity, based on concentration generates concern for environment safety.

Prokaryotic microbes are abundantly present in the soil and constitute a major fraction and biomass of microbial diversity of the soil. Rhizospheric microbes significantly influence properties of a soil ecosystem by regulating geochemical cycles through their enzymatic machinery. Microbial activities are responsible for nutrient transformation, decomposition, protection against pathogens and formation of soil structure (Bowles et al., 2014). Study of microbial population and their function in the soil is required to understand specific changes in microbial diversity of an ecosystem under specific conditions. Soil microorganisms can be used as bioindicator to test soil quality as they play crucial role in biogeochemical cycling of essential nutrients like carbon, nitrogen, zinc and phosphorus (Ghimire et al., 2014).

PGPR (Plant Growth-Promoting Rhizobacteria) are used as bioinoculants in agriculture system to enhance plant growth by enhancing nutrient accessibility (Bhardwaj et al., 2014; Bergkemper et al., 2016). Microbes of a soil ecosystem rapidly respond to environmental changes. Changes in the activities of microbial communities have been correlated with physical and chemical of soil under the stress of heavy metal or pesticides (Cai et al., 2015; Chen et al., 2016; Asad et al., 2017). PGPR like *Bacillus, Pseudomonas, Pantaoe agglomerans, Acinetobacter* and *Paenibacillus* are commonly found in the rhizospheric soil and reported to enhance plant growth through nutrient acquisition, biocontrol activities and developing resistance in plants (Zhang et al., 2017; Chaudhary and Sharma, 2019).

Extracellular enzymes secreted by soil microbes like cellulases, lipases and phosphatases are found responsible for the improvement of soil health (Glenn, 1976). FDA hydrolysis is directly related to major and active microbial activity of the soil. Dehydrogenases, the main indicator enzymes are involved in redox reactions and oxidative activities of microbes in the soil (Tabatabai, 1982). Alkaline phosphatases of microbial origin are involved in phosphorus cycling. Phosphatases and phytases are reported to enhance the availability of phosphorus in the rhizosphere. Microorganisms mineralize organic P by release of organic acids, anions and enzymes (Tarafdar and Yadav, 2011).

Bacterial diversity in the environmental samples could be studied by using cultivable and uncultivable practices. Metagenomic approach provides better picture of the microbial community in comparison to culture based approaches (Delmont et al., 2011). It is necessary to figure out the behaviour of nanocompounds in the soil and under stressed environmental conditions. Objective of this study was to examine the impact of two nanocompounds (nanozeolite and nanochitosan) in the presence of a bioinoculant on the bacterial community of maize rhizosphere through cultivable and uncultivable techniques. Results of this study may provide useful information on the behaviour of two nanocompounds on microbial population and soil health of maize rhizosphere.

2. Materials and methods

2.1. Bacterial strain and nanocompounds

Bacillus sp. (PS10-KX650179) used in this study was originally isolated from agriculture field (used exclusively for nanocompounds) of Crop Research Centre, G.B. Pant University, Pantnagar, India. According to Khati et al. (2019a), the organism was Gram positive spore former and showed PGPR properties like production of Indole acetic acid (IAA), siderophore and ammonia and phosphate solubilization. Two nanocompounds used in the experiment were purchased from Intelligent Materials Pvt.Ltd., India. Nanozeolite and nanochitosan with stock number NS6130–09-905 and NS6130–09-918 respectively, had size of <80 nm, pH 7–8 and 7–9, refractive index 1.47 and purity were 99.9% (Khati et al., 2019b).

2.2. Experimental design and field experiment

A field experiment was conducted on maize in 2017 (June to September) using nanocompounds and a bioinoculant at CRC, G.B.Pant University of Agriculture and Technology, Pantnagar. This region falls under subtropical climatic zone and situated at an altitude of 243.84 above mean sea level, 29^0N latitude and 79.3°E longitude. The summers are hot with maximum temperature more than 35^0C (June and July) whereas minimum temperature remains 23^0C during September and October. During experimental period, maximum rainfall was received during the month of July. Agriculture field used in the present study was under the practice of application of different nanocompounds since five years.

For field trial, four treatments: control (T1: without bacterial culture and nanocompounds), *Bacillus* sp. (T2: PS10), nanozeolite and nanochitosan along with PS10 (T27 and T30) were used in randomized block design (RBD). Three replications of each treatment were used in a plot size of 3.5m×4.2m (width and length) , where distance between row to row was 60 cm and plant to plant was 20 cm. Nanocompounds and the bioinoculant were applied through seed bacterization. Maize seeds were soaked in bacterial suspension having population of 2×10^8 cfu seed^{-1} along with nanochitosan and nanozeolite (50 mg L^{-1}) in nutrient broth for 10 min. Treated maize seeds (dried aseptically under room temperature) were sown in field.

2.3. Collection of soil samples

Sampling of test soil was done after 60 days of the experiment. Control and treated soil samples were collected from a depth of 15 cm, randomly from each plot and mixed properly. After sieving through 2 mm mesh sieve, soil samples were kept in sterile plastic bags in a cool box and stored at —20^0C in a deep fridge for molecular studies. Sub samples used for physico-chemical and enzyme activities were stored at 4^0C.

2.4. Physicochemical analysis of soil

Soil samples were air-dried for physicochemical analysis of soil. Soil pH was estimated by making soil solution (1:3, soil to distilled water) with the help of a pH meter. Organic Carbon (OC) was estimated according to Black (1965). Method of Jackson (1973) was used to determine total nitrogen. Available phosphorous and potassium content were measured as per the method described by Jackson (1958).

2.5. Enumeration of total bacterial population

Enumeration of total viable bacterial counts was done by using standard protocol as described by Messer and Johnson (2000). For total bacterial counts, soil samples were diluted upto 10^{-4} dilutions and pour plating was done using nutrient agar. Inoculated plates were incubated at $30^0 \pm 1$ °C in a BOD incubator for 24 h. Bacterial colonies were counted and expressed as log cfu g^{-1} of soil. This experiment was performed in triplicate.

