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Effect of Jhum Cycles on *Acidobacteria* and *Burkholderia* Community in the Rhizosphere of Mixed Crops of Different Jhum Fields

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Abstract In the present study, culture-dependent and independent [Denaturing gradient gel electrophoresis (DGGE)] approach were used to analyze the effect of fallow cycle on bacterial populations associated with mixed crops selected from three different jhum fallow cycles (5-, 10- and 20- years) of Changki village, Nagaland. Two important bacterial phyla viz. *Acidobacteria* and *Burkholderia* were selected for DGGE analysis. According to the DGGE banding profile, *Acidobacteria* showed more richness compared *Burkholderia*. The presence and absence of few of the bands in the profile indicate the community shifts in the selected jhum fields. Shannon diversity index was higher in *Acidobacteria* than *Burkholderia*. Multivariate analysis showed that *Acidobacteria* community structure was similar among the crop plants of the same fallow cycle. *Burkholderia* community did not show clear separation among fallow cycles although crop of same fallow cycle tend to group together. ANOSIM and PERMANOVA showed significant differences in rhizosphere community pattern among fallow cycles and the crop plants of same fallow cycles indicated that both fallow cycle and crop plants are factors determining the *Acidobacteria* and *Burkholderia* community composition in jhum soils which was also confirmed by the culture-dependent analysis. Culturable bacterial counts were dependent on media used and significantly different at different fallow cycles. Bacterial communities (both culturable and DGGE patterns) were correlated with soil nutrients suggesting their importance in stability of jhum soil. The present study revealed that both fallow cycle and host plant may be the key factors in shaping many bacterial communities in jhum soils.

Keywords: *Acidobacteria, Burkholderia, bacterial community, denaturing gradient gel electrophoresis (DGGE), jhum*

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1. Introduction

Jhum or shifting cultivation is a traditional form of agriculture in which a piece of land or forest is cleared through slashing and then burning of dried biomass, cultivated for a short period of time, then leaving the land abandoned (fallow) allowing the soil to restore its fertility, while the cultivator moves on to another plot. The nutrients lost during cultivation are approximately replaced during fallowing period [1]. The process of shifting cultivation from slashing to burning has shown to have detrimental effects on soil properties and on the diversity and abundance of different microbial populations in the soil. Microbial diversity of soil is important to sustainable agriculture because microbes mediate many processes that support agricultural productions and even may indicate disturbances or beneficial effects of amendments or management strategies [2].

Shifting cultivation in India is mostly practiced in the hilly areas by the tribal groups of the North Eastern states of India like Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, and Nagaland, Sikkim and Tripura [3]. Mixed cropping system is mostly practiced in these jhum fields. In our study we have selected four crop plants viz. rice (*Oryza sativa)*, colocasia (*Colocasia esculenta)*, maize (*Zea mays)* and Perilla (*Perilla frutescens)*, that were grown and common to all the fields (5-, 10- and 20- years), except for *P.frutescens* which is absent in 10 years fallow field.

The rhizosphere is a "hot spot" of intense microbial activity in the soil surrounding the roots, and is the focus in the field of plant stress response [4]. These microbes in the rhizosphere play crucial roles in the rhizosphere ecosystem which includes metabolic processes such as nutrient cycling and organic matter decomposition which exert positive effects on plant health and growth [5] and they also play a crucial role in helping plant to adapt to various environmental stresses [6]. Plant roots produce

various exudates that help in shaping variety of microbes such as bacteria, fungi, algae, and protozoa [4]. Although products released by roots comprise an important pool of organic compounds for soil microorganisms, their composition and quality can vary according to the plant species, soil type and plant developmental stage [7]. *Acidobacteria* is one of the most abundantly distributed bacterial groups in the environment [8]. The abundance of *Acidobacteria* in soils and their ability to withstand extreme and polluted environments suggest that they serve functions that are important in the environment and that are potentially quite varied [9]. The phylum *Burkholderia* is also among the most abundant bacteria in the environment. The plasticity of their genomes and their capacity to adapt to changing conditions allow them to colonize diverse environmental niches. Some *Burkholderia* interact with host plants, resulting in beneficial effects and can also be used as biofertilizers, either by fixing nitrogen or by releasing iron or phosphorus from rock phosphates, to benefit crops cultivated in low-fertility soils, while other scan potentially be utilized as powerful pesticides in control of soil borne diseases, thus, becoming ecologically important [10]. Since there is capability of *Acidobacteria* and *Burkholderia* for maintaining environment and agricultural stability purposes (such as nitrogen fixation, plant growth stimulation, biological control) we therefore investigate *Acidobacteria* and *Burkholderia* communities which may play major role in sustaining crop plants in jhum soils.

Plant associated microbial communities, such as *Acidobacteria* and *Burkholderia*, and their effects on plant growth and development in many disturbed, undisturbed, extreme and even stressed environments, have been done by many researchers around the world, however it has not been studied in jhum soil. Since plant and their surrounding microbes are living in association with each other, it is crucial to understand how these microbes are affected by the land use pattern. In a disturbed or stressed environment, soil nutrients and soil physical and biochemical properties also changes which will directly or indirectly affect plant growth and development. Therefore, this study aimed at analyzing the *Acidobacteria* and *Burkholderia* community associated with mixed crops under jhum soils using molecular approaches. DGGE fingerprints of 16S rRNA gene fragment were generated so as to investigate the two phylum community structures in the selected plant microsites (rhizosphere, loosely adhered soil and bulk) and to gain information as to whether these communities were altered or affected by different jhum fallow cycles (in this case 5-, 10- and 20-years). The study may help us to understand the effect of fallow length and crop plant on the recruitment of rhizosphere community in jhum soils.

2. Material and Methods

2.1. Collection of Soil Samples

The crop plants and soil samples were collected at their growing stage from 5-, 10- and 20-years fallow cycles in triplicates. Rhizospheric soil was collected by gently brushing the soil attached to the roots. Loosely adhered soil was removed by shaking the roots. The replicate soil sample of each plant (separately for rhizosphere, loosely adhered and bulk soil) were then mixed together to make composite sample. The resulting soil samples were stored at -20 ℃ for soil DNA extraction.

2.2. Bacterial Counts

Isolation of bacteria from rhizosphere soil was done using serial dilution method on Tryptic Soy Agar (TSA) and Reasoner's 2Agar (R2A) culture media in triplicates. For isolation of root endophytes, fresh roots of the selected crop plants were washed in running tap water to remove any remaining soil attaching to the root surface and air dried for 3 to 4 hours. Dry weight per gram root sample was also taken.1 g of root was weighed and cut to 1 cm length. The roots were subjected to surface sterilization using 70% Ethanol and 2% Sodium hypochlorite solution. The roots were treated in 70% Ethanol for 1 minute followed by 2% Sodium Hypochlorite for 5 minutes. The roots were then treated in 70% Ethanol for about 30 seconds and finally followed by three rinses in sterile distilled water. The roots were transferred to sterile mortar pestle and grinded in 1X Phosphate buffer saline. The resulting suspension (both rhizosphere soil and root tissue) was serially diluted upto 10^{-5} dilution. Dilutions 10^{-3}, 10^{-4} and 10^{-5} were plated onto TSA and R2A agar media in triplicates. Plates were then incubated at 30±1˚C, observed at 24, 48 and 72 hours and counted. Colonies Forming Units (CFU) g^{-1} fresh soil or root was calculated. Two-way analysis of variance (ANOVA) was also performed using SPSS 16 software to compare the effects of crop plant and fallow cycles as well as their interaction effects on the rhizobacteria and root endophytes CFU. CFU was also correlated with soil properties using Pearson's correlation.

