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ORIGINAL PAPER

Effects of organic and inorganic fertilization on soil bacterial and fungal microbial diversity in the Kabete long-term trial, Kenya

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Abstract The effects of crop manure and inorganic fertilizers on composition of microbial communities of central high land soils of Kenya are poorly known. For this reason, we have carried out a thirty-two-year-old long-term trial in Kabete, Kenya. These soils were treated with organic (maize stover (MS) at 10 t ha^{-1}, farmyard manure (FYM) at 10 t ha^{-1}) and inorganic fertilizers 120 kg N, 52.8 kg P (N_2P_2), N_2P_2+MS, N_2P_2+FYM, a control, and a fallow for over 30 years. We examined 16S rRNA gene and 28S rRNA gene fingerprints of bacterial and fungal diversity by PCR amplification and denaturing gradient gel electrophoresis separation, respectively. The PCR bacterial community structure and diversity were negatively affected by N_2P_2 and were more closely related to the bacterial structure in the soils without any addition (control) than that of soils with a combination of inorganic and organic or inorganic fertilizers alone. The effect on fungal diversity by N_2P_2 was different than the effect on bacterial diversity since the fungal diversity was similar to that of the N_2P_2+FYM and N_2P_2+MS-treated. However, soils treated with organic inputs clustered away from soils amended with inorganic inputs. Organic inputs had a positive effect on both bacterial and fungal diversity with or without chemical fertilizers. Results from this study suggested that total diversity of bacterial and fungal communities was closely related to agro-ecosystem management practices and may partially explain the yield differences observed between the different treatments.

Keywords Organic and inorganic amendments · Microbial diversity

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Introduction

In large parts of Kenya, food crop yields are very poor due to declining soil fertility as a result of continuous cropping with little or no replenishment of nutrients removed through either crop harvests or other losses such as leaching and soil erosion (Kibunja 2007). With the liberalization of trade and introduction of Structural Adjustment Programs, unfavorable crop fertilizer prices and financial constraints have greatly contributed to the low level of fertilizer use in many smallholder farms in the country (Waswa et al. 2007). The Eastern and Central highlands smallholder farmers in particular have been experiencing declining soil fertility and crop productivity. This has compelled researchers to

place a high priority on increasing agricultural productivity and alleviation of poverty among smallholder farmers. Attainment of this goal calls for an Integrated Soil Fertility Management, with management practices that necessarily include the use of fertilizer, organic inputs, and improved germplasm combined with the knowledge on how to adapt these practices to local conditions, aiming at maximizing agronomic use efficiency of the applied nutrients and improving crop productivity (Vanlauwe et al. 2010). In response to these problems, researchers, in collaboration with farmers, have set up trials on various soils management techniques for combating soil nutrient depletion. Technologies being actively and vigorously promoted to farmers in the region include: the combined use of soil amendments, organic materials, and mineral fertilizers to replenish the soil nutrients and improve the efficiency and cost-effectiveness of external inputs. These technologies have been found to be both technically feasible and socially acceptable (Sanchez and Jama 2000; Jama et al. 1999). The emerging dilemma with this approach, however, is that the impacts of these resources on soil biota, which are key functional groups influencing agricultural production and ecosystem services, are not yet well documented.

Intensification of agriculture has focused on the use of chemical and mechanical inputs, often at the expense of biologically mediated processes. Soil biota is indispensable for key soil function such as decomposition of soil organic matter, nutrient cycling, and formation of soil aggregates. The total mass of living microorganisms therefore has a central role as a source, sink, and regulator of the transformation energy and nutrients in the soil. Both organic and inorganic sources of fertilizer have residue effects in the field. These effects are a vital component of sustainability because they smooth season-to-season variations in soil fertility and crop productivity, but they are difficult to assess quantitatively. Therefore, it is advantageous to undertake well-characterized medium-to-long-term experiments rather than single-season trials and to detail the interactions rather than averaging the responses over different seasons and environments (Tandon and Kanwar 1984). Long-term implies that primary objectives, treatment, and management are not changed during the period under consideration, often regarded as at least 10 years (Laryea et al. 1995). An experiment was established in 1976 at Kabete, and the primary objective of the trial was to identify appropriate methods for maintaining and improving the productivity of the soil through repeated use of inorganic fertilizers (in particular, N and P), farmyard manure, and crop residues under continuous cropping following the husbandry practices of small-scale farmers. The challenge now remains to provide some understanding on the impact of these resources on soil microbial communities. The work described here was carried out in the long-term field experiment at the National Agricultural Research Laboratories (NARL-KARI) Kabete, near Nairobi, Kenya, which provides a unique resource to investigate the impact of long-term fertilization of organic and inorganic resources on the soil bacterial and fungal communities.