2.6. Soil enzyme activity

Immediately after harvesting, soil samples from each plot were used to estimate activities of three important indicator enzymes viz. fluorescein diacetate hydrolysis, dehydrogenase and alkaline phosphatase. Hydrolysed products of each enzyme were analysed by taking optical density using a spectrophotometer and reading was compared with the standard curve(s). All the enzyme assays were conducted in triplicate.

2.6.1. FDA enzyme activity

For FDA analysis, 1 g soil was placed in 150 ml flask containing 50 ml sodium phosphate buffer (pH -7.6). After adding FDA solution (0.5 ml), flasks were incubated in an orbital shaker for 1 h at 24 °C. 2 ml acetone was added to terminate the enzyme reaction. Soil suspension was centrifuged at 8000 rpm for 5 min. Obtained supernatant was filtered through Whatman No.2 filter paper. Absorbance of the filtrate was measured at 490 nm and FDA hydrolysis was expressed as μg fluorescein/g dry soil/h (Schnurer and Rosswall, 1982).

2.6.2. Dehydrogense enzyme activity

To analyse dehydrogenase activity, 5 g soil was added in Triphenyl Tetrazolium Chloride (TTC) solution (5 ml) in 150 ml flask. Flasks were incubated at 120 rpm for 8 h at 37 °C. After adding 25 ml acetone, contents were mixed and centrifuged at 4500 rpm for 10 min at 4 °C. Obtained supernatant was filtered through Whatman No.2 and absorbance of the filtrate was taken at 485 nm using spectrophotometer. Dehydrogenase activity was expressed as μg TPF/5 g dry soil/8 h (Casida et al., 1964).

2.6.3. Alkaline phosphatase activity

One gram of test soil was put in a test tube in which 250 μl of toluene, modified universal buffer (4 ml, pH- 11) and 1 ml *p*-nitrophenyl phosphate (pNpp) (25 mM) were added. Tubes were incubated at 37 °C for 2 h. After incubation, $CaCl_2$ (1 ml) and Tris buffer (0.1 M, pH 12) (4 ml) were added to the soil mixture. Soil suspension was allowed to develop colour and then filtered through Whatman no.1 paper. Intensity of the colour was determined at 400 nm. Enzyme activity was expressed as μg pNP/ g dry soil /h (Tabatabai and Bremner, 1969).

2.7. Soil DNA extraction and PCR amplification

Extraction of DNA from rhizospheric soil samples of different treatments (control, nanozeolite+PS10 and nanochitosan+PS10) was carried out within 24 h of sample collection using Power Soil™ DNA isolation kit of Mobio Lab.Inc., USA. Quantity and purity of DNA was quantified by taking absorbance of DNA samples at 260 and 280 nm as well as through 1.2% agarose gel electrophoresis. Purified DNA was stored at -20 °C for further use. Variable V3 and V4 regions of 16S rDNA genes were amplified using primers (341F-5'CCTACGGRRBGCASCAGGKVRVGAAT; 785R5'GGACTACNVGGGTWTCTAATCC).

2.8. Illumina sequencing

Composition and distribution of prokaryotic microorganisms in the test soil was analysed by amplifying two variable regions of 16S rDNA (V3-V4) by Illumina MiSeq sequencing which generated 300 bp paired end reads. Paired end reads were processed and checked for quality parameters like base calling and preliminary quality analysis, average base content and GC distribution and merged using Bcl2fastaq (v2.17.1.14). Multiple filters like conserved region and mismatch filters were placed to generate high quality V3-V4 region sequences with an average contig length of ~350 to ~450 bp. Pre-processed reads were pooled and clustered from all the samples to access Operational Taxonomic Units (OTUs) using QIIME program at 97% similarity (Caporaso et al., 2010). Taxonomic classification of each representative OTU was operated using SILVA 16S RNA gene database (DeSantis et al., 2006). Phylum, class, genus and species level distribution for different samples based on OTU were identified. Alpha diversity analysis of the species in each sample was determined through a series of statistical indices like ACE, Shannon and Simpson index, Chao1 and good coverage. These indices were estimated with the help of QIIME software (Version 1.9.1). Rank Abundance Curve is used to analyse diversity which reflects species abundance and species uniformity using R software for graph generation based on OTUs analysis. Rarefraction curve was used to predict the species abundance using QIIME software. Weighted Unifrac approach was used to measure the differences (β- diversity) in species in different samples. PCoA (Principal Co-ordinate Analysis) visualizes the similarity and difference of data. PCoA analysis was performed and plotted based on Brary-Curtis distance matrix. PCA (Principal Component Analysis) is a statistical technique and used to analyse the distribution of functional genes of different samples. NMDS (Non-metric Multidimensional Scaling) analysis is used to position each object in multidimensional space based on its functional classification information and to calculate the distances between different points as a measurement of their difference which are used to obtain the spatial position map. Graph is generated using vegan package in R based software on beta diversity distance matrix. UPGMA (Unweighted Pair Group Method with Arithmetic Mean) cluster analysis was performed for evolutionary information of sample sequences to calculate whether samples in a specific environment are significantly different from evolutionary lineage in microbial communities. Clustering method was used to cluster the samples (tree) based on the Brary-Curtis distance matrices for all the samples.

2.9. Statistical analysis

Statistical analysis of soil enzyme activities and bacterial counts was carried out by One way analysis of variance (ANOVA) using SPSS, ver. 16.0 software. Significant differences among means were tested with Duncan's Multiple Range Test (DMRT) at $P < 0.05$. The data represented in the figures are expressed as means of three replicates ± standard deviation (SD).

2.9.1. Accession number

Metagenomic sequencing data were submitted to NCBI Sequence Read Archive with accession number PRJNA548041.

