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Soil Amendments Mitigate GHGs and Affect the Functional Community Structure of Soil Micro Flora

Aradhana Mishra^{1*}, Richa Shukla¹, Madhuree Kumari¹, Ashmita Tandon¹, Poonam C. Singh¹, Sanjay Dwivedi¹, Ramakant Bajpai² and Vivek Pandey¹

¹ CSIR-National Botanical Research Institute, Rana Pratap Marg, Lucknow, 226001, India

² Department of Soil Science, Indira Gandhi Krishi Vishwavidyalaya, Krishak Nagar, Raipur, 492012, India

E-mail: mishramyco@yahoo.com

Abstract: This study was conducted to elucidate effects of long-term fertilization practices on plant growth, GHGs emission (methane and carbon dioxide), and microbial diversity in rice field soil. The rice field selected for experiments represented balanced and imbalanced fertilization practices. Among five treatments [Control (SC), 100% urea (SU), NPK-Zn (NPK), 50% NPK-Green manure- *biofertilizer* (NGMBF) and biofertilizer (BF)], a significant increase in root growth parameters was observed in NGMBF treatments which was closely followed by BF treatments. Similarly, further analysis of yielding attributes also exhibits the same pattern of growth where an increase in different yielding parameters was evident in NGMBF treatment. Soil incubation studies showed highest CH₄ and CO₂ emission on day3 with NPK treated soil in submerged condition whereas it was least in BF treated soil. Dehydrogenase activity in soil was also analyzed and it was recorded highest in BF treated soil. The structure of the microbial community in different soil amendments was analyzed through the sole-carbon-source utilization profiles using ECO Biolog plates. As compared to SC, maximum changes in the community structure was recorded in the BF treatment which was followed by NGMBF, SU, and NPK. These findings suggest that sustainable management practices like amendment with BF in the soil are needed to mitigate CH₄ and CO₂ emission from rice cultivation.

Abbreviations: GHG: Green house gasses; CLPP: Community Level Phylogenetic Profiling; BF: Bio-fertilizer

Keywords: Climate change, fertilizer, methane emission, soil microbial diversity

INTRODUCTION

During the periods of green revolution, the indiscriminate use of chemical fertilizers and pesticides, to improve the agricultural productivity has caused detrimental impact on environment. The prolong use of chemical fertilizer destroy critical soil microbes, contribute to desertification and global climate change, and saturate farmlands with toxic pesticides, herbicides and fertilizers that then migrate into ground water, rivers, lakes and oceans.

India is one of the world's largest producers of rice, accounting for 20% of all world rice production and occupies 42.24 m. hr. in the Indian subcontinent, the largest in Asia (Cheng *et al.* 2014). Apart from its reputation in fulfilling the nutrition requirement of human as food, rice fields had a reputation as one of the major GHG sources in the agricultural sector as it emits CH₄ and CO₂ either in aerobic or anaerobic condition depending on soil and water content (Liu *et al.* 2014; Ogle *et al.* 2014). On an average, the agricultural sector emits about 15-20% of total global anthropogenic CH₄ emission (Mishra *et al.* 2012). CH₄ and CO₂ emission is affected by fertilizer management and has been studied by various workers (Majumdar 2003; Tang *et al.* 2015). With an increase in world rice harvested area, it is expected that the emission of CH₄ would also increase. Hence, there is an urgent need of mitigation strategies.

The richness, abundance, and activity of microbial communities are influenced by chemical properties of soil such as organic matter content and nutrient availability (Armstrong *et al.* 2014; Wang *et al.* 2014). Farmyard manure and inorganic fertilizers (NPK) have been reported to have a positive effect on the microbial activities (Liang *et al.* 2012). The majority of the soil microorganisms are heterotrophs, and utilization of carbon sources by microbial communities is the driving force for soil functioning. Using this driving force, Biolog technique has been widely employed to differentiate bacterial

community-level structure from different habitats (Mishra *et al.* 2011).

There are many microorganisms which are reported to enhance plant growth promotion and are used widely in agriculture. Among them, the species of genus *Trichoderma* are known for their biocontrol abilities in improving plant growth worldwide (Cornejo *et al.* 2016; Mahfooz *et al.* 2016). Apart from their biocontrol abilities, some species of *Trichoderma* are known to accelerate the process of rice straw decomposition and produce more soil-available nutrients such as labile organic matter and inorganic N/P/K (Tripathi *et al.* 2015; Yadav *et al.* 2009); hence reducing the intake of chemical fertilizers. Hence, the present study was conducted to (i) to study the effect of different fertilization practices on CH₄ and CO₂ emissions and (ii) to examine the microbial community level phylogenetic profiling (CLPP) using substrate utilization pattern.

MATERIALS AND METHODS

Field site, treatments and soil sampling procedures

A long-term continuous field trial was established at the CSIR-National Botanical Research Institute, Banthra, Lucknow, Uttar Pradesh, India (26°42'17"N, 80°49'53"E). Lucknow has a warm humid subtropical climate with cold-dry winters from December to February along with dry-hot summers from April to June. It receives an average rainfall of 896.2 mm (35.28 inches) from the southwest monsoon winds. Most of the rainfall occurs between mid-June and mid of September months. Summers are extremely hot with the temperature rising up to 48°C (113°F). Soil type was classified as sandy loam. Total field size was 28m x 20.10m whereas plots were 5m x 4m in size. Each treatment was replicated thrice. During the present study, five treatments consisted of SC- Control, SU- Farmers Practice (Urea only- 120 kg ha⁻¹), NPK- NPK Zn (120:80:60:20 kg ha⁻¹), NGMBF-NPKZn (120:80:60:20 kg ha⁻¹) + *Sesbania*