Materials and methods

The study site description

The study was superimposed on the ongoing long-term field experiment at Kenya Agricultural Research Institute (KARI) at the National Agricultural Research Laboratories station at Kabete, located at 36°41′ E and 01°15′ S and at an altitude of 1,737 m above sea level (Kibunja et al. 2010). These soils are mainly humic Nitisols (FAO 1990) that are deep and well weathered. The mean annual rainfall is about 980 mm and is divided into two distinct annual rainy seasons—the long rains between mid-March and June and the short rains between mid-October and December. The average annual maximum and minimum temperature is 23.8°C and 12.6°C, respectively.

Experimental design and choice of treatments

The field experiment was established in 1976 to investigate the effect of continuous application of farmyard manure, crop residues, and N and P fertilizers as calcium ammonium nitrate and triple super phosphate, respectively (once a year) in a maize–bean rotation. Triple super phosphate is applied at planting while calcium ammonium nitrate is top-dressed at 7 weeks after planting. The experiment was established as a randomized complete block design with 18 treatments replicated four times (four blocks). For this study, only seven treatments were monitored for a period of 1 year from March 2007 to February 2008 and included one maize and bean-growing season. Each plot is a rectangle with an area of 30 m^2 (4×7.5 m^2). The selection of the seven treatments (Table 1) was based on the crop yield data from the KARI yield data base. The averages of the soils characteristics are as follows: organic matter (3.67%), total N (0.16%), total C (2.13%), and available P (163 mg of P kg^{-1}).

Experimental design for bacterial and fungal communities diversity

Soil samples were collected 6 weeks after planting following a “W” design across the plots receiving treatments of interest (Table 1) by pushing a sterile soil auger at

Table 1 Selected treatments from the Kabete long-term experiment

Treatment	Fertilizer treatment	Organic amendments	Crop
1. Control	0 kg N, 0 kg P	0	Maize
2. Maize stover (MS)	0 kg N, 0 kg P	Maize stover returned	Maize
3. FYM_2	0 kg N, 0 kg P	10 t/ha farmyard manure	Maize
4. N_2P_2	120 kg N, 52.8 kg P	0	Maize
5. N_2P_2+MS	120 kg N, 52.8 kg P	Maize stover returned	Maize
6. N_2P_2+FYM	120 kg N, 52.8 kg P	10 t/ha farmyard manure	Maize
7. Fallow	Fallow	Fallow	Fallow

a depth of (0–10 cm). Six soil cores per plot were taken and well mixed to make a composite sample of 20 g. This was repeated for the four blocks. The soils were put in ziplock plastic bags and transported in cooler boxes to the laboratory where they were frozen at −80°C degrees and analyzed within 3 weeks.

DNA extraction, PCR amplification, and DGGE

Total genomic DNA was extracted from all soil samples by the direct lysis extraction procedure (Martin-Laurent et al. 2001). Soil (0.25 g) was treated using 0.5 g glass beads (106 μm diameter, Biospec Products) and 1 ml lysis buffer (100 mM Tris–HCl (pH 8.0), 100 mM NaCl, 1% (*w*/*v*) polyvinylpolypyrrolidone, and 2% (*w*/*v*) sodium dodecyl sulfate in a 20-ml Eppendorf tube. The Eppendorff tubes were homogenized for 30 s at 1,600 rpm using a Biospec Mini-Bead Beater cell disruptor and centrifuged at 14,000×*g* for 1 min at 4°C. The supernatant was collected, 1/10 volume of 5 M sodium acetate was added and incubated on ice for 10 min, then centrifuged at 14,000×*g* for 5 min. The DNA was precipitated with one volume of ice cold isopropanol overnight. The DNA was washed with 70% ethanol and dissolved in double distilled water.

PCR amplifications were performed using the forward primer 338f with a GC clamp and reverse primer 518r (Øvreås and Torsvik 1998). The total reaction mixture (25 μl) contained 2 μl of pure total DNA extract, 1× freeze-dried bead (Ready-to-Go PCR beads, Pharmacia Biotech) containing 1.5 U of Taq polymerase, 10 mM Tris–HCl, (pH 9 at RT), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, and 1.0 μM of each primer and sterile distilled water. Two replicates were performed for each sample. A Primus 96 ^{plus} PCR system (MWG AG Biotech) thermal cycler was used for PCR amplification with 2 min at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C. The first 20 cycles had an annealing temperature of 65°C, which decreased every cycle until a touchdown at 55°C. The primer extension was carried out at 72°C for 15 min. The PCR product (approximately 180 bp) was checked on a 1% agarose gel stained with ethidium bromide. PCR amplifications were performed using the forward primer 662f with a GC clamp and reverse primer 314r as described by (Sigler et al. 2002).