3. Results

3.1. Soil physicochemical analysis

In the present study experimental soil was Mollisol, sub order –udoll, great group – Hapludoll (Deshpande et al., 1971). pH for control, *Bacillus* sp., nanozeolite+PS10, nanochitosan+PS10 treated soil was 7.2, 7.5, 7.9 and 7.8 respectively. Organic carbon (%) was 0.75, 0.77, 0.79 and 0.78 for control, *Bacillus* sp., nanozeolite+PS10 and nanochitosan+PS10 treated soil respectively. Available nitrogen was 212.89, 214, 219 and 224.12 kg ha^{-1} for control, *Bacillus* sp., nanozeolite+PS10 and nanochitosan+PS10 treated soil respectively. Values of available phosphorus were 20, 23, 25.12 and 25.55 kg ha^{-1} for control, *Bacillus* sp., and nanozeolite + PS10 and nanochitosan + PS10 treatments respectively. Available potassium was 133.20, 136.88, 139.23 and 140.12 kg ha^{-1} for control, *Bacillus* sp. (PS10), nanozeolite+PS10 and nanochitosan+PS10 respectively (Table 1). Different macronutrients regulate the nutrient level of the soil. We observed enhanced level of macronutrients in the treated soil.

3.2. Total bacterial population of soil

Total bacterial population in rhizospheric soil of nanozeolite+PS10, nanochitosan+PS10, *Bacillus* sp. and control were found to be 2.56×10^6, 2.53×10^6, 2.44×10^6 and 2.12×10^6 cfu g^{-1} (Colony Forming

Table 1
Physicochemical properties of the experimental site.

S. No	Particulars	Control	*Bacillus* sp.	Nanozeolite + *Bacillus sp.*	Nanochitosan + *Bacillus sp.*
1	pH (1:3) Soil: water ratio	7.2	7.5	7.9	7.8
2	Organic carbon (%)	0.75	0.77	0.79	0.78
3	Available nitrogen (Kg ha^{-1})	212.89	214	219	224.12
4	Available P_2O_5 (Kg ha^{-1})	20.00	23	25.12	25.55
5	Available K_2O (Kg ha^{-1})	133.20	136.88	139.23	140.12

Unit) respectively. Results show significant difference in bacterial counts in different soil samples.

3.3. Soil enzyme activities

Significant difference ($p < 0.05$) in the enzyme activities of different treatments was observed. Fluorescein diacetate activity was maximum (43.45 µg fluorescein g^{-1} h^{-1}) in nanozeolite+PS10 treated soil followed by 41.54 µg fluorescein g^1 h^{-1} in nanochitosan+PS10 treated soil, 31.25 µg fluorescein g^1 h^{-1} in *Bacillus* sp. treated soil and 17.45 µg fluorescein g^{-1} h^{-1} in control. More than two fold increase in FDA activity was observed in the treated soil over control. Two fold increase in dehydrogenase activity in nanozeolite+PS10 (7.79 µg $TPFg^{-1}$ h^{-1}), nanochitosan+PS10 treated soil (7.75 µg $TPFg^{-1}$ h^{-1}) than the control (3.77 µg $TPFg^{-1}$ h^{-1}). Highest alkaline phosphatase activity (479.50 µg PNP g^{-1} h^{-1}) was found in nanochitosan+PS10 treated soil followed by nanozeolite+PS10 treated soil (463.00 µg pNP g^{-1} h^{-1}), *Bacillus* sp. treated soil (423.33 µg pNP g^{-1} h^{-1}) and control (219.33 µg pNP g^{-1} h^{-1}) (Fig. 1). On the basis of total bacterial population and enzyme activities combined treatment of nanozeolite and nanochitosan along with *Bacillus* sp. showed best performance hence further used for metagenomic studies.

3.4. Statistics of metagenome sequencing of V3-V4 region of 16S rDNA and overall diversity of bacterial communities in the maize rhizosphere of three treatments

Targeting the hypervariable (V3-V4) region, total 2,22,110 (T1), 1,97,562 (T27) and 2,15,336 (T30) reads of 300 bp sequence length were obtained using Illumina MiSeq platform (SM 1). From all of the samples, 2,36,012 high-quality V3-V4 sequences (76,535 for T1, 75,749 for T27 and 83,728 sequences for T30) with an average contig length of 454, 455 and 452 bp were obtained after filtering the low-quality reads, chimeras and attachment sequences. The effective of sequences were found to be 68.92%, 76.68% and 77.76% with a GC content of 54.51%, 56.20% and 55.85%.

