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Effect of organic and inorganic fertilization on soil bacterial diversity associated with sole crop (Pigeon pea) and crop rotation (Green gram-Sorghum)

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Abstract
An idea behind the status of soil microbial structure under organically and inorganically managed cropping systems is necessary for the development of suitable and sustainable crop production systems. We determined the bacterial community structure of the rhizosphere soils under the influence of organically and inorganically managed fields of sole crop and crop rotation using denaturing gradient gel electrophoresis. Shannon diversity index and range weighted richness indicated that the microbial communities are influenced by the management practices whereas there is no much difference between the cropping pattern. Irrespective of the crop or management, the bacterial diversity was higher during the vegetative stage of the crops. Species diversity and richness seen to be higher in organically managed fields as compared to inorganic management. The long term organic management of the fields will be useful in maintenance of soil health as well as the sustainable crop production.

Keywords: Cropping system, organic fertilization, growth stages, bacterial diversity, denaturing gradient gel electrophoresis (DGGE)

Introduction
The basic goal of cropping systems is the food security with efficient use of land and farm inputs; however the biological activity in the soil also plays a role in output of the cropping systems which is not well understood. The organic and inorganic amendments provide nutrient rich environment for both microbial communities (Crecchio *et al*. 2007) [3] as well as for the crops. The use of organic/inorganic amendments results into the change in the microbial community structure (Calbrix *et al*. 2007) [2], which in turn influences the soil quality and plant nutrition (Bulluck III *et al*. 2002; Celler *et al*. 2014) [1]. Understanding the amount of microbiota added to the soil through the addition of organic or inorganic amendments over a period of time will help us to evaluate whether the practices, maintain or improve the quality of soil for cultivation. Several studies identified significant differences among the microbial communities arising from different long-term cropping and management practices, while some have studied the effects of crop rotation on soil microbiota (Marschner *et al*. 2004; Crecchio *et al*. 2007; Suzuki *et al*. 2012) [9, 3, 19]. For the development of improved crop production systems we need a better understanding of the relationships among cropping systems and the resultant changes in soil microbial ecology under organically and inorganically managed field. Therefore, we used denaturing gradient gel electrophoresis (DGGE) to analyze the impacts of crop rotation and crop type on the bacterial communities in experimental field plots that were kept under sole Pigeon pea and green gram – sorghum crop rotation. The field plots were all located in the same region and was managed using the same tillage system, with the fertilizer applications adjusted for each crop type.

Materials and Methods
Treatment details
For the investigation, two cropping systems were selected: sole pigeon pea and green gram – sorghum, under 100% organic and 100% inorganic fertilization were selected.

Soil sampling
The soil samples were collected near the root zone of three different crops namely pigeon pea, green gram and sorghum at different crop growth stages (before sowing, vegetative, flowering and maturity). The samples were taken at a depth of 10-15 cm from five random spots, then pooled together and labeled before storage at 20 ^{0}C till further processing.

Soil DNA extraction and PCR-DGGE

Total DNA was extracted by lab based protocol developed at the IABT, University of Agricultural Sciences, Dharwad. Briefly, 200 mg soil was mixed with 1ml of extraction buffer [100mM Tris-Cl (pH-9.0), 100 mM NaEDTA (pH- 9.0), 1.5M NaCl and 100 mM $CaCl_2$] and 200 µl of 20% sodium dodecyl sulphate followed by vigorously vortexing for 30 seconds. The samples were then incubated on thermomixer at 70 ^{0}C and 1400 rpm for one hour. The supernatant was collected by centrifugation at 13000 rpm for 10 minutes. Nucleic acid was separated from other contaminants by adding equal volume of Choloro form: isoamyl alcohol (24:1) and centrifugation at 13000 rpm for 15 minutes at room temperature. The upper aqueous phase was transferred to a fresh micro centrifuge tube and equal volume of pre-chilled isopropanol was added and incubated overnight at 20 ^{0}C for precipitation. After overnight incubation, centrifugation at 13000 rpm for 10 minutes at 4 ^{0}C was followed. The pellet thus obtained was washed with 70% alcohol; air dried and dissolved in TE buffer.

