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# Soil Bacterial Diversity Analysis of Cotton Field under Organic and Inorganic Management using DGGE

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**Microbial diversity is one of the important microbial parameters that would reflect the fertility and health of soil. The effect of inorganic (conventional) farming on soil microbial structure and function is not very definite as yet. Organic farming is known to improve soil microbial diversity and leads to sustainability. Bacterial community structure of short term organic and inorganic farm soil was studied using culture independent approach and denaturing gradient gel electrophoresis. The species richness, diversity and the distribution of bacterial species improved in organic farming, but the change is subtle. We expect that this difference will be significant under long term application of organics.**

**Key words:** Organic farming, metagenomics, diversity, denaturing gradient gel electrophoresis.

Soil contains large number of diverse organisms, of which microbes forms the major part wherein a gram of soil is known to contain $10^6$ different species. They play an important role in maintaining the structure and function of soil. In turn, they are affected by edaphic[1], environmental[2] and anthropogenic factors[3] and to a large extent by soil management practices[4]. Conventional (inorganic) and organic are the two major forms of land management practices. Organic farming is known to be potential in improving soil structure, soil biodiversity, tolerating environmental stress and improving food quality[5-8]. However, the impacts of organic inputs on soil microbial community structure and function can vary widely[8]. Build up of large amount of active molecules is necessary for the sustainable productivity in organic farming. Soil microbes have a major role to play in these processes. They act as transient sink as well as release the nutrients from organic matter which will be taken up by plants[10]. On the other hand, the information about long term effect of inorganic fertilizers on soil microbial diversity is conflicting. Goyal *et al*. (1992)[11] showed that inorganic fertilizers improve microbial biomass. However, Sarathchandra *et al*. (2001)[12] reported that it had no significant effects on soil microbial population but reduced the functional microbial diversity. Here, we analysed the diversity and distribution of bacterial species in short term organic and inorganically managed farm soil using the concept of metagenomics coupled with the denaturing gradient gel electrophoresis.

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## MATERIALS AND METHODS

### Soil type and sampling

The soil samples of vertic (clay 58.8%, silt 19.61%, sand 20.61%, pH 7.55, EC 0.35 $dSm^{-1}$, CEC 58.90 me/100g and 0.41% organic carbon) were collected from seven year old fixed-field trials of

organic and inorganic farming, Main Agricultural Research Station, University of Agricultural Sciences Dharwad. Nutrient and pest management was done by chemical fertilizers and pesticides in inorganic farming. Vermicompost, green leaf manure and enriched compost were used as a source of nutrient whereas neemcake, *Nomuraea rileyii* and *Trichoderma* were used for pest and disease management in organic farming. In addition, *Azospirillum* and panchagavvya (a concoction prepared by mixing dung, urine, milk, curd and ghee of cow) was used as bio-fertilizer and plant growth promoter respectively in organic farming. The samples weighing approximately 10 gram were collected near to the root zone of cotton at vegetative and flowering stages at a depth of 10 cm. Sample was taken from five spots in each plot randomly, pooled, labelled and stored at 4°C until further processing.

**Nucleic acid extraction**

Soil microbial community DNA was extracted by direct lysis method. Briefly, 250 mg soil was mixed with 1 ml of soil DNA extraction buffer {100mM TrisCl (pH 9.0), 100mM NaEDTA (pH 9.0), 100mM Sodium phosphate buffer (pH 9.0), 1.5M NaCl and 100mM $CaCl_2$} and 200µl of 20% sodium dodecyl sulphate and vortex rigorously for 2-3 Minutes. The sample was later incubated on a thermomixer on 1400 rpm for 1 hour 30 minutes at 75°C. The supernatant was collected by spinning at 10000 rpm for 10 minute at 25°C. Nucleic acid was separated from other contaminants by adding equal volume of chloroform, isoamyl alcohol (24:1) and spinning at 10000 rpm for 10 minute at room temperature. The upper clear aqueous layer was transferred to a fresh microcentrifuge tube and the DNA was precipitated by adding equal volume of pre-chilled isopropanol and spinning at 10000 rpm for 10 minute at 4°C. The pellet was washed, dried and dissolved in TE buffer.

**PCR amplification and DGGE**

The hypervariable region (V3) of 16S rDNA was amplified using PRBA338 and PRUN518 primers[13] with GC clamp. Each PCR reaction contained 1X PCR buffer, 1.2 mM $MgCl_2$, 250µmoles of each dNTP, 5 µM of each primer, 1 unit Taq DNA polymerase, and 100 ng template DNA. The template DNA was denatured at 95°C for 5 min followed by 32 cycles of denaturation at 94°C for 50 sec, annealing at 57.4 °C for 30 sec and extension at 72°C for 50 sec. 1200ng of PCR product (210 bp) was separated in 12% polyacrylamide gel with 20% to 80% denaturant (40% formamide and 7M urea corresponds to 100% denaturant)[14]. The gel was run in 1X TAE buffer for 18 hours in Ingeny PhorU unit at 170 volts and stained using silver staining.