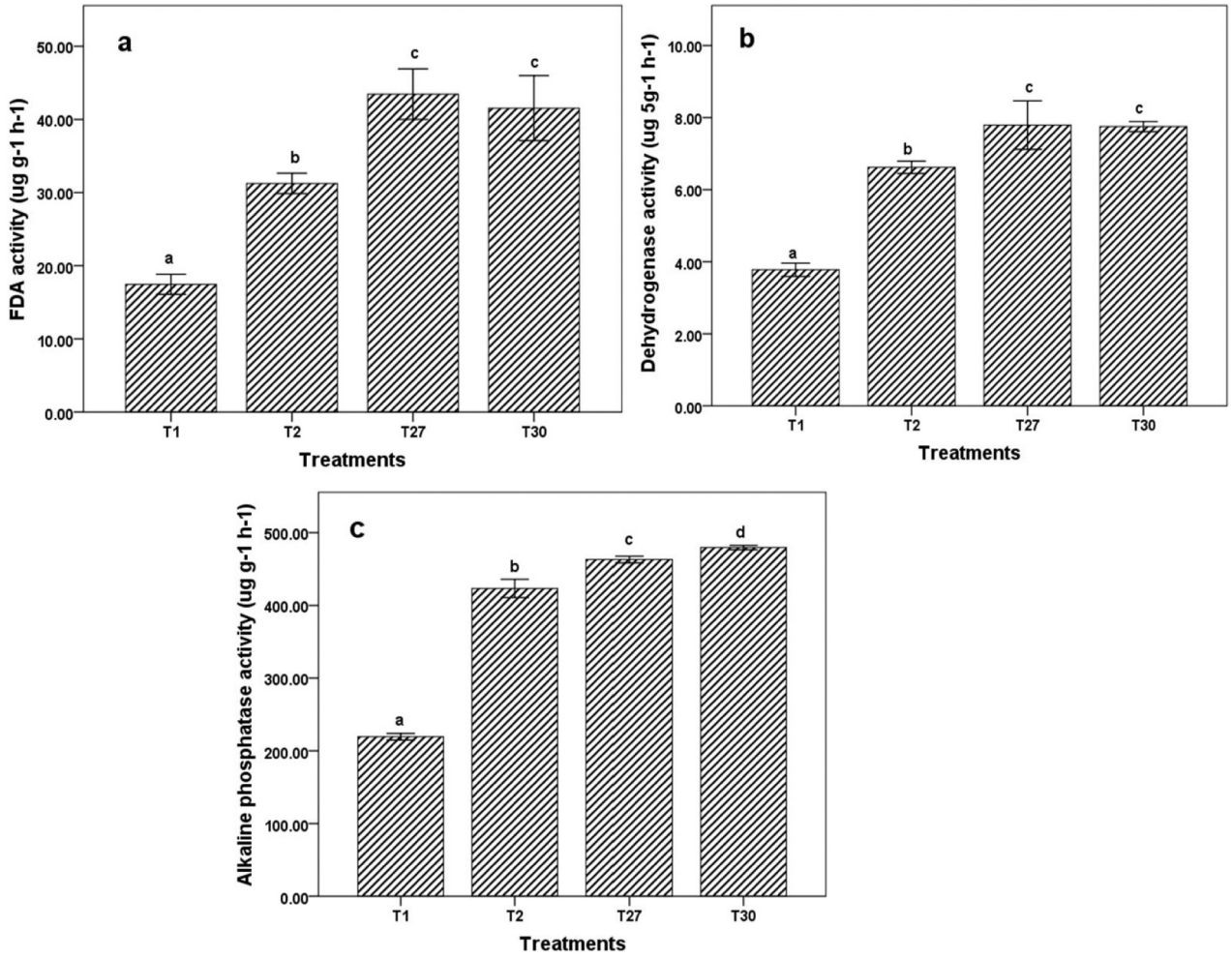

Fig. 1. Enzyme activities of rhizospheric soil treated with nanocompounds and *Bacillus* sp. (a) Fluorescein diacetate, (b) Dehydrogenase and (c) Alkaline phosphatase.

3.4.1. Richness and diversity of bacterial communities

Nanocompounds treated soil affected richness of bacterial community as T1 had 734.43, T27 had 855.58 and T30 had 867.81. Richness of nanozeolite+PS10, nanochitosan+PS10 and control was 850.346, 858.571 and 728.023 respectively. Diversity indices for bacterial communities displayed different trends in control and treated soil. Shannon index of bacterial community in soils under the treatment with nanozeolite+PS10 (6.89), nanochitosan+PS10 (7.33) were significantly higher than the control (5.24). Simpson indices values of samples T1, T27 and T30 were 0.909, 0.957 and 0.979. Nanochitosan+PS10 treated soil showed greatest coverage and the Chao1, Shannon index, as well as the Simpson index. β-diversity of these samples was determined using UniFrac distance matrix (Fig. 2). Three samples were aligned into two clusters. Control (T1) sample formed independent cluster whereas nanozeolite+PS10 (T27) and nanochitosan+PS10 (T30) samples joined in the same cluster (Fig. 3).

3.4.2. Effect of treatments on bacterial diversity

UniFrac-weighted PCA, based on the OTU composition also demonstrated variations among different soil samples, with the first two axes explaining 85.46% and 14.54% total alteration in bacterial diversity (Fig. 4). Nanozeolite+PS10 and nanochitosan+PS10 treatments were distinctly separated from the control. Alteration in community structure was figured out using Principal Component Analysis (PCoA). Two axis explained 59.31% and 40.69% variation in this study. Bacterial community in maize planted soil with nanocompounds was different from the control soil (Fig. 4). NMDS showed that each point represents a sample, and the distance between the points indicates the degree of difference. Samples of the same group are represented by the same colour. Stress <0.2 indicates that NMDS can accurately reflect the difference between the samples.

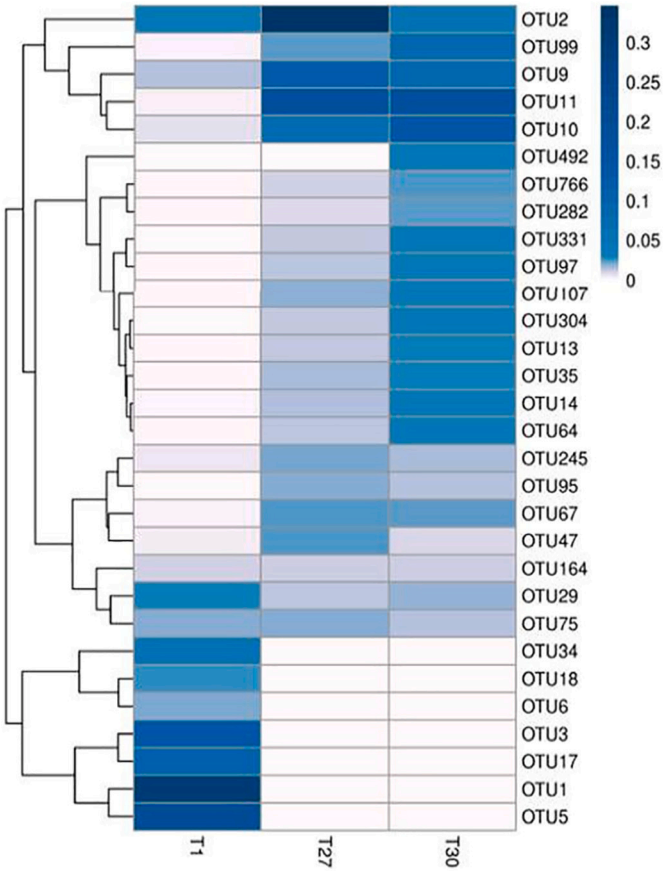

Fig. 2. Top 30 OTUs abundance clustering heatmap of treated soil: T1 (Control), T27 (nanozeolite+ *Bacillus* sp.) and T30 (nanochitosan+ *Bacillus* sp.). The left side of the figure is OTU cluster tree, the top is sample cluster tree. The value of each coloured box is the relative abundance of the OTUs.