2.3. Soil DNA Extraction

DNA extraction was done using PowerSoil^{tm} DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA) with a modification of additional incubation at 65 ℃ for 10 minutes at the cell lysis step. DNA quality was checked at $A_{260/280}$ under NanoDrop 2000 spectrophotometer.

Table 1. List of primers used in this study for PCR amplification

Phylum	Primer used	Sequence	Region	Refrences
Acidobacteria	31F	5'-GATCCTGGCTCAGAATC-3'	V6-V8	[11,12]
Burkholderia	Burk3	5′-CTGCGAAAGCCGGAT-3′	V6-V8	[13,14]
	Burk3-GC*	5′- CTGCGAAAGCCGGAT-3′	V6-V8	
	BurkR	5′-TGCCATACTCTAGCYYGC-3′	V6-V8	
Bacteria	F984GC*	5'-AACGCGAAGAACCTTAC-3'	V6-V8	[15]
	1378R	5'-CGGTGTGTACAAGGCCCGGGAACG-3'	V6-V8	
*GC- clamp	5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3'			[16]

2.4. 16SrRNA Gene Amplification and DGGE Fingerprinting

The primers used in this study are listed in Table 1. Optimization of PCR primers was done using gradient PCR. Amplification of *Acidobacteria* and *Burkholderia* 16S rRNA gene fragment was done using PCR-DGGE approach. The first round PCR primer pair for *Acidobacteria* used was 31F and 1378R and for second round PCR was F984GC and 1378R. Primer pair of *Burkholderia* used in first round PCR was Burk3 and 1378R and Burk3-GC and BurkR for the second round (Table 1). Approximately, 20 ng (1 µl) aliquot of each sample DNA was used for a 25 µl PCR reaction. The PCR reaction contained 1X PCR buffer (New England Biolabs, NEB), 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.2 µM forward and reverse Primer, 0.1 μg^{-1} µl BSA (Bovine serum albumin), and 3U Taq Polymerase (New England Biolabs). The PCR parameters were as follows: initial melt at 94 ℃ for 3 min followed by 35 cycles of 94 ℃ for 45 sec, annealing at 52 ℃ (*Acidobacteria*)/ 58 ℃ (*Burkholderia*) for 45 sec and 72 ℃ for 1 min and a final hold at 72 ℃ for 7 min. For PCR amplification, a blank was also loaded along with the samples. The PCR amplified products were analysed using agarose (0.8%) gel electrophoresis. The same PCR condition was run for the second round PCR at annealing temperature of 62.2 ℃.

2.5. Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was performed with the IngenyPhorU mutation detection system. PCR products were loaded onto polyacrylamide gel 6% (wt/vol) Acrylamide/Bisacrylamide (ratio of Acrylamide to bisacrylamide is 37.5:1) in 0.5X TAE buffer (pH 8) with a denaturant gradient (100% denaturant consist of 7M Urea and 40% (wt/vol) deionized formamide) of 35% to 70%and 30% 65% for *Acidobacteria* and *Burkholderia*. The stacking gel consists of 6% Acrylamide/Bisacrylamide (37.5:1) and 0.5X TAE buffer, pH 8.0. The wells were loaded with roughly equal amounts of DNA and electrophoresis was carried out in 0.5X TAE buffer at 90V for 16 hours at 60 ℃. The gels were silver stained as follows and photographed.

2.6. Silver Staining of DGGE Gel

Briefly, silver staining was carried out by soaking the gel in fixing solution (10% ethanol and 1% acetic acid) for 10 minutes and washed using distilled water. The gel was pre-treated using the pre-treatment solution (1.5% Nitric acid) for about 3 minutes under constant shaking and rinsed. The gel was stained using 0.2% silver nitrate impregnation solution for about 20 minutes and under constant shaking and rinsed. The gel was developed by applying initially 250 ml of pre-cold (12 ℃) developing solution (30 g of Sodium carbonate and 0.54 ml of 37% formaldehyde. The solution was kept at 12 ℃ before use) and gently shaking until the solution became dark. The solution was drained and the tray was again filled with 750 ml fresh cold solution for 4-7 minutes until the bands appeared with desirable intensity. The gel was transferred to new tray containing stop solution (5% acetic acid) to stop the reaction and kept for 5 minutes. The gel was washed in distilled water. The gel was air-dried and photograph.

2.7. Analysis of DGGE Banding Pattern

The bacterial community profiles generated were transformed to a matrix based on the presence/absence of bands and further scored based on the intensity of the band. Scoring was made from 0 to 5. Strongest bands were scored 5, least intense band 1 and absence of band 0. The band intensity score matrix was further expressed as relative abundance matrix [(number of a specific band/ sum of all the bands in the sample) x 100]. The species richness from the DGGE banding profile was expressed as the total number of bands for both *Acidobacteria* and *Burkholderia*. Species diversity and evenness was calculated using the Shannon diversity index (H′) [17] using band intensity.

$$Shannon\ diversity\ index\ (H) = -\Sigma pi \ln(pi)$$

$$Evenness\ (E_H) = H / \ln S$$

Where,
H = Shannon's diversity index
S = total number of species in the community (richness)
pi = proportion of *S* made up of the *i*th species
E_H = equitability (evenness)

Multivariate analysis was performed using PAST3 software. Non-metric multidimensional scaling (nMDS) and Principal Component Analysis (PCA) was done for similarity community composition between samples, and cluster/dendrogram was constructed using the Unweighted pair group method with arithmetic mean (UPGMA) algorithm. ANOSIM (analysis of similarity) (100,000 permutations) and PERMANOVA (Permutational multivariate analysis of variance) (999 permutations) was done for test of similarity of community composition of different fallow cycles [18]. SIMPER was also used to estimate the similarity or dissimilarity percent of community composition among the fallow cycles. All multivariate analysis was done based on the Bray-Curtis similarity indices. Pearson's correlation was done SPSS16 to correlate soil properties and bacterial community. All statistical analysis was performed using the relative abundance matrix except for the Pearson's correlation it was done using the presence and absence matrix.

2.8. Soil Physico-chemical and Biochemical Properties

Physicochemical properties of soil were determined as per the standard procedures described by Page *et al.* [19]. Soil Moisture content (MC) was determined gravimetrically by oven drying fresh soil samples at 105 ℃ to constant weight (wt.). Air dried soil samples (passed through 0.5 mm sieve) were used for determination of soil pH and soil organic carbon (SOC). Soil pH was measured in 1:2.5 soil: H_2O suspension using a standard pH meter (Mettler Toledo, Switzerland). Air-dried and finely ground soil (<0.1 mm, 0.5 g) was used for determination of SOC by the potassium dichromate wet oxidation method [20].