**Analysis of DGGE profiles**

The DGGE profile was 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 as their proportion in the population. Species richness was calculated by range-weighted richness {$Rr=(N^2 \times Dg)$}[15], 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 {$H=-\leq PiLn(Pi)$} [16], where $H^{|}$ is Shannon diversity index, Pi is the proportional intensity of each band and Ln(Pi) is the natural logarithm of proportional intensity of each band. The distribution pattern of species in each sample was analysed by Pareto-Lorenz evenness curve[17]. The functionality of cotton ecto-rhizosphere soil under organic and inorganic management was estimated by Pareto-Lorenz curve[17]. After scoring, the bands in each lane were arranged from high to low intensity. The total intensity of all the bands in a lane was calculated and proportional intensity of each band was computed (normalized). Cumulative normalized intensity for each band was calculated, used as Y axis and plotted against the cumulative normalized number of bands on X axis. The Y axis projection of respective intercept with the vertical 20% X axis line was used to interpret the result numerically[18]. Statistical analysis for Shannon diversity index was performed according to Hutcheson's modified t test[19]. Per cent bacterial species shared between vegetative and flowering stage was calculated by Sorenson's similarity index[13].

## RESULTS

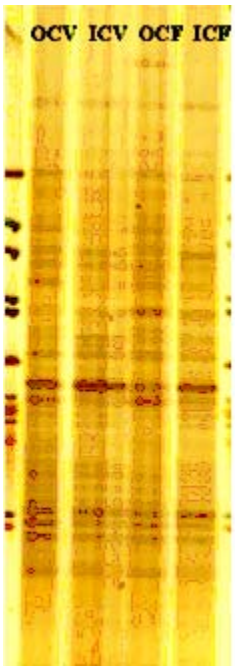
Denaturing gradient gel electrophoresis profile of all the samples was distinctly different and reproducible (Figure1). Most of the OTU's were common to all the samples. Few OTU's of very faint and very good intensity specific to soil management and crop growth were also observed. Overall, 35 and 32 OTU's were observed in organic and inorganic soil samples respectively. 30 OTU's were observed at both vegetative and flowering stages of cotton in organic soil, whereas 31 and 27 OTU's were found in inorganic soil at vegetative and flowering stages respectively (Table1).

The highest (213) and lowest (161) range weighted richness was found at flowering stage in organic and inorganic soil respectively. But at vegetative stage, the range weighted richness was more in inorganic soil (212) compare to organic soil (202). Overall range weighted richness of 294 and 226 was observed in organic soil. Shannon diversity index of 3.11, 2.97, 3.05 and 2.86 was observed in organic and inorganic soils at vegetative and flowering stages respectively. But the difference in Shannon diversity index between organic and inorganically managed soil is significant only at flowering stage. The Pielou's evenness index of 0.91 and 0.90 in organic soil and 0.86 and 0.87 in inorganic soil at vegetative and flowering stages respectively showed that the

**Table 1.** Soil bacterial richness, diversity and distribution under organic and inorganic management of cotton

| | Vegetative | | Flowering | | Overall | |
|---|---|---|---|---|---|---|
| | Organic | Inorganic | Organic | Inorganic | Organic | Inorganic |
| Number of OTU | 30 | 31 | 30 | 27 | 35 | 32 |
| Range weighted richness (Rr) | 202 | 212 | 213 | 161 | 294 | 226 |
| Shannon diversity index ($H^l$) | 3.11 | 2.97 | 3.05* | 2.86 | 3.12* | 2.98 |
| Effective number of species | 22.42 | 19.5 | 21.12 | 17.46 | 22.65 | 19.69 |
| Pielou evennes index (E) | 0.91 | 0.86 | 0.90 | 0.87 | 0.88 | 0.86 |

Values in bold with asterisk mark are significant between soil management at p=0.05 calculated by Hutcheson t test (for Shannon diversity index)




**Fi. 1.** DGGE profile of organic (OC) and inorganic (IC) soil bacteria at vegetative (V) and flowering (F) stages of cotton. 16S rDNA amplified by PRBA338GC and PRUN518 primers was separated in 12% poyacrylamide gel containing 20%-80% denaturant and silver stained