PCR amplifications were carried out in 25-μl reaction volumes with a Primus 96 ^{plus} PCR system (MWG AG Biotech). The reaction mixture was slightly modified by reducing the quantity of template DNA from 2.0 μl to 1.0 μl. Cycling conditions were as follows: initial denaturation at 95°C for 10 min followed by 49 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min followed by a final extension phase at 72°C for 10 min. The PCR product (approximately 348 bp) was checked for size on a 1% agarose gel stained with ethidium bromide.

PCR products were separated using denaturing gradient gel electrophoresis (DGGE) with a D-Code Universal Mutation system (Bio-Rad Laboratories). The PCR products were loaded on 8% (*w*/*v*) polyacrylamide (acrylamide–bisacrylamide [37.5:1]) gels with a denaturing gradient of 40–60% (100% denaturant contains 7 M urea and 40% formamide). The gels were run for 16 h at 75 V in Tris-acetate–EDTA buffer (1×) at 60°C with a pre-run of 10 min at 20 V. After migration, the DGGE profiles were stained with EtBr for 20 min, destained for 45 min, and then scanned with Gel Doc^{TM} XR (Bio-Rad Laboratories). The gel images were captured using Quantity One software version 4.6.0 (Bio-Rad Laboratories).

The DGGE patterns were transformed to graphs by the Bio-Rad Quantity One^{TM} software where each resolved band of the gel lanes was converted to a trait whose intensity is related to the amount of the corresponding DNA fragment. Total Lab 120 version 2006 software (Nonlinear Dynamics Ltd) was then used to calculate the percentage of similarity among lanes, taking into account the migration distance and the relative intensity of all bands. The DGGE profiles were compared and unweighted-pair group method cluster analysis was used to produce the dendograms by using the total lab 120 version 2006 software.

Diversity measurements and data analysis

The Shannon–Weaver indices (H') (Shannon and Weaver 1963) were calculated using the following equation:

$$H' = -\sum p_i \log p_i$$

Where $p_i = n/\sum n$

Where n is the volume of a single band per lane and $\sum n$ is the total volume of all bands per lane.The Shannon indices were used to perform ANOVA analysis using GENSTAT Release 7.2 (Lawes Agricultural Trust 2007).

Results

Genetic fingerprinting by DGGE of eubacteria 16S rRNA-amplified fragment showed few dominating bands and a number of faint unresolved bands (Fig. 1a) compared to the PCR amplification of fungal 28S rDNA gene where most of the bands were distinct (strong) and well resolved (Fig. 1b). However, the number of bands was higher in the eubacteria 16S rRNA gene DGGE profiles than in the fungal 28S rDNA gene DGGE profiles, an indication of high bacteria numbers compared to the fungal populations in the various treatments. Cluster analysis of PCR-DGGE patterns generally distinguished the fungal and bacterial communities in two major clusters (Fig. 2). In the bacteria community, cluster analysis showed great similarity (65%) with two major clusters, those with mineral fertilizers only, fallow and control in one and those with organic in one cluster or without inorganic fertilizers in another cluster (Fig. 2a), while for the fungal communities, a moderately lower percentage (55%) with two major clusters was also observed, organically treated soils clustered away from those with combined inorganic and organic inputs (Fig. 2b). Within the group of the combined inorganically and organically as well as that of inorganic treatments, the control and fallow treatments further grouped into a smaller cluster indicating different community structures in the control and the fallow land. Shannon indices indicated a higher total diversity of fungal and bacterial communities in the N_2P_2+FYM treatments. The lowest diversity in the bacterial community was in the N_2P_2-treated soils (Fig. 3), while for the fungal communities, the lowest diversity was observed in the fallow and control treatments.

Statistical analysis of Shannon indices indicated that there were no significant differences ($p \leq 0.05$) between N_2P_2+R, N_2P_2+FYM, and fallow treatments for bacterial communities (Table 2). A significant difference was however noted between these treatments and N_2P_2, control, MS, and FYM (Table 2). For the fungal communities, no significant differences ($p \leq 0.05$) were found between N_2P_2 +MS, N_2P_2+FYM, but significant differences were noted between fallow, control, MS, FYM, and N_2P_2.