Microbial biomass carbon (MBC), Microbial biomass nitrogen (MBN) and Microbial biomass phosphorus (MBP) were determined by chloroform-fumigation method [21]. Available phosphorus (AvP) in soil was measured following Bray's method [22]. Available potassium (AvK) was extracted using neutral ammonium acetate [23]. Soil available nitrogen (AvN) was determined by the method described by Subbiah and Asija [24]. Exchangeable Ca+Mg (Ex Ca+Mg) were estimated using the EDTA method. Breifly, 10 g of air-dried soil was extracted using 50 ml of neutral ammonium acetate and shaken for 10 minutes. The extraction mixture was then filtered through Whatman No.1 filter paper.10 ml of aliquot was taken and 0.5 ml of ammonium chloride-ammonium hydroxide buffer was added. 3-4 drops of Erichrome black T indicator was added. This solution was titrated with 0.01N EDTA till the colour changes to bright blue or green and no tinge of wine-red colour remains behind.

Soil dehydrogenase activity (DHA) was measured in terms of the amount of triphenylformazan (TPF) produced during incubation of fresh soil sample with 2,3,5-triphenyltetrazolium chloride (TTC) at 37±1 ℃ for 24 hours, and expressed as µg (TPF) g^{-1} (dry weight) soil h^{-1} [25]. Soil acid-phosphomonoesterase activity (PHA) was determined in terms of amount of p-nitrophenol (PNP) produced during incubation (37±1 ℃ for 1 hour) of fresh soil sample with p-nitrophenyl phosphate (p-NPP) in the presence of a modified universal buffer (MUB, pH 6.5), and was expressed as mg PNP g^{-1} (dry weight) soil h^{-1} [26]. β-Glucosidase activity (GSA) of soil was determined based on the method described by Eivazi and Tabatabai [27]. One gram soil was incubated with 0.25 ml Toluene, 4 ml MUB (pH 6) and 1ml of PNG (p-Nitrophenyl-β-D-glucoside) solution, pH 6. After 1 hour incubation, 0.5 M $CaCl_2$, 4 ml of 0.1 M THAM (Tris (hydroxymethyl aminomethane) buffer, pH 12, were added, swirled and then filtered. The amount of p-nitrophenol released during incubation was expressed in µg p-nitrophenol g^{-1} dry wt. soil h^{-1}.

3. Results

3.1. Bacterial Counts

Table 2 showed the bacterial CFU of rhizosphere (g^{-1} fresh soil) and root endophyte (g^{-1} root) of *O.sativa*, *C.esculenta*, *Z.mays* and *P.frutescens* from different jhum cycles (5-, 10- and 20- years). CFU g^{-1} soil and CFU g^{-1} root recorded was higher on R2A than TSA media. Highest CFU was seen in the 20 years and minimum in 5 years fallow cycle in all the rhizosphere samples. However, for root endophyte CFU was highest in 5 years and lowest in 20 years fallow cycle in all the root samples. Crop plant and fallow cycle effect on rhizosphere CFU were statistically significant at $p<0.001$ and $p<0.05$, respectively (Table 3). Crop plant effect on root endophyte CFU was statistically different at $p<0.01$. The main effect of crop plant on rhizosphere and root endophyte yielded an effect of 89% and 81.6%, respectively. The effect of fallow cycle on rhizosphere bacteria was 53%. The interaction of crop plant and fallow cycle was not statistically significant for both rhizosphere and root endophytes CFU.

3.2. *Acidobacteria* and *Burkholderia* Community Richness and Diversity

Figure 1 and Figure 2 depicts DGGE gel profile images of *Acidobacteria* and *Burkholderia*, respectively. Banding profile revealed varied banding pattern among the crop plant samples of the same and different fallow lengths. DGGE analysis revealed richness of 11-21 and 2-7 distinct bands in *Acidobacteria* and *Burkholderia*, respectively. *Acidobacteria* showed more number of bands compared *Burkholderia* indicated that *Acidobacteria* is highly diverse. Band A, B, C and D were some of the bands that showed *Acidobacteria* community shifts among the crop plants while band E was present only in bulk sample of 10 years fallow cycle (Figure 1). *Burkholderia* community pattern (Figure 2) showed few numbers of bands in all the samples. Band A was present in all the crop samples except in the 10 years *Z.mays* rhizosphere, whereas B to F were few of the bands (dominant) that are likely to be selectively present only in some of the samples. Example, band B was present in loosely adhered of 5 years *O.sativa* as well as in the rhizosphere and loosely adhered soil of 20 years *Z.mays*. Band C was seen in the 5 and 10 years bulk sample, 5 years loosely adhered *O.sativa*, 10 years rhizosphere of *O.sativa* and 20 years rhizosphere and loosely adhered soil of *Z.mays*.

Average Shannon diversity index was higher in *Acidobacteria* (H = 2.67±0.07) than *Burkholderia* (H = 1.23±0.34). *Acidobacteria* showed highest diversity at fallow cycle 20 and highest evenness at fallow cycle 5 in all the crop samples (Table 4). *Burkholderia* community showed highest diversity as well as evenness in 5 (*O.sativa* and *Z.mays*) and 10 (*C.esculenta* and *P.frutescens*) years fallow cycles (Figure 3).

Table 2. Bacterial CFU g^{-1} rhizosphere soil ($x10^6$) and bacterial CFU g^{-1} root ($x10^4$) of selected crop plants

	Culture Media	*O.sativa*			*C.esculenta*			*Z.mays*			*P.frutescens*	
		5	10	20	5	10	20	5	10	20	5	20
Rhizosphere	**R2A**	5.1±1.6	5.2±0.6	5.5±0.7	4.1±1.5	4.2±0.6	4.87±0.7	3.99±0.5	4.02±0.4	4.22±1.3	1.73±1.4	3.1±0.2
	TSA	4.5±0.3	4.6±0.9	4.9±0.4	3.1±1.3	3.5±3.3	3.9±0.7	3.6±1.8	3.8±1.7	3.9±0.6	1.5±2.6	3.1±1.2
Root Endophyte	**R2A**	4.9±0.4	4.6±0.7	4.2±0.3	3.8±1.7	3.5±0.04	3.2±0.1	4.6±1.2	4.3±0.8	4.1±0.7	2.8±0.7	1.8±0.4
	TSA	4.01±1.1	3.99±0.2	3.8±0.8	2.3±0.4	2.1±0.2	1.8±0.09	4.5±0.1	4.2±1.2	4.05±0.2	2.5±0.3	1.1±0.4

Values showing mean values of three replicates ±std deviation.

Table 3. ANOVA table for CFU of rhizobacteria and bacterial root endophyes of selected crop plants from different jhum cycles

Source	Rhizosphere				Root Endophyte			
	Df	Mean Square	F	Effect Size	Df	Mean Square	F	Effect Size
Crop plant	3	5.22	29.72***	0.89	3	5.92	16.07***	0.816
Fallow Cycle	2	1.09	6.21*	0.53	2	0.90	2.44	0.304
Crop x Fallow Cycle	5	0.18	1.03	0.32	5	0.08	0.21	0.089

*significant at p<0.05; **significant at p<0.01; ***significant at p<0.001.