3.4.3. Predominant bacterial taxonomic composition at phylum, class, genus and species level

Composition of the bacterial communities at phylum level varied among different treatments (Fig. 5). Total number of phyla observed in control, nanozeolite+PS10 and nanochitosan+PS10 were 14, 16 and 18 respectively. Two representative bacterial phyla *Proteobacteria* and *Actinobacteria* were dominant in all the treatments. Abundance of *Proteobacteria* was more in control (73.90%) as compared to treated soil T27 (36.11%) and T30 (54.92%). We observed significantly higher abundance of sequences affiliated with *Actinobacteria* (T1–14.02%, T27–22.12% and T30–15.66%), *Firmicutes* (T1–7.91%, T27–25.72% and 6.26%), *Bacteroidetes* (T1–0.43%, T27–3.91% and T30–8.92%), *Acidobacteria* (T1–1.32%, T27–3.35%, and 5.70%), *Chloroflexi* (T1–1.40%, T27–3.19% and T30–3.12%), *Gemmatimonadetes* (T1–0.59%, T27–2.24% and T30–3.45%), *Cyanobacteria* (T1–0%, T27–1.62% and T30–0.05%), *Nitrospirae, Planctomycetes, Verrucomicrobia* and *Saccharibacteria* as compared to control. T27 treatment had higher number of sequences affiliated to *Firmicutes* and *Chloroflexi*.

Among the treatments, pattern of taxonomic distribution was more apparent at class level. Six most abundant bacterial classes found in nanocompounds treated soil were *Alpha proteobacteria*, *Gamma proteobacteria*, *Bacilli*, *Actinobacteria*, *Thermoleophilia* and *Sphingobacteria* (Fig. 5). Nanochitosan+PS10 treated soil had significantly greater abundance of sequences affiliated with *Alpha proteobacteria* (38.57%) than nanozeolite+PS10 treated (22.24%) and control (31.38%). Control (T1) had maximum abundance of sequences affiliated with *Gamma proteobacteria* (39.75%) than the other treatments. T27 and T30 had 11.31% and 13.23% abundance of sequences respectively which were affiliated to *Gamma proteobacteria*. T27 treatment had higher number of sequences related to *Bacilli* (25.56%) than the other two treatments as T30 had 6.15% and control had 7.68% abundance of sequences related to bacilli.

Based on relative abundance, four dominant bacterial genera viz. *Sphingomonas, Bacillus, Rhizobium and Serratia* frequently occurred in different treatments (Fig. 6). The relative abundance of *Sphingomonas, Bacillus* and *Serratia* was 11.24%, 23.64%, 8.52, 26.60%, 4.98%, 8.42% and 3.02, 5.53%, 0.24% in T27, T30 and T1 treated soil samples, respectively. Abundance of sequences for *Rhizobium* was more in control than the treated soil.

Four dominant bacterial species in various treatments were *Bacillus drentensis*, *Ambiguous_taxa*, *Bacillus luciferensis* and *Bacillus simplex* (Fig. 6). Relative abundance of these species was more in T27 (17.06%, 5.25%, 1.17% and 1.06%) and T30 (2.9%, 5.04%, 0.08% and 0.05%) in treated soil samples as compared to control T1 (2.71, 4.71%, 0.05 and 0.02%), respectively.

3.4.4. Difference in microbial community in treated and untreated soil

Total number of unique bacterial taxa was 987 in all the three treatments. Out of which, 582 phyla were common (Fig. 7). 151 bacterial taxa were shared in nanochitosan and nanozeolite treated soils while 28 and 35 bacterial taxa of control were shared between nanozeolite+PS10 and nanochitosan+PS10 treated soil.

4. Discussion

Soil is a very complex system consists of various components where soil microorganisms play very important role to maintain its health and sustenance. Soil health that depends on balanced nutrient status is a matter of biochemical reactions performed by soil microbes. According to Kumar et al. (2019) microbial population of the soil is highly sensitive towards nanoparticles and needs proper optimization and assessment of different nanocompounds before their application in agricultural practices. Some reports on the role of nanoparticles and PGPR on plant

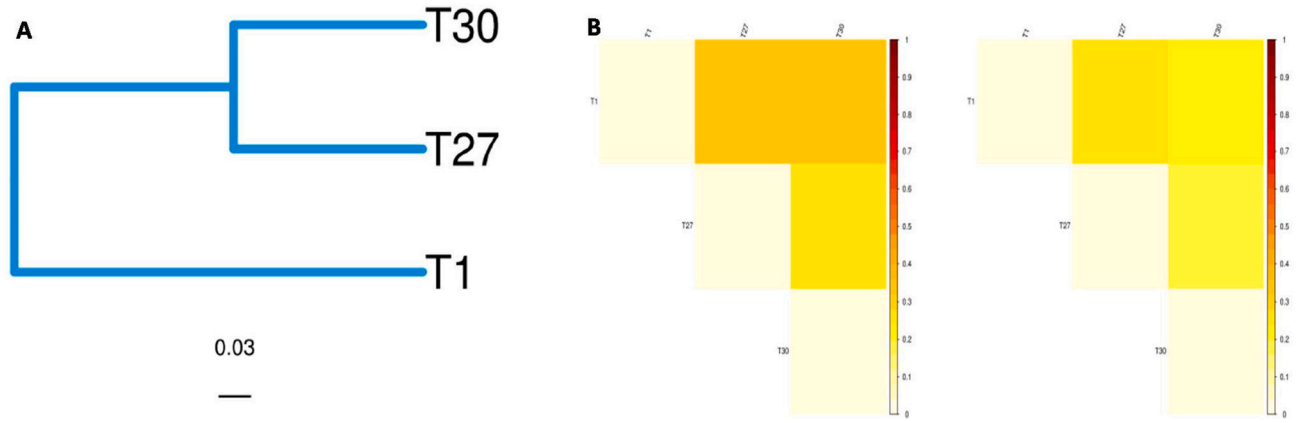

Fig. 3. (A) UPGMA tree each branch in the figure represent a sample (B) Un weighted unifrac distance matrix heat map.