Hypervariable region (V5) of 16S rDNA was amplified using primer pair E783 with GC clamp and E926 (Wang & Qian, 2009) [21]. Each PCR reaction contained 1X PCR buffer, 1.2mM $MgCl_2$, 250 µmoles of each dNTP, 5µM of each primer, 1 unit of Taq DNA polymerase and 50 ng template DNA. The template DNA was denatured at 95 ^{0}C for 5 min followed by 30 cycles of denaturation at 94 ^{0}C for 45 seconds, annealing at 55 ^{0}C for 30 seconds and extension at 72 ^{0}C for 45 seconds. 1500 ng of PCR product was separated in 12% polyacrylamide gel with 30% to 70% denaturant (40% formamide and 7M urea corresponds to 100% denaturant). The gel was run in 1X TAE buffer for 18 hours in Ingeny PhorU unit at 100 Volt and stained using silver staining (Torsvik and Ovreas, 2002) [20].

Analysis of DGGE profiles

The DGGE gel profiles were documented in Syngene G box gel documentation unit and processed by GeneTools software (Syngene). The faint band in marker is scored as 10 and used as reference for the densiometric based scoring of bands in sample. The number of bands was taken as measure of different operational taxonomic units (OTU's) and the respective intensity. Species richness was calculated by range-weighted richness {Rr=(N2 x Dg)}(Marzorati *et al*, 2008) [10], where Rr is range weighted richness, N is number of bands and Dg is the range of denaturant gel in which the uppermost and lowermost bands were obtained. Bacterial diversity was calculated by Shannon diversity index {H| =-ΣPiLn(Pi)} (Shannon, 1948), where H| is Shannon diversity index, Pi is the proportional intensity of each band or OTU and Ln(Pi) is the natural logarithm of proportional intensity of each band (OTU). Statistical analysis for Shannon diversity index was performed according to Hutcheson's modified t test (Hutcheson, 1970) [5]. The distribution pattern of species in each sample was analysed by Pielou's evenness index ($J^1 = H^1/Hmax$), where J1 is Pielou evenness index, H^1 is Shannon diversity and Hmax is natural logarithm of species richness (Pielou, 1966) [16]. Pielou evenness index ranges between 0 (highly uneven distribution) to 1 (highly evenly distributed). Similarity between the samples was calculated by Sorenson's similarity index (Sorensen, 1948) [18] [Cs = 2j/(a+b), where j is the number of OTU's common for both samples, a and b are the number of OTU's present in first and second samples respectively]. The shift in bacterial community structure during growth periods of crops was studied by moving window analysis (Nauhaus *et al*., 2007) [13]. Similarity in microbial community composition between two sampling points was calculated by Sorenson similarity index (Nakatsu *et al*., 2000) [12]. Percent change in microbial community between two sampling points was calculated by subtracting percent similarity from 100. This was done for consecutive sampling points over experimental time period. The % change value matrix was used to perform moving window analysis by plotting the values between consecutive sampling points.

Results

To identify the effect of organic and inorganic fertilization on the soil bacterial diversity, DGGE analysis based on 16S rRNA genes was performed. Based on the DGGE profile of different crops under organic and inorganic fertilization (Fig. 1) diversity index and species richness was calculated. The Shannon diversity index calculated at different growth stages of the crop rhizosphere samples indicated that there was no significant difference between the organic or inorganic fertilization; however the soil bacterial diversity was higher in organically managed rhizosphere as compared to inorganically managed soil rhizosphere of the given crops at all the stages. The Shannon diversity index was higher at vegetative stage of all the crops irrespective of the soil management practices. Highest Shannon diversity index was recorded at vegetative stage of organically managed sole crop pigeon pea (2.91) whereas lowest Shannon index was observed at maturity stage of inorganically managed sole crop pigeon pea (2.37).