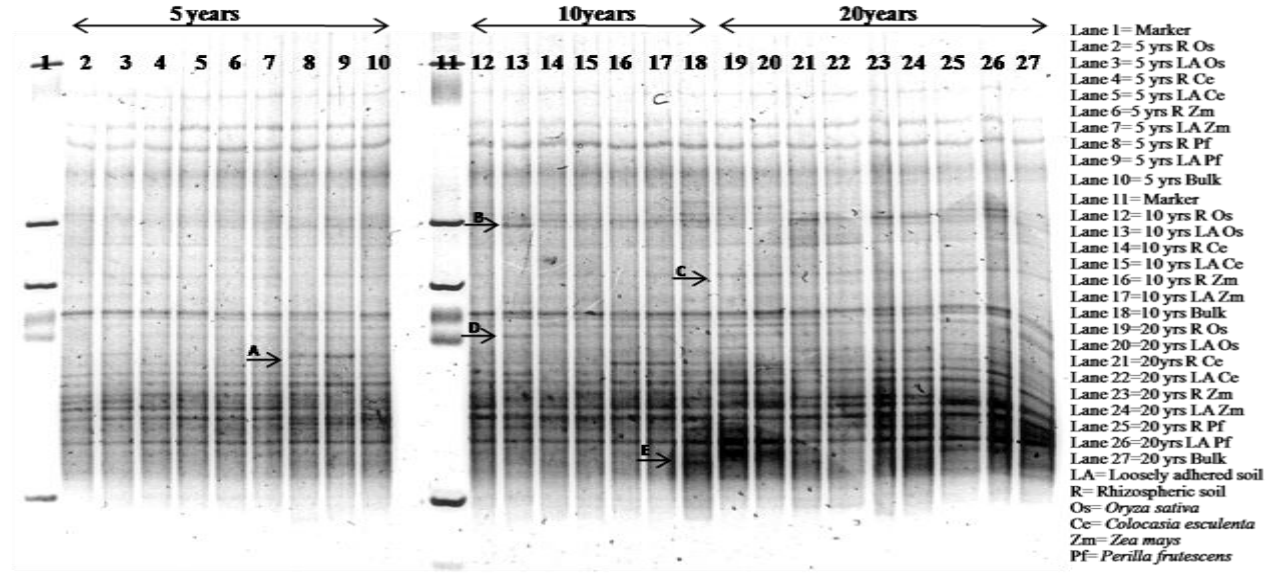

Figure 1. DGGE pattern of *Acidobacteria* community in the microsites of selected crop plants of three fallow cycles

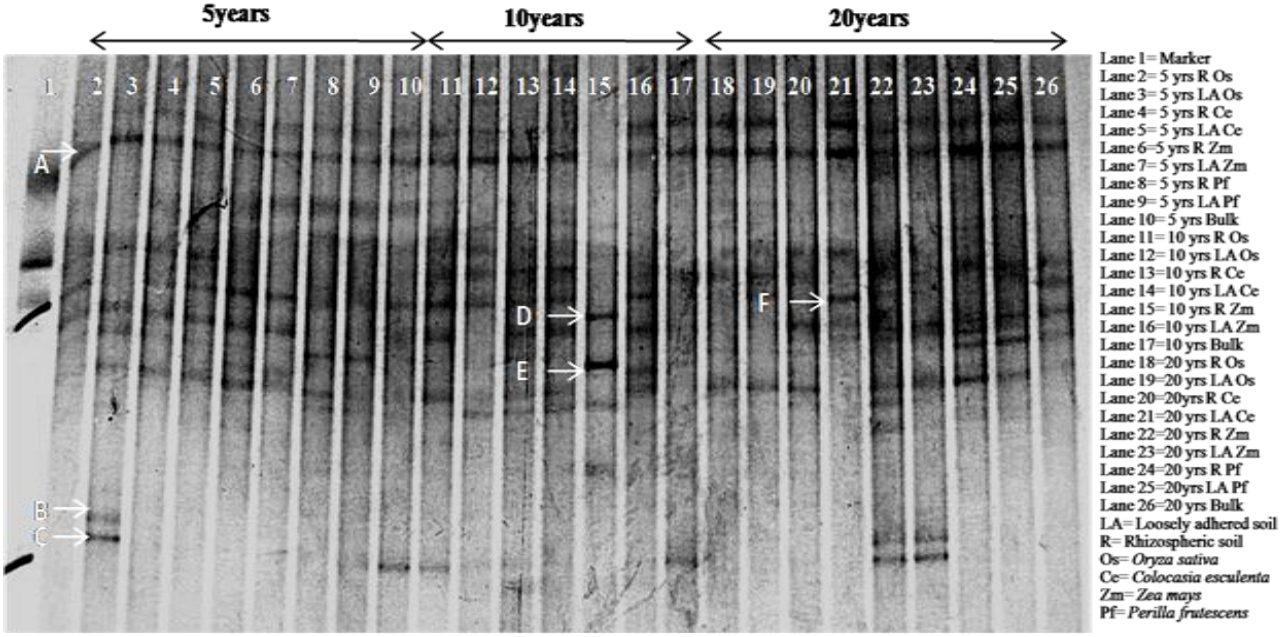

Figure 2. DGGE pattern of *Burkholderia* community in the microsites of selected crop plants of three fallow cycles

Table 4. Shannon diversity index and evenness of rhizosphere soil of 5-, 10- and 20 years fallow cycles of selected crops

		O.sativa			*C.esculenta*			*Z.mays*			*P.frutescens*	
		5	**10**	**20**	**5**	**10**	**20**	**5**	**10**	**20**	**5**	**20**
Acidobacteria	**Shannon Index (H)**	2.25	2.51	2.8	2.59	2.74	2.92	2.72	2.56	2.79	2.75	2.79
	Evenness (H/lnS)	0.86	0.82	0.82	0.89	0.86	0.88	0.89	0.86	0.82	0.87	0.81
Burkholderia	**Shannon Index (H)**	1.58	1.89	0.45	1.75	1.31	1.01	1.47	0.69	1.33	1.28	1.17
	Evenness (H/lnS)	0.81	0.94	0.78	0.96	0.92	0.92	0.87	1	0.95	0.89	0.8

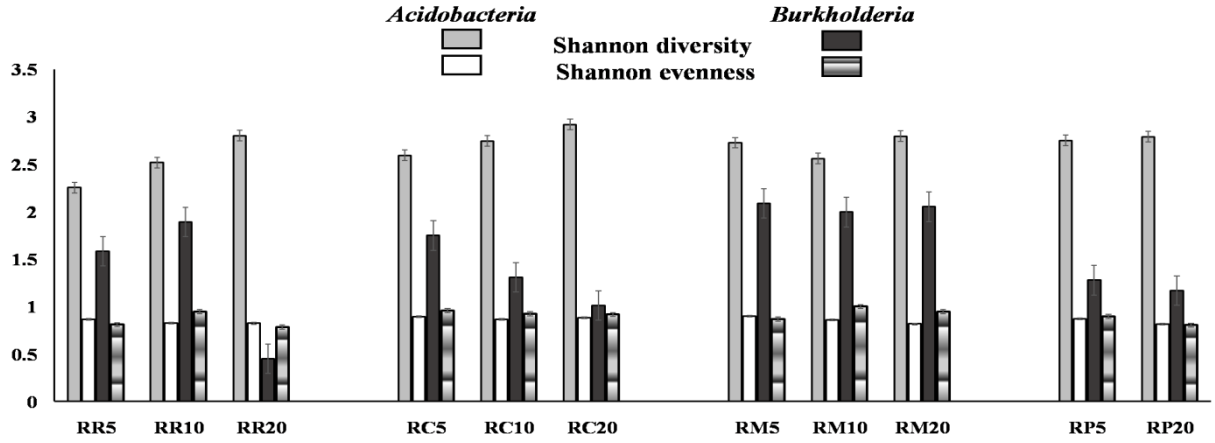

Figure 3. Shannon diversity index and evenness of *Acidobacteria* and *Burkholderia* in the rhizosphere of crop plants of the three fallow cycles. RR5/10/20 = rhizosphere of *O.sativa* of fallow cycle 5/10/20 years; RC5/10/20 = rhizosphere of *C.esculenta* of fallow cycle 5/10/20 years; RM5/10/20 = rhizosphere of *Z.mays* of fallow cycle 5/10/20 years; RP5/20 = = rhizosphere of *P.frutescens* of fallow cycle 5/20 years