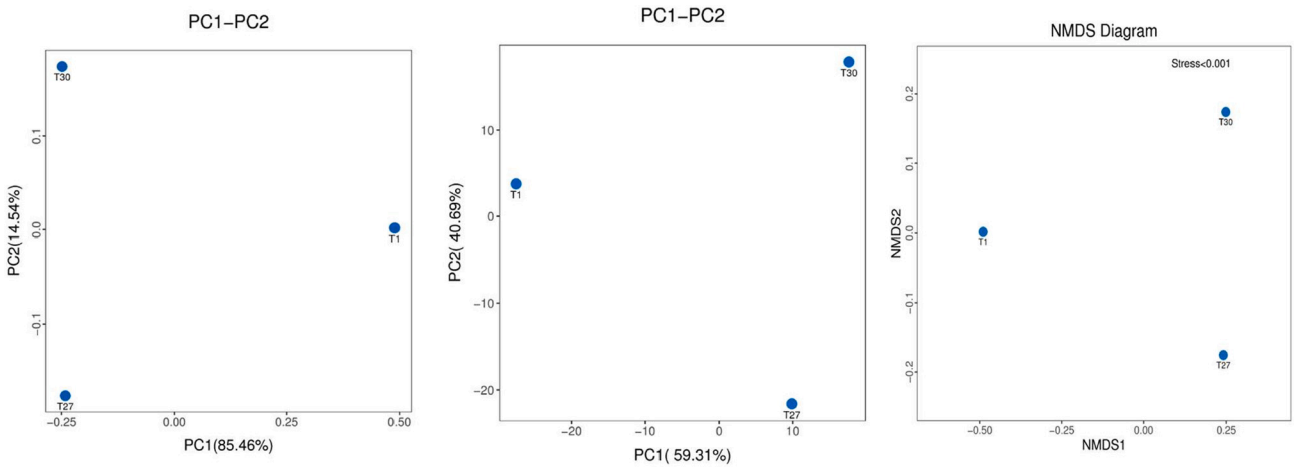

Fig. 4. Principal component analysis, Principal co-ordinate and NMDS analysis of T1 (Control), T27 (nanozeolite +*Bacillus* sp.) and T30 (nanochitosan+ *Bacillus* sp.)

growth and soil health are available, but combined effect of nanocompounds and bioinoculants with plant growth promontory characters has not been studied much. In this study, attempts have been made to observe the effect of two nanocompounds (nanozeolite and nanochitosan) with a bioinoculant (*Bacillus* sp.) on the status of macro/micro nutrients of the soil, total bacterial population, soil health indicator enzymes and on the composition of bacterial communities of the experimental soil.

In the present study application of nanocompounds improved the macronutrient status of the soil which was also found responsible for improved microbial population in the soil. According to Khati et al. (2017, 2018) nanozeolite and nanochitosan (50 mg L^{-1}) improved the physicochemical properties of the soil under the cultivation of maize crop in a pot experiment. Similarly both the nanocompounds were found to improve microbial population of the soil which might have indirectly enhanced maize health and productivity (Parul, 2019). The bacterial population was higher in treated soil as compared to control. These results indicated that the nanocompounds (50 mg L^{-1}) improved rhizospheric microbial population through efficient nutrient management. Chai et al. (2015) observed the effect of ZnO (Zinc oxide), SiO_2 (Silicon Dioxide), CeO_2 (Cerium Dioxide) and TiO_2 (Titanium Dioxide) nanoparticles (1 mg g^{-1}) on the microbial population of experimental soil and found that the functional bacterial counts were low in ZnO and TiO_2 treated soil but SiO_2 boosted the bacterial counts and activity of the soil enzymes.

Different soil enzymes, involved in cycling of carbon, nitrogen and sulphur immensely dependent on the abundance of bacterial population in soil. We observed two fold increase in enzyme activities over control in the presence of nanozeolite and nanochitosan. Activity of FDA, dehydrogenase and alkaline phosphatase enzymes of the experimental soil was significantly different in treated and untreated samples. Proteases, Esterases and lipases are involved in FDA hydrolysis in soil. Presence of the significant higher level of FDA activity in the test soil demonstrated that nanocompounds did not pose any toxic effect on microbial population of the soil. Dehydrogenases are intercellular respiratory enzymes and regulate various redox reactions in the microorganisms and serve as index of total microbial activity (Trevors, 1984). Enzyme activity is highly sensitive to the presence of xenobiotics like heavy metals and high level of pollution which can alter the microbial activity and thus population. Thus availability of enhanced level of different soil enzymes in the presence nanocompounds might be due to positive regulation of genes involved in metabolic activities of bacterial population. Application of nanocompounds in agriculture practices may support secreation of P- mobilizing enzymes by soil microbes which are involved in conversion of unavailable P to available form to be absorbed by plants. Similar observations were reported by Raliya and Tarafdar (2013) when Cluster bean (*Cyamopsis tetragonoloba*) was treated with ZnO (10 mg L^{-1}). Two fold increase in dehydrogenase activity of the soil was observed when treated with nano CuO (10 mg Kg^{-1}) in glass container (Jośko et al., 2019). They observed that a long term exposure of soil to engineered nanocompounds did not cause any significant changes in the enzyme activities and in the population size of tested group of microbes. A positive influence of Cu can be correlated with their function as a cofactor involved in metabolic processes. Similarly, Kwak et al. (2017)