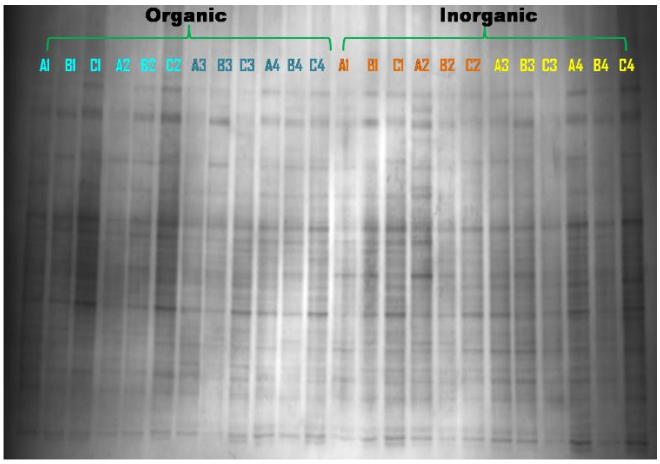

Fig 1: Silver stained DGGE profile of organically and inorganically managed rhizosphere soil samples of different crops (A-Green gram; B-Sorghum; C-Pigeon pea) at different growth stages (before sowing-1, vegetative-2, flowering-3 and maturity-4).

Table 1: Diversity, richness and distribution of soil bacteria under organically and inorganically managed rhizosphere of green gram (A), sorghum (B) and pigeon pea (C) at various crop growth stages.

Growth Stages	Organic											
	Before sowing			Vegetative			Flowering			Maturity		
Particulars	A1	B1	C1	A2	B2	C2	A3	B3	C3	A4	B4	C4
Shannon index	2.67	2.60	2.66	2.74	2.81	2.91	2.63	2.74	2.81	2.61	2.70	2.78
Range weighted richness	124	112	136	176	149	149	136	112	175	101	91	124
Pielou's eveness index	0.88	0.87	0.86	0.85	0.90	0.93	0.85	0.91	0.87	0.89	0.93	0.91
Number of OTU's	21	20	22	25	23	23	22	20	25	19	18	21
Growth Stages	**Inorganic**											
	Before sowing			**Vegetative**			**Flowering**			**Maturity**		
Particluars	A1	B1	C1	A2	B2	C2	A3	B3	C3	A4	B4	C4
Shannon index	2.60	2.56	2.74	2.77	2.70	2.87	2.62	2.58	2.45	2.57	2.45	2.37
Range weighted richness	103	115	138	126	152	151	94	93	114	92	91	64
Pielou's eveness index	0.88	0.85	0.89	0.91	0.84	0.92	0.91	0.89	0.82	0.89	0.83	0.88
Number of OTU's	19	20	22	21	25	23	18	18	20	18	19	15

Species richness was higher at vegetative stage of all the crops irrespective of management practice except in case of organically managed pigeon pea, where species richness was observed higher at flowering stage (175). Highest (176) and lowest (64) range weighted richness was found at vegetative stage of organically managed green gram and at maturity of inorganically managed pigeon pea respectively. Pielou's eveness index under greengram – sorghum cropping system is more evenly distributed as compared to sole crop pigeon pea both in organic and inorganic management (Table 1). Irrespective of organic or inorganic management, the functionality of the soil bacterial structure was highly organized; overall percentage of individuals in organic and inorganic green gram was 83.17% and 83.25%; 84.32 % and 86.51 % in organic and inorganic sorghum whereas, 84.78 % and 84.77% of the individuals in organic and inorganic pigeon pea belonged to only 20% of the total species observed (data not shown).

The rate of change 65.98 ± 6.35% and 50.89 ± 8.76% in organic and inorganic green gram; 47.49 ± 4.42% and 52.29 ± 9.89% in organic and inorganic sorghum; 46.67 ± 0.87% and 60.06 ± 4.98% in organic and inorganic pigeon pea respectively was observed through moving window analysis of the DGGE fingerprint. The moving window analysis indicated that ecto-rhizosphere bacterial community structure reduced over the growth stages in organic management of the given crops, whereas under inorganic management the rate of the change in the bacterial communities increased with the growth stages (Fig. 2).