Discussion

While the significance of the microbial community shifts following soil management practices remains to be recognized in the tropical regions, the results of this study indicate that the long-term combination of organic and inorganic amendments enriches the soil bacterial and fungal community and promotes diversity. Zhong et al. (2010) showed how long-term fertilization of organic manure (with or without NPK application) led to C utilization pattern shifts and increased soil microbial functional diversity. Meanwhile, Joergensen et al. (2010) showed that the long-term application of farmyard manure in combination with organic farming practices led to an increased accumulation of bacterial residues. Previous work in the same site in KARI-Kabete showed that use of manure, NP fertilizers,

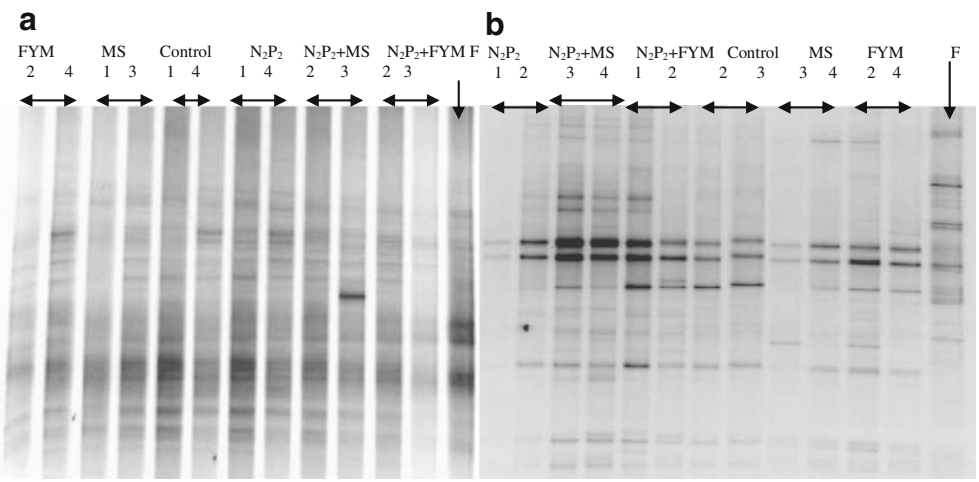

Fig. 1 DGGE profiles of 16S rRNA gene (**a**) and 28S rDNA gene (**b**) of soil samples from Kabete long-term experiment in 2007. *Numbers 1*, *2*, *3*, and *4* represent selected treatment replicates

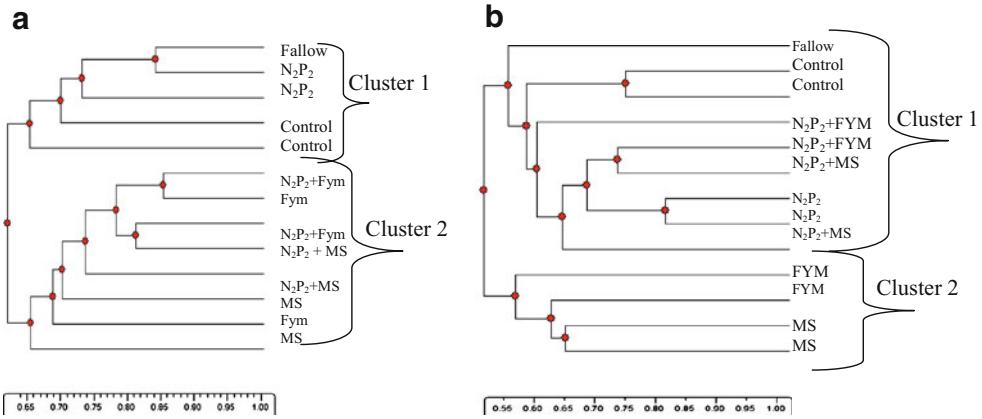

Fig. 2 Dendograms revealing relatedness of PCR-DGGE fingerprints from the gels in Fig. 2 **a** (bacterial) and **b** (fungal) clusters using Dice's similarity coefficient

and crop residue retention caused least organic C loss from the soil and raised the total soil N significantly compared to the control (Kapkiyai et al. 1998). Allison et al. (2007) found that N fertilization alone reduced fungal taxonomic richness and altered community structures, N deposition reducing significantly total fungal diversity in soils (mainly by reducing decomposer fungi). This has been confirmed by our results. Malý et al. (2009) showed that long-term mineral fertilization increases the proportion of *r*-strategists in soil. The authors assume that these results are probably due to increases in available P and rhizodepositions. Meanwhile, Kibunja et al. (2010) found that continuous application of chemical fertilizers in this site led to net loss of soil organic matter and a drop in soil pH explaining the decline in bacterial communities in this treatment. Our results also showed that bacterial community population was generally higher than that of the fungal community in all treatments. Zhong et al. (2010) suggested that soil bacteria were sensitive indicators of soil fertility while Lesueur et al. (unpublished data) suggested that indicator based on the fungal communities might not be suitable as microbial indicator for soil quality. On the other hand, appearance of less numerous but strong bands in the fungal DGGE profiles would substantiate the hypothesis that a limited number of dominant and ecologically well-adopted fungal types were present in the soil of the long-term experiment.