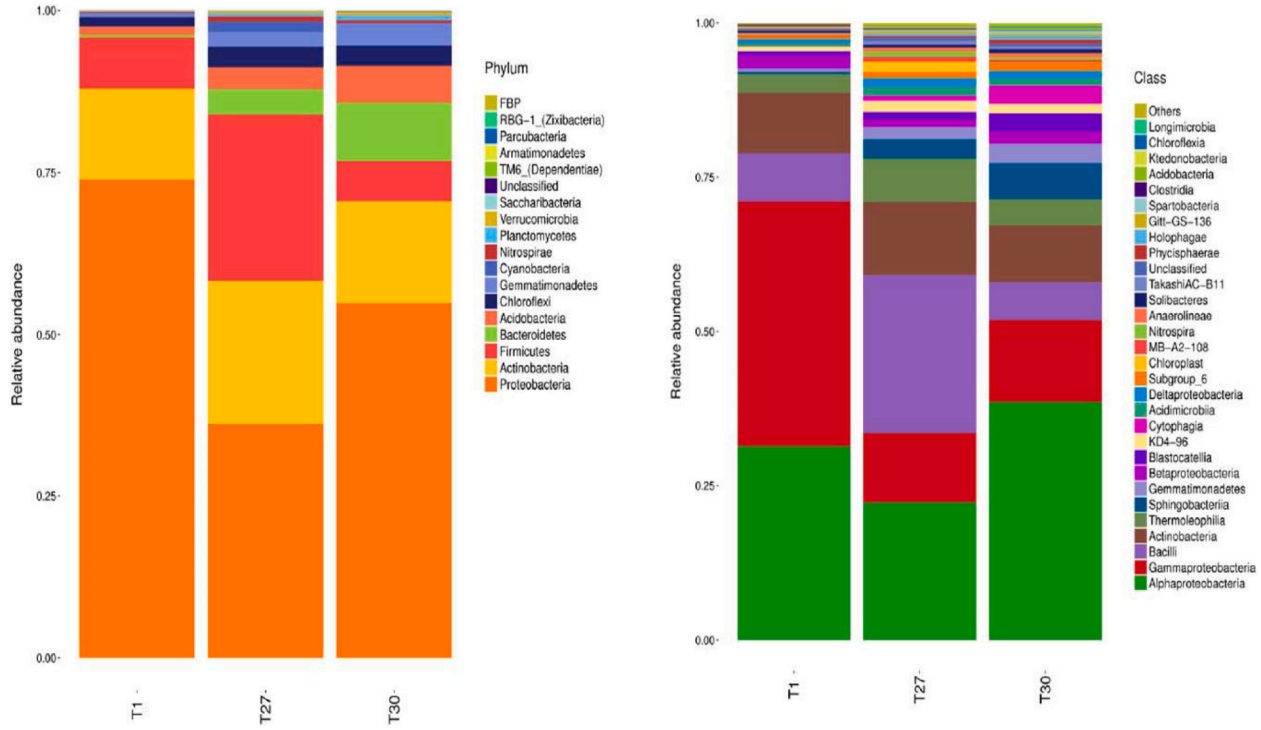

Fig. 5. Taxonomic distribution of bacterial Phyla and Class in rhizospheric soil of maize treated with nanocompounds and *Bacillus* sp. . T1 (Control), T27 (nanozeolite + *Bacillus* sp.) and T30 (nanochitosan + *Bacillus* sp.).

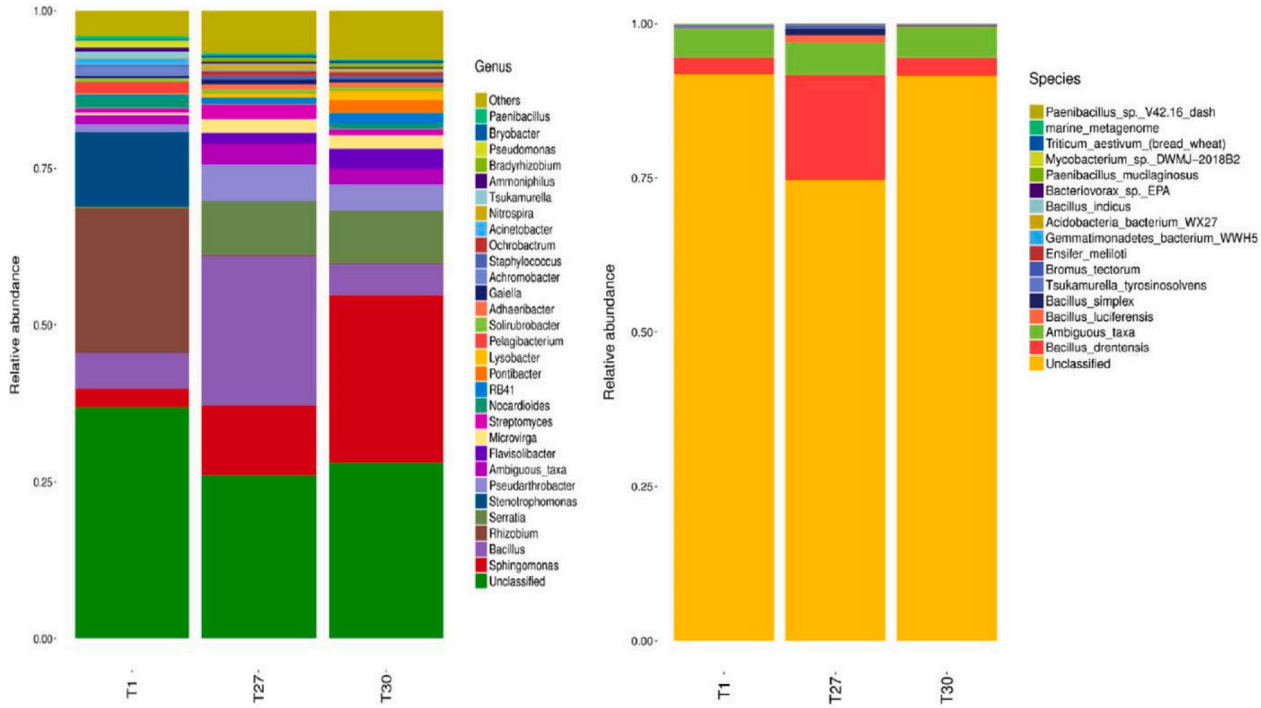

Fig. 6. Taxonomic distribution of bacterial Genus and Species in rhizospheric soil of maize treated with nanocompounds and *Bacillus* sp.. T1 (Control), T27 (nanozeolite + *Bacillus* sp.) and T30 (nanochitosan + *Bacillus* sp.).

observed increase in dehydrogenase activity of the soil when treated with zinc oxide nanoparticles. On the other hand Sillen et al. (2015) reported less FDA activity in the soil of maize rhizosphere treated with nanosilver (100 mg kg^{-1}) as compared to control. McGee et al. (2017) reported that silver nanoparticles reduced the dehydrogenase activity at 1 mg Kg^{-1} in the experimental soil. Kukreti et al. (2020) reported 1.5–2 fold increases in dehydrogenase activity when maize soil was treated with PGPR and nanosilicon dioxide.