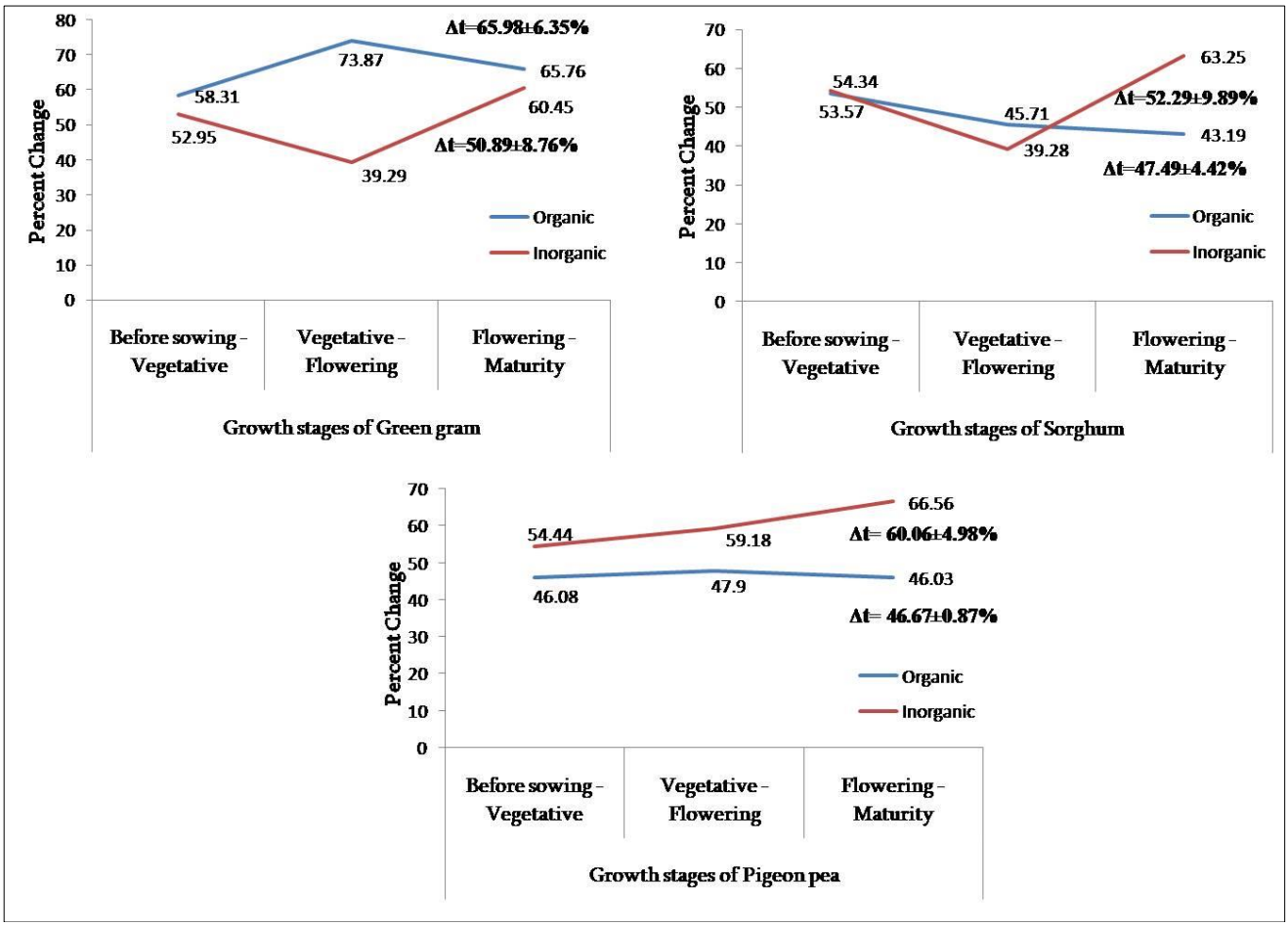

Fig 2: Moving window analysis of Green gram (a), sorghum (b), and pigeon pea (c) rhizosphere bacterial community composition analyzed by DGGE profiling.