Shifts in bacterial community structure following adaptation of soil management practices have been reported by different studies (Diacono and Montemurro 2010). The distinct clustering of treatments with and without organic and inorganic inputs suggested a direct effect of organic and inorganic applications on total diversity of bacterial and fungal communities as the percentage similarity in cluster analysis was greater than 18% in the microbial communities. It is always difficult to distinguish between the direct and the indirect effects of an amendment on the behavior of soil microorganisms. However, Murphy et al. (2007) showed that organic material such as compost or manure is slowly decomposed in the soil, and the continuous release of nutrients can sustain the microbial biomass population for longer periods of time, compared with mineral fertilizers. Our results are in accordance with such study. As a general rule, the quantity and quality of organic material added to soils are the major factors in controlling the abundance of different microbial groups and the activity of microorganisms involved in nutrient cycling (Diacono and Montemurro 2010). Acosta-Martinez et al. (2008)

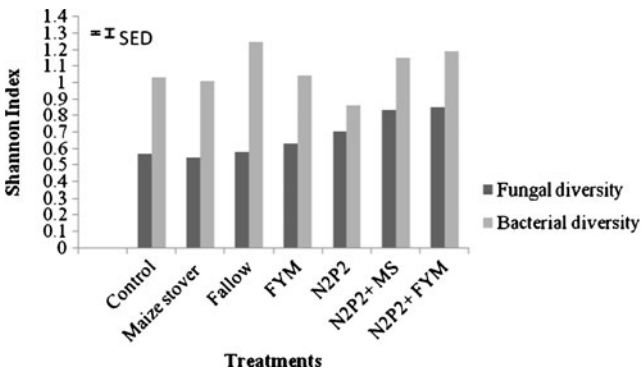

Fig. 3 Fungal and bacterial communities' diversity as affected by different treatments

Table 2 Means of Shannon–Weaver index for the Kabete long-term trial study on microbial diversity

Treatment	Fungal	Bacterial
MS	0.545 a	1.007 b
Control	0.565 a	1.030 b
Fallow	0.580 a	1.245 d
FYM	0.627 b	1.045 bc
N_2P_2	0.705 c	0.860 a
N_2P_2+MS	0.835 d	1.152 cd
N_2P_2+FYM	0.852 d	1.192 d

Means followed by same lower case letters are not significantly different from each other (Duncan multiple range test, $p<0.05$)

demonstrated that 30–50% C reduction in soil resulted in a community structure with lower fungal populations and lower enzyme activities compared with undisturbed pastures. Soil C content may be one of the key factors influencing soil microbial diversity. Considering that agricultural use of inorganic fertilizers unavoidably decreases microbial activity, Ge et al. (2010) recommend that combined use of organic manure with inorganic fertilizers should be considered based on the balance between crop demand and soil supply of available nutrients. This is totally in accordance with Manna et al. (2007) who showed that if a balance fertilizer, either alone or in combination with manure application, had a positive effect on crop yields in a cereal-based cropping system, application of balanced fertilizer with manure had a great impact on soil fertility improvement.

Conclusion

Considering the long-term effects of continuous application of farmyard manure, plant residues, and N and P fertilizers, we showed that the total diversity of bacterial and fungal communities was considerably affected by the input type. Combination of organic and inorganic resources increased soil bacterial and fungal diversity and resulted in a more even distribution than that in soil treated with inorganic fertilizers and in untreated control soils. These alterations were linked with the availability of organic sources in inorganically treated soils. Further investigations using sequencing should be undertaken to identify the specific communities affected by chemical fertilizations or combined use of organic and inorganic inputs. It will give us useful information about the soil functional diversity. Therefore, it may be relevant to test the effects of applications of other organic matter resources such as *Tithonia diversifolia*, *Senna spectabilis*, and *Calliandra calothyrsus* residues as they are easy to get locally and are commonly used by farmers for several purposes.

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