Bacterial population of a soil is an important bio indicator to determine the soil health. Only 1–2% bacterial diversity can be estimated using cultivable methods like plate counts which usually prefer only fast growing microbes (Siqueira Jr. et al., 2017). To overcome this problem,

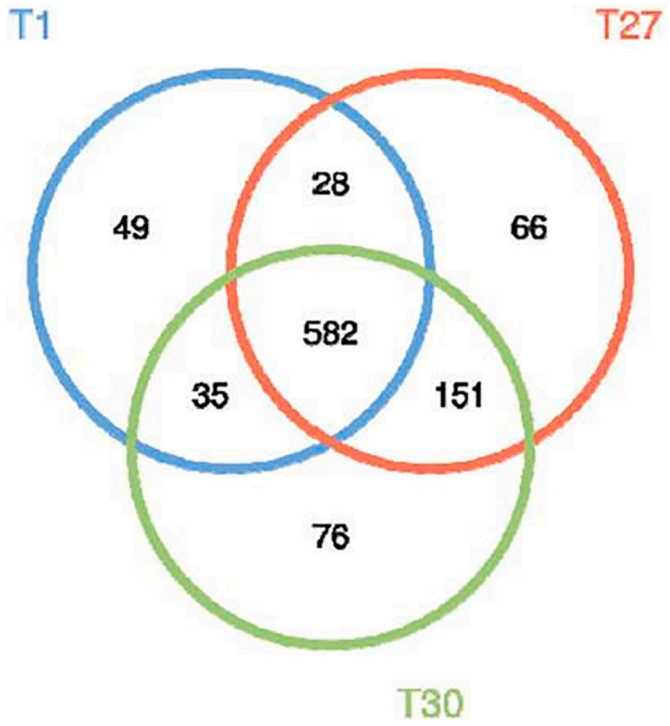

Fig. 7. OTU Venn diagram of different sample the numbers in the figure represent the numbers of OTUs unique or common to each sample or group. Each petal represents a sample. The numbers on the petals represent the number of OTUs unique to the sample, and the white circle in the middle represents the number of OTUs shared by all samples and groups.

culture independent approach can be used to evaluate the diversity and ecological aspect of uncultivable microorganism. In the present study α diversity index of bacterial population in nanozeolite and nanochitosan treated soil was higher than the control. Similarly Chao1 indices, Shannon and Simpson index values were also higher for treated soil than the control. *Proteobacteria* and *Actinobacteria* were predominant phyla in the rhizosphere which play important role in ecological and metabolic functioning of the soil due to their involvement in nitrogen fixation, decomposition and humus formation (Johnston-Monje et al., 2016; Mashiane et al., 2018). A positive correlation of nanocompounds with *Firmicutes, Bacteroidetes, Chloroflexi* and *Cyanobacteria* was observed. According to Liu et al. (2017), 16S rRNA Illumina sequencing of wheat-planted soil showed the abundance of nitrogen fixing sp. of *Cyanobacteria, Nitrospirae* and *Chloroflexi.* According to Khati et al. (2019b) application of nanozeolite under wheat cultivation showed increase in *Actinobacteria, Bacteroidetes, Chloroflexi* and *Cyanobacteria*. Timmusk et al. (2018) also reported that titania nanoparticles along with PGPRs improved the beneficial microbes around the roots and supported wheat growth. Similarly we observed high abundance of *Bacillus* sp. in nanozeolite treated soil. Our result showed a positive correlation between nanozeolite and bacilli. Similar observations were reported by Frenk et al. (2013), when soil was exposed to metal oxide nanoparticles (1%) concentration. They reported abundance of *Bacilli* but decreased population of *Rhizobium* and *Sphingomonas* in treated soil. Andreazza et al. (2011) reported that *Bacilli* persist in copper treated soil due to their resistance mechanism. In this study decrease in rhizobial population may be due to the higher abundance of *Bacilli* in the treated soil. *Pseudomonas* and *Bacillus* sp. are considered as promising biocontrol agents because of their ability to produce antibiotics (Moeinzadeh et al., 2010). They are the most common plant growth promoting rhizobacteria and help in improvement of plant growth via suppressing plant diseases (Kinsella et al., 2009). Gatahi et al. (2016) reported that application of chitosan nanocomposite increases the biocontrol efficacy of *Bacillus subtilis* and *Trichoderma viridae*. According to Kumari et al. (2020), application of nanocompounds improves nutrient status of the soil which may further help in propagation of overall microbial population. Besides, some observable positive impact of nanocompounds on soil structure and plant health, concern for toxicity of these nanocompounds should not be neglected. Sillen et al. (2015) reported that nanosilver had toxic effect on bacterial and fungal population. Similarly Juan et al. (2017) also reported that application of silver nanoparticels (100 mg g^{-1}) showed decrease in microbial community. Shao et al. (2015) reported that application of silica fertilization and nano-MnO_2 decreases the population of *Actinobacteria, Cyanobacteria* and *Chloroflexi* in paddy soil.

In this study, we propose that the application of nanocompounds (nanochitosan and nanozeolite) along with a bioinoculant (*Bacillus* sp.) in maize crop under field condition could be helpful to improve plant and soil health. These nanocompounds can be used to make bio-formulations to support the survival of PGPR for longer time which may offer an ecofriendly and sustainable approach for the farmers.

5. Conclusion

Application of nanozeolite and nanochitosan along with *Bacillus* sp. enhanced the bacterial population in the treated soil and helped in maintenance of soil health. The underlying mechanisms related to improved soil microbial population may comprised of better availability of nutrients, water use efficiency of microbes and plants under the influence of nanocompound. These nanocompounds improved the growth of *Bacillus* sp. and other beneficial bacterial population in the soil which consequently supported growth and development of plants. Though the application of bio-inoculants is in practice but their survival and performance under field conditions are still a matter of great concern. Some agriusable nanocompounds may offer assistance to the exogenous bio-inoculants for their long survival and performance. A deep understanding of the mechanisms involved among nanocompounds, microbes, plants and soil are needed to get maximum benefits in agricultural practices.

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Declaration of competing interest

Authors declares that they have no conflicts of interests.

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