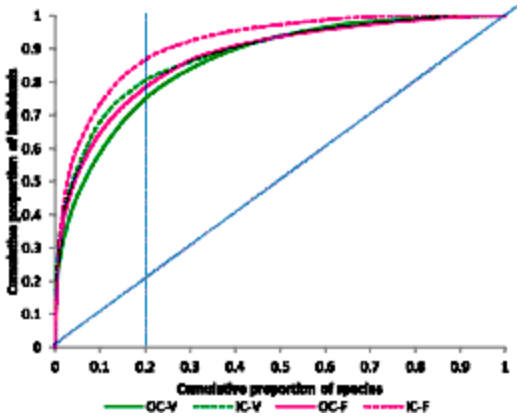



**Fig. 2.** Pareto-Lorenz evenness curve of organic (OC) and inorganic (IC) cotton soil bacteria at vegetative (V) and flowering (F) stages. Large number of individuals belongs to only few species reflecting these soil bacteria are highly functionally organised.

species are more evenly distributed in organically managed soil (Table1). Irrespective of management practice, the functionality of soil bacteria was observed to be highly organised; 72.8% and 76.5%, of individuals at vegetative and flowering stages of organic cotton and 78.9% and 85.2% of individuals at vegetative and flowering stages of inorganic cotton belonged to only 20% of the total species observed (Figure2). 74.63% and 76.19% of the bacterial species are shared between vegetative and flowering stages in organic and inorganic soil respectively.

## DISCUSSION

Microbes play an important role in soil ecosystem. They improve soil physical and chemical properties, degrades organic matter and supply nutrients to plants. However, they respond quickly to changes in soil properties like soil pH, salinity, temperature, organic matter composition, nutrient availability and presence of toxic compounds[20]. The effect of farming system (organic and inorganic) on soil bacterial community structure was studied by careful selection of sample. The effect of vegetation, edaphic and environmental factors was kept negligible by taking the soil from same patch of land (black clay soil) near the root zone of cotton. The DNA isolated from the soil microbial community by direct lysis method was free from inhibitors like humic acid which was confirmed by amplification of 16S rDNA from the vegetative and flowering stage samples by *Taq* DNA polymerase. Separation of bands in wide range of denaturant observed in this study is an indicative of unbiased analysis of soil bacterial community structure.

All the soils analysed, irrespective of soil management and crop growth stage support large number of genetically divergent species as indicated by the high range weighted richness. The range weighted richness indicates the habitability of an ecosystem, wherein more the range weighed richness, more habitable the environment is[15]. Addition of organic amendments to soil is known to increase the microbial biomass and their activity[21]. The overall number of OTU's, bacterial carrying capacity (range weighted richness), diversity, distribution and the effective number of bacterial species observed in organic soil is slightly more than inorganic soil. But the difference in diversity is significant only at flowering stage. The lack of significant difference could be due to the history of land use management[4]. The experiment of organic farming in this study was hardly seven year old (which was previously under inorganic cultivation). No significant difference was observed in Shannon diversity index, species richness and evenness in bacterial community between organic and inorganic soil of four to five year old[22], more than five year old[23] and twenty year old[24] experiments.

Rhizosphere microbial community composition is specific to plant root exudates[25]. The composition of root exudates depends on the plant species and cultivar, its developmental stage and stress[26]. 75% of the bacterial community composition remains same at vegetative and flowering stages of cotton. The change in one fourth of the species could be due to stage specific plant root exudates or it could be due to environmental and antropogenic factors[27]. There were intensive agronomic practices like weeding, inter-cultivation, application of nutrients and management of pest and disease during the crop production in both organic and inorganic farming which disturbed the soil and hence may be the reason for higher dynamics in bacterial community composition observed in both the management[22]. Mathew *et al* (2012)[28] showed that less disturbance to the soil improved both physico-chemical and microbiological properties of soil. Due to lack of information about plant stage specific root exudates and their effect on bacterial growth, we could not differentiate between the effect of plant root exudates and environmental factors on soil bacterial diversity. The shift in bacterial community composition between vegetative and flowering stage remains same under organic and inorganic management, indicating the effect of seasonal shift (temperature and moisture) and soil disturbance is more than application of organics and inorganics on soil bacterial community dynamics.

The bacterial community structure is more meaningful when it is correlated with the functionality of the ecosystem. The Pareto-Lorenz evenness curve[17] to assess the functionality of organic and inorganic soil ecosystem indicated that both the ecosystems showed high functional organisation. Irrespective of management both the

soils have specialised community, only few species are dominant and rest of the species are only in very low number. This could be again due to short period of organic farming (on previous inorganic farm) and also the continuous disturbance to the soil by human intervention. This type of organization is highly influenced by external disturbance[15]. Since there is less buffering (functional redundancy), a slight disturbance in high functionally organized ecosystem may lead to loss in functionality from the ecosystem.

## CONCLUSION

The present study showed minor difference in the soil bacterial richness, diversity, distribution and their functional organisation between organic and inorganic farming practices. The soil was under organic management for short period of time and it was continuously disturbed. We assume that it will take more than seven years (the period under which the field under study was subjected to organics) to realize the positive effects of organics on soil bacterial richness, diversity and their composition.

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