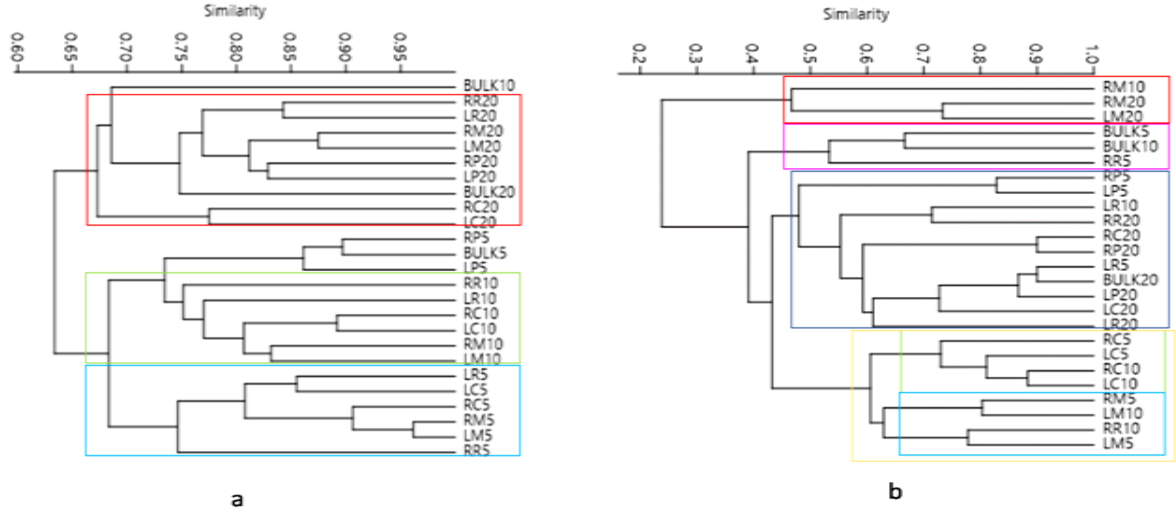

Figure 4. Cluster analysis of (a) Acidobacteria and (b) Burkholderia community composition of the rhizosphere, loosely attached and bulk soil of selected crops of three fallow cycles generated from relative abundance matrix using UPGMA algorithm

3.3. *Acidobacteria* and *Burkholderia* Community Composition

Dendrogram was constructed based on the relative abundance matrix generated from the community pattern on the gel for both *Acidobacteria* and *Burkholderia* (Figure 4). The dendrogram of *Acidobacteria* community structure resulted in three clusters which separate the three fallow cycles. The three clusters showed similarity of ~65% to ~75%. The crop samples showed similarity of ~75% to ~95%. *Acidobacteria* as well as *Burkholderia* in the rhizosphere and loosely adhered soil of the same crop plant also tend to group together. *Burkholderia* community showed similarity of ~50 to ~90% among the crop samples but do not show clear separation between fallow cycles nor among rhizosphere, loosely adhered and bulk soil. Crops from 5 years and 10 years fallow cycle tend to group together. Rhizosphere of *Z.mays* 10 and 20 years group together. Rhizosphere and loosely adhered soil of *C.esculenta* of 5 and 10 years grouped together. On the other hand, rhizospheric soil of *O.sativa*, *C.esculenta* and *P.frutescens* of 20 years grouped together. Similarly, loosely adhered soil of *O.sativa*, *C.esculenta* and *P.frutescens* of 20 years grouped in the same cluster which indicates that rhizosphere and loosely adhered soil the fallow cycle 20 years are closely similar to each other.

The nMDS and PCA (Figure 5) plots showed grouping of fallow cycles 5-, 10- and 20 years separately for *Acidobacteria*. *Burkholderia* community structure did not show clear separation between fallow cycles although the crop plants of same fallow cycle tend to group together. nMDS plot for *Acidobateria* and *Burkholderia* was computed and shown in two co-ordinates with 2D stress value of 0.15 and 0.19 respectively.

The ANOSIM (Table 5) of *Acidobacteria* showed significant differences between fallow cycles of ($R=1$, $p<0.01$) and crop plants ($R=0.67$, $p<0.01$). *Burkholderia* community pattern also showed significant differences between fallow cycles ($R=0.67$, $p<0.01$) and crop plants ($R=0.42$, $p<0.01$). However, no significant differences were observed between rhizosphere, loosely adhered and bulk soil. PERMANOVA analysis (Table 5) indicated that fallow cycle was a determining factor of *Acidobacteria* ($F=8.16$, $p<0.01$) and *Burkholderia* ($F=1.12$, $p<0.05$). Crop plants were also found to be the determining factor for both *Acidobacteria* ($F=1.82$, $p<0.01$) and *Burkholderia* ($F=2.07$, $p<0.01$) communities. SIMPER analysis showed highest

Acidobacteria dissimilarity between 5 and 20 years fallow cycle (37.77%) followed by 10 and 20 years (33.43%) and 5 and 10 years (33.15%). Similarly, in the case of *Burkholderia*, dissimilarity was highest between 10 and 20 years fallow cycles (61.90%) followed by 5 and 20 years (56.60 %) and least between 5 and 10 years fallow cycle (54.55%).