Discussion

Microbial community structure is very much important for the soil ecosystem, as they are involved in various biological processes such as degradation of organic matter, nutrient cycling, formation of soil aggregates and improving soil properties. The microbial communities respond quickly to intensive agronomic practices such as land management, fertilizer management, crop rotation, pest and disease management both in the organic and inorganic farming (Pasha *et al*., 2015) [15].

To analyze the soil bacterial diversity between sole cropping and crop rotation denaturing gradient gel electrophoresis (DGGE) was used. DGGE separates the pool of different sequences containing variations but have same molecular size on vertical gel electrophoresis in which the gel contains the denaturant in increasing concentration from top to bottom of the gel. For unbiased analysis, the samples were run in a wide range of denaturant which can be noticed through the separation of the bands observed in the DGGE profile (Fig. 1).

Previous studies of crop rotation system and its effect on microbial communities in bulk soils have yielded different results, where it was reported that the microbial communities are influenced by the crop rotation system (Ngosong *et al*. 2010; Yin *et al*. 2010; Suzuki *et al*. 2012) [14, 23, 19]; on the other hand few found that the crop rotation system had no significant effects on the microbial communities (Govaerts *et al*. 2008; Wang *et al*. 2010) [4, 22].

The soils which were analyzed in this study, irrespective of crop or management practice support good amount of species richness indicating the habitability of the given ecosystem. However it can be seen that the range weighted richness was higher at vegetative stages of the studied crops in both management practices. But the richness was decreased at consecutive growth stages of the crops under inorganically managed ecto-rhizosphere. The number of OTU's, species richness and diversity was slightly higher in organically managed crops than inorganically managed ones. The significant difference between the organic and inorganic management of crop was not seen, which could be due to the medium term of this given study. Shannon diversity index between the organic and inorganic cultivation of crops didn't shown any significant difference, but the diversity index of organically managed crops was slightly higher and uniform as compared to inorganic applications. The diversity index of organically managed sole pigeon pea was higher than the green gram and sorghum at both management practices indicating over a period, change in the crop type may cause disturbance in the bacterial community structure. Mathew and his coworkers reported that the fewer disturbances to the soil improved the soil microbial properties (Mathew *et al*. 2012) [8]. Insufficient information regarding the crop growth stage specific exudation of roots and its influence on bacterial structure, the differentiation between soil bacterial structure affected by exudation and environment is difficult. The bacterial community structure shifts didn't shown any kind of distinct pattern in case of cropping system, however irrespective of crop, there was increased change in bacterial community structure over the crop growth period in case of inorganic management. Application of organics has positive increment in nutrient status, microbial activity and productive potential of soil as compared to use of chemical fertilizers in the cropping system, which resulted in a poor microbial activity and reduced productive potential of soil (Kang *et al*., 2005) [6]. Irrespective of the management practices or cropping system, the ecosystem has shown highly organized functionality. Very few species belonged to dominant groups and rest of them was found in low number as seen in Pareto-Lorenz evenness curve (Pareto, 1897; data not shown). The possible reason for this could be the shorter period of organic management of the field and other disturbances due to human activities. As described by Marzorati and his team that the community which is highly functionally organized are fragile to changes due to external interference and may lead to longer period for recovery (Marzorati *et al*. 2008) [10].

Conclusion

The present investigation reports that there have been minor differences in bacterial community structure and their functional organization between the organically and inorganically managed cropping systems. To obtain the significant effects of organic farming and their influence on crop growth, it may require long term organic management of the ecto rhizosphere under the different cropping systems. For sustainable crop production, the maintenance and enrichment of the soil fertility through organic agriculture is of utmost priority due to climate changes. This study might help in analysis and interpretation of huge number of DGGE fingerprints to bring out meaningful results as well as it would help in narrowing the number of samples to be taken forward for metagenomic sequencing and metatranscriptome studies.

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