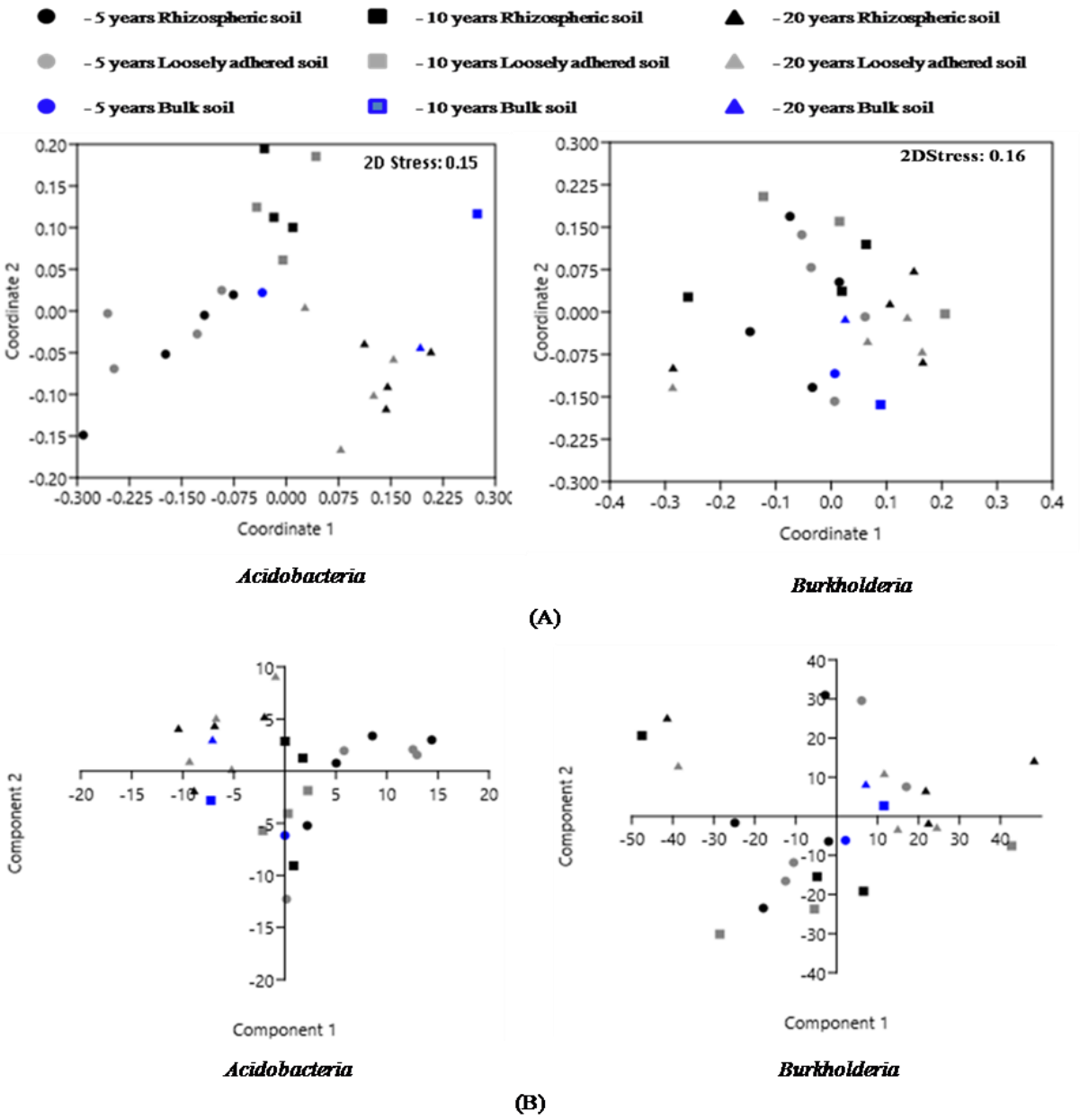

Figure 5. nMDS plots (A) and PCA plots (B) of Acidobacteria and Burkholderia community composition of selected crop plants generated from relative abundance matrix

Table 5. ANOSIM and PERMANOVA between fallow cycles, crop plants and plant microsites

	Factor	ANOSIM (R)	df	PERMANOVA (F)
Acidobacteria	**Fallow cycle**	1**	2	8.16**
	R/L/B	0.05	2	0.77
	Crops samples	0.67**	4	1.82**
Burkholderia	**Fallow cycle**	0.67**	2	1.117*
	R/L/B	-0.06	2	0.53
	Crops samples	0.42**	4	2.073**

*significant at p<0.05
**significant at p<0.01
R: Rhizosphere soil
L: Loosely adhered soil
B: Bulk soil

3.4. Correlation of CFU (Rhizobacteria and Bacterial Root Endophytes with Soil Physico-chemical and Biochemical Properties)

Physical, chemical and biochemical properties of soil were analysed (Supplementary Table S1). All soil parameters analyzed were found to be higher in 20 fallow cycles, except all the three soil enzymes were found to be higher in the 5 years fallow cycle. Pearson's correlation (r) of soil properties, bacterial counts, *Acidobacteria* and *Burkholderia* community pattern which was based on the presence/absence of bands were analysed (Supplementary Table S2). CFU of rhizosphere soil was found to be positively correlated with soil pH ($r=0.67$; $p<0.05$), SOC ($r=0.81$; $p<0.01$), MBC ($r=0.84$; $p<0.01$), MBN ($r=0.85$; $p<0.01$), MBP ($r=0.80$; $p<0.01$), AvN ($r=0.68$; $p<0.05$), AvK ($r=0.93$; $p<0.01$) and ExCa+Mg ($r=0.69$; $p<0.05$) and negatively correlated with DHA ($r=-0.66$; $p<0.05$), PHA ($r=-0.71$; $p<0.05$), GSA ($r=-0.67$; $p<0.05$). CFU of root endophyte was found to be negatively correlated to pH ($r=-0.97$; $p<0.01$), SOC ($r=-0.72$; $p<0.05$), MBC ($r=-0.63$; $p<0.05$), MBN ($r=-0.62$; $p<0.05$), MBP ($r=-0.88$; $p<0.01$), AvK ($r=-0.63$; $p<0.05$) and CFU of rhizosphere soil ($r=-0.75$, $p<0.01$) and positively correlated with GSA ($r=0.73$; $p<0.05$).

Acidobacteria was positively correlated with soil pH ($r=0.67$; $p<0.05$), SOC ($r=0.863$; $p<0.01$), MBC ($r=0.74$; $p<0.01$), MBN ($r=0.74$; $p<0.01$), MBP ($r=0.78$; $p<0.01$), AvN ($r=0.71$; $p<0.05$), AvK ($r=0.66$; $p<0.05$) and Ex Ca+Mg ($r=0.61$; $p<0.05$) and negatively correlated to soil PHA ($r=-0.61$; $p<0.05$). *Burkholderia* was positively correlated to soil DHA ($r=0.61$; $p<0.05$) and negatively correlated with soil MBC ($r=-0.63$; $p<0.05$). *Acidobacteria* was positively correlated with CFU of both rhizosphere ($r= 0.65$; $p<0.01$) and negatively correlated with root endophyte ($r=-0.78$; $p<0.01$).

4. Discussion

In this study, we have used both culture-dependent and culture-independent technique for studying the effect of fallow cycle and crop plants on bacterial communities in soil. We used conventional method of isolation and enumeration of bacterial population in the rhizosphere and root tissue. PCR-DGGE has been used in molecular microbial ecology for about a decade [28]. DGGE fingerprinting is the most widely used and appropriate method for analysis of multiple samples. Since its introduction into microbial ecology, it has been adapted in many laboratories for assessment of microbial diversity in natural samples and is reproducible and sensitive [29]. Numerous samples can be analyzed simultaneously allowing the monitoring of microbial communities and whether they are affected by any environmental parameter [30]. The first silver staining of polyacrylamide gels was introduced by Switzer *et al*. [31]. We have used this technique as it offers sensitivity, is rapid and easy to use for identification of nucleic acids. Silver staining also reduced the cost of testing by reducing the PCR mixture volume and provides a permanent record of result [32]. Different statistical strategies have been applied for analyzing DGGE fingerprinting data and multivariate analyses of DGGE patterns were confirmed by Muylaert *et al*. [33] and incorporation of both the methods (DGGE and multivariate analyses) has become a powerful tool in molecular microbial community's studies.

The choice of matrix between presence/absence and relative abundance depends on whether the specific aim of the study is quantitative (presence/absence) or quantitative (relative abundance) and that relative abundance is recommended over presence/absence matrix to investigate complex bacterial community composition and to reveal the full extent of the changes in microbial community composition [18]. In our study, we have analyzed all the data based on the relative abundance matrix except for correlation we have used the presence/absence matrix.

Number of CFU (both rhizosphere and root tissue) of *O.sativa*, *C.esculenta*, *Z.mays* and *P.frutescens* from different jhum cycles (5-, 10- and 20- years) were comparatively higher on R2A than TSA media indicating the ability of R2A media to provide nutritional requirements and support microbial growth [34]. 89% and 53% effect size were attributed to individual crop plant and fallow cycle indicating that crop plant and fallow cycle are likely the factors affecting the culturable bacteria in the rhizosphere of selected crop plants. 81.6% of the variance in culturable endophytic bacterial population was explained by crop plant pointing to the ability of plant species to attract and recruit special bacterial endophytes that may help them cope with the surrounding environment. However, there is no combine effect of crop plant and fallow cycle on rhizobacteria and root endophytes indicating their effect were independent of each other. Endophytic community of a plant is strongly influenced by host plant and different plant species growing in the same soil can have different endophytic communities and same plant species growing in different agricultural soil can have different endophytic bacteria [36].

The community composition of soil *Acidobacteria* and *Burkholderia* was altered in shifting cultivation system as revealed by the DGGE finger printing. In this study, both the DGGE banding patterns were complex and showed higher number of species especially with *Acidobacteria* community. This was also seen from the Shannon diversity index where *Acidobacteria* had the higher average diversity index which was due to higher number of bands present. Thus, indicating the dominating nature of this group in the selected jhum plots. Community shifts as seen by presence and absence of bands between crop plants of same and different fallow were most likely due to changes of root exudation patterns at different fallow cycles due to abiotic and biotic stresses at different fallow cycles suggesting selection of different species in this environment. Many biotic and abiotic stresses can alter rhizosphere microbial community structures because some microbial communities can sense plant signal molecules under stresses which can trigger some microbial populations to increase or decrease [37,38]. In this study, Shannon evenness was variable yet higher in all the rhizosphere of the selected crop plants reflecting to functional resistant of dominant species to any environmental change. Cluster analysis showed that there was similarity in the

Acidobacteria community associated with crop plants of the same fallow cycle. There were similarities in few crop species and also among fallow cycles in the case of *Burkholderia* population. The effects of both plant microsites (rhizosphere and loosely adhered soil) and fallow cycle seemed to be stronger on *Acidobacteria* compared to *Burkholderia*. Our results have also showed significant differences in *Acidobacteria* and *Burkholderia* population at different fallow cycles and among the selected crop plants within the same fallow cycle indicating that both fallow cycle and plant species are determining factor in shaping the community structure of both bacterial groups. For *Acidobacteria*, fallow cycle had a stronger effect than crop species and plant microsites, whereas in the case of *Burkholderia*, the effect of crop species is slightly higher than fallow cycles but the difference is low, pointing that may be both these factors were having equal effect on shaping *Burkholderia* community. This was also confirmed by our culture-dependent analysis which showed the effect of crop plant and fallow cycle on bacterial population. Site dependent community analysis of bacteria was also studied by Costa *et al*. [39] which showed that the effects of plant and site on bacterial communities using PCR-DGGE approach. His study showed that plant roots played a more important role than the sampling site for determining the rhizospheric microbial community. Plant effects on soil microbial communities have been frequently observed in the rhizosphere, which refers to the soil directly influenced by root exudates [40,41]. However, the composition of root exudates varies from plant to plant and affects the relative abundance of microorganisms in the vicinity of the roots [2004]. According to Marschner [43], the interaction between plant species, soil type and root zone affect the bacterial community composition.

It was already known that bacterial population in soil is greatly affected by soil properties. This study also pointed to the relationship between soil parameters and bacterial population in soil. It was seen that culturable rhizobacteria and root endophytes were correlated to a number of soil parameters such as pH, SOC, PHA, DHA, GSA, MBC, MBN, MBP, AvN, AvP, AvK and Ex Ca+Mg. Correlation was also found between CFU of rhizobacteria and CFU of root endophytes suggesting that the surrounding rhizobacteria may be controlling the endophyte population and diversity or vice versa by releasing root exudates. *Acidobacteria* community was correlated with rhizobacteria and root endophyte population suggesting that *Acidobacteria* may play a role in contributing or controlling the population of rhizobacteria and root endophytic bacteria through interaction with each other or with other organisms and also with the environment and hence shape the diversity. Among the soil properties that correlated with bacterial population in soils, pH was most prominent and also a strong predictor of *Acidobacteria* abundance and community composition in soil. The highest incidences of *Acidobacteria* were found in soils with the lowest pH [44] and phylogenetic clustering of *Acidobacteria* communities became stronger as soil pH departed from neutrality [45]. Preference for an acidic pH has been found to be a trait of other subdivision *Acidobacteria* [46] but is probably not characteristic of the entire phylum [9], whereby, multiple members of the phylum *Acidobacteria* have been found to be abundant in alkaline soils [47,48]. Higher SOC in longer fallow cycle increase the microbial population, particularly *Acidobacteria* in this case, due to accumulation and build up of organic matter during the long fallow period. Yang *et al*. [49] also found that SOC was the main driving factor changing the bacterial communities. Higher MBC, MBN and MBP in the soil of 20 years fallow cycle indicates the high rate of decomposition of plant, animal and soil organic matter thus releasing carbon dioxide and plant available nutrients in these soil. Higher microbial biomass is an indication of higher abundance of bacterial population. PHA is involved in phosphorus cycle and has been reported to be governed by soil microclimate, SOC and AvP [50]. The relative stress in younger fallow cycle may have caused the higher activity in soil enzymes which could be due to higher plant and microbial secretion in these soils. The high Ex Ca+Mg in the rhizosphere of selected crop plants revealed the soil ability to provide sufficient nutrients required by plants, although a wide range of other factors influence the exchange capacity.

Therefore, it was seen that the bacterial population in the soil and root can be greatly affected by plant species and fallow cycle and their dependent on soil nutrients as revealed by both culture-dependent and culture-independent technique. Due to this variation in exudation, different plant species growing in the same soil type are known to select divergent bacterial communities [2,7,51]. However, when the microbial communities associated with the same plant species growing in different soil types are analyzed, the soil type may exert a large influence on microbial diversity [7,52]. Keeping in mind that several other factors like climate, plant genotypes, age, root exudates, etc. are also responsible for shaping the bacterial population. The changed microbial community composition during stress or during disturbance in jhum soil may have implications for plant survival and health. There is a need to identify root associated microbial communities that thrive under adverse environments and can confer stress tolerance and potentially be advantageous to the host.

5. Conclusion

This study reveals that under relative stressed environments like jhum ecosystem, along with other factors such as soil type, the factor determining the microbial community structure may likely be due to the length of fallow cycles which exert pressure to the plant to secrete root exudates that attract special microbial communities that can confer resistance and tolerance to the host plant from biotic and abiotic stresses. Plant species is also a determining factor for recruiting *Acidobacteria* and *Burkholderia* communities that may help the individual crop plant to survive in jhum soil especially in shorter fallow cycles. This study also revealed that community patterns of *Acidobacteria* and *Burkholderia* responds differently to the length of the fallow cycles. This study may also help in formulating ecorestoration strategies by targeting dominant strains (as revealed in DGGE fingerprinting) that may help in plant growth and their survive ability, and developing appropriate bio-inoculant that is confined to mixed crops under jhum agro-ecosystem for improving crop productivity and reduce the environmental impacts

caused by chemical fertilizers. The symbiotic associations of microbes and plants if targeted can provide a lot more of information where we can improve the soil health status in jhum fields and improve crop productivity in these systems.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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Supplementary

Table S1. Physico-chemical and biochemical properties of soil under different crop plants

Mixed crops	Jhum cycle (in years)	MC (%, w w^{-1})	pH (1:2.5 soil water suspension)	SOC (%, w w^{-1})	DHA (µg TPF g^{-1} soil h^{-1})	PHA (mgpNPg^{-1} soil h^{-1})	GSA (µgpNPg^{-1} soil h^{-1})	MBC (µgg^{-1} soil)	MBN (µgg^{-1} soil)	MBP (µgg^{-1} soil)	AvP (kg ha^{-1})	AvN (kg ha^{-1})	AvK (kg ha^{-1})	Exch.Ca+Mg c mol (P^{+}) kg^{-1}
Oryza sativa	5	28.9 ± 0.05	4.6 ± 0.12	2.06 ± 0.07	7.02 ± 1.5	1.27 ± .05	24.2 ± 1.2	368 ± 26.2	74 ± 9.2	14 ± 2.5	8.61 ± 0.0	254.33 ± 4.9	156.69 ± 0.2	0.07 ± 0.00
	10	30.7 ± 0.11	5.10 ± 0.14	2.40 ± 0.11	5.94 ± 0.3	1.23 ± 0.07	22.1 ± 1.4	429 ± 2.2	76 ± 10.5	17.80 ± 1.2	13.66 ± 1.1	288.51 ± 0.00	211.29 ± 0.6	0.08 ± 0.02
	20	31.4 ± 0.12	5.5 ± 0.18	2.50 ± 0.14	4.96 ± 0.2	0.85 ± 11.0	20.2 ± 1.3	591 ± 10.52	118 ± 14.5	20 ± 1.4	23.71 ± 0.2	291.65 ± 4.4	262.98 ± 1.3	0.21 ± 0.02
Colocasia esculenta	5	25.6 ± 0.14	4.5 ± 0.16	2.33 ± 0.04	6.9 ± 0.3	1.18 ± 0.08	29.8 ± 1.1	334 ± 13.5	79 ± 15.3	15.6 ± 3.3	11.08 ± 0.1	297.55 ± 4.4	159.66 ± 0.7	0.08 ± 0.02
	10	27.3 ± 0.4	4.9 ± 0.14	2.51 ± 0.01	5.86 ± 0.2	1.12 ± 0.07	25.2 ± 2.0	471 ± 15.2	88.51 ± 19.2	18 ± 1.27	37.18 ± 0.0	323.11 ± 0.00	186.14 ± 3.0	0.23 ± 0.02
	20	28.9 ± 0/04	5.4 ± 0.17	2.82 ± 0.16	5.15 ± 0.1	0.65 ± 0.09	21.5 ± 2.3	567 ± 9.11	137 ± 20.1	24 ± 2.5	50.71 ± 0.1	345.23 ± 4.4	255.53 ± 1.3	0.37 ± 0.06
Zea mays	5	27.5 ± 0.07	4.4 ± 0.14	2.32 ± 0.16	5.21 ± 0.2	1.20 ± 0.06	25.4 ± 1.6	373 ± 16.8	74.1 ± 11.8	12.66 ± 3.5	4.66 ± 0.0	306.14 ± 0.0	148.951 ± 0.6	0.15 ± 0.03
	10	32.9 ± 0.60	5.0 ± 0.15	2.47 ± 0.09	4.43 ± 0.1	1.19 ± 0.07	23.5 ± 0.9	434 ± 25.4	72.5 ± 7.5	15.91 ± 4.4	13.72 ± 0.1	310.4 ± 4.4	169.9 ± 1.0	0.17 ± 0.02
	20	37.9 ± 0.09	5.1 ± 0.20	2.87 ± 0.09	3.63 ± 0.1	1.08 ± 0.11	19.9 ± 1.1	576 ± 29.8	105.6 ± 21.3	25.56 ± 5.1	13.34 ± 0.1	328.51 ± 8.9	261.3 ± 3.5	0.19 ± 0.05
Perilla frutescens	5	31.9 ± 0.14	5.3 ± 0.12	2.31 ± 0.15	6.61 ± 0.2	1.30 ± 0.14	23.2 ± 1.2	397 ± 22.1	68.7 ± 6.5	18.11 ± 1.5	23.32 ± 0.1	269.7 ± 26.6	138.71 ± 0.8	0.04 ± 0.00
	20	32.4 ± 0.15	5.9 ± 0.15	2.62 ± 0.14	5.26 ± 0.3	1.23 ± 0.12	20.6 ± 1.5	439 ± 20.5	97.7 ± 5.2	24.78 ± 5.5	14.83 ± 1.1	315.83 ± 4.4	208.38 ± 1.5	0.12 ± 0.02

Mean value ± standard error (n=2, for DHA n=3) are shown.

Table S2. Pearson's correlation between soil properties, CFU of culturable bacteria and *Acidobacteria* and *Burkholderia*

	MC	pH	SOC	DHA	PHA	GSA	MBC	MBN	MBP	AvP	AvN	AvK	Ex Ca+Mg	A	B	C	D
MC																	
pH	0.51																
SOC	0.52	0.56															
DHA	-0.69	-0.34	-0.77														
PHA	0.01	-0.33	-0.63	0.40													
GSA	-0.77	-0.77	-0.57	0.63	0.36												
MBC	0.53	0.58	0.80	-0.70	-0.78	-0.76											
MBN	0.18	0.55	0.76	-0.47	-0.93	-0.55	0.85										
MBP	0.61	0.79	0.87	-0.54	-0.49	-0.72	0.74	0.74									
AvP	-0.14	0.42	0.51	-0.07	-0.73	-0.23	0.57	0.67	0.46								
AvN	0.18	0.30	0.88	-0.70	-0.60	-0.28	0.59	0.64	0.61	0.53							
AvK	0.47	0.58	0.79	-0.64	-0.76	-0.72	0.92	0.88	0.79	0.44	0.57						
Ex Ca+Mg	0.01	0.26	0.72	-0.55	-0.89	-0.33	0.74	0.83	0.47	0.76	0.82	0.66					
A	0.36	0.67*	0.83**	-0.59	-0.61*	-0.56	0.74**	0.74**	0.78**	0.56	0.71*	0.66*	0.61*				
B	-0.37	-0.50	-0.50	0.61*	0.53	0.45	-0.63*	-0.50	-0.38	-0.45	-0.45	-0.39	-0.59	-0.59			
C	0.42	0.67*	0.81**	-0.66*	-0.71*	-0.67*	0.84**	0.85**	0.80**	0.44	0.68*	0.93**	0.69*	0.65**	-0.51		
D	-0.51	-0.97**	-0.72*	0.45	0.39	0.73*	-0.63*	-0.62*	-0.88**	-0.48	-0.50	-0.63*	-0.38	-0.78**	0.53	-0.75**	

*Correlation is significant at p<0.05 (2-tailed)
**Correlation is significant at p<0.01(2-tailed)
A: *Acidobacteria*
B: *Burkholderia*
C: CFU of rhizobacteria
D: CFU of bacterial root endophyte