green manuring + FYM 10 t ha⁻¹ + Biofertilizer, and BF- Biofertilizer. For green manure, *Sesbania* was grown in the field for one month. Later, it was plowed and mulched in the field and left for 45 days to decompose. Liquid suspension (10g/liter) of *T. reesei* (MTCC5659) was used to inoculate rice seedlings for 30 min before transplantation. Commonly cultivated rice variety NDR-359 was grown and three seedlings were transplanted per hill after 30 days of the nursery by dibbling procedure while keeping a distance of 20×10 cm (Singh *et al.* 1999) and later harvested at maturity. The emerging weeds were controlled manually and also with the application of weedicide during cropping period. The plots were submerged in water with a brief aeration to create aerobic condition for *Trichoderma* through ploughing. Soil samples (four) from each plot were collected at a depth of 15 cm after harvesting and were pooled to make a composite sample. Altogether, 20 soil samples (four samples for each treatment) were collected in sealed polybags for physico-chemical, microbiological, and biochemical studies.

Estimation of CH₄ and CO₂ Emission

To observe the effect of moisture on CH₄ and CO₂ emission, soil was adjusted at partially (60%) and fully submerged (100%) condition and incubated in airproof containers. Emitted CH₄ and CO₂ was collected from the airproof containers on day3, day20, day40, day60 and day90 and quantified. The gas (1.0 ml) in the head space was withdrawn and injected into a glass column (0.26 mm-2.0 m) which was packed with Porapak Q (80/100 mesh) on a Thermo Trace Gas Chromatograph (600, India) equipped with a thermal conductivity detector and a flame ionization detector. Column temperature was set at 70°C, injection and detector temperatures were set at 80°C and 150°C. Methane concentration was calculated from a head space volume. Collection and analysis of CO₂ was conducted by following a procedure described by Anderson (1982). Analysis of variance (ANOVA) and Duncan's multiple range

test (DMRT) were performed to determine the significant difference between treatments by using SPSS 17.0 software.

Community-level physiological profiles

The patterns of potential carbon source utilization by soil microbial communities under different fertilizer treatments were assessed by Biolog Eco and MT plates (Biolog, Inc., Hayward, CA, USA) as described earlier (Campbell *et al.* 1997). A final dilution up to 10⁻³ of 1.0 g of individual rice rhizosphere soil was prepared and 150 µl of the sample was inoculated at 30°C in each well of Biolog plates. The carbon utilization rate is measured by the reduction of tetrazolium (redox indicator dye), which alters from colorless to purple. Data were recorded during the study period (day 1-15) at 590 nm as described earlier (Mishra and Nautiyal 2009). Microbial activity was expressed and determined as average well color development (AWCD) in each micro-plate (Garland 1996). Microbial evenness indexes and diversity were calculated as per Mishra and Nautiyal (2009). Principal component analysis (PCA) was done on data divided by AWCD (Garland and Mills 1991).

Estimation of dehydrogenase activity

Dehydrogenase activity was measured spectrophotometrically using characteristics of TTC (2,3,5-triphenyltetrazolium chloride) reduction to TPF (triphenylformazan) as earlier described (Casida *et al.* 1964). Briefly, TTC was prepared in 100mM of Tris-HCl (pH 7.7). Thereafter, 5g of soil was mixed with 5ml of TTC solution and kept at 28°C for 24 h in the dark condition. After incubation, 40 ml of acetone was added into each bottle and it was further incubated for 24 h in dark at regular shaking. Later, the supernatant was taken, centrifuged and its absorbance was measured at 540nm. Tris-HCl (5 ml) without TTC served as control. Dehydrogenase activity was expressed in micrograms of formazan per gram of sample (µg TPF/g soil/day).

Fourier transform infrared spectroscopy of humic acid (HA) and fulvic acid (FA)

Extraction of humic acid and fulvic acid from soil was carried out to fractionate the status of passive pool of organic carbon by using the method described by Naidja et al., (2002). 20 gram of air-dried soil was passed through a 2-mm sieve and shaken with 200 ml of 0.5 N NaOH on a rotary shaker for 12 hr at 30°C. The supernatant was then separated from the insoluble residue by centrifugation at 10,000 rpm for 10 min, acidified with 3N HCl to pH 1.5 and allowed to coagulate for 16 hr. The new yellow supernatant (FA) was then separated from the residue (a dark brown HA gel) by centrifugation at 10, 000 rpm for 15 min. HA was washed 3 times with deionized distilled water to remove residual HCl. FA was adsorbed on a column (H1-Amberlite IR-120 to remove Na cations) and it was freeze-dried with HA and FA fractions. Fourier transform infrared spectroscopy analysis of soil samples were done with Perkin Elmer Spectrum RX1. To obtain these spectra, 0.5 mg of the freeze-dried humic and fulvic acid fractions was mixed with 80 mg KBr and grounded in an agate mortar. The KBr-technique was applied for absorption spectra of humic and fulvic acid in a range of wave numbers between 4000 and 450 cm⁻¹.

DATA ANALYSIS

- (i) The net emission of CH₄ from soil incubation studies was calculated by the following equation:

Emission of CH₄ (μ mole g⁻¹ of soil) = factor x area of sample peak

Where

$$\text{Factor} = \frac{\text{Headspace diameter of Syringe}}{\text{Area of CH}_4 \text{ standard peak} \times \text{weight of incubated soil} \times \text{injected volume of air sample}}$$

Standard of CH₄ was calibrated against CH₄ obtained from CSIR-NPL (National Physical Laboratory, New Delhi, India).

- (ii) The amount of CO₂ produced under laboratory incubation is expressed as μ mole g⁻¹ of CO₂ based on the following equation-

$$\text{Emission of CO}_2 \text{ (mg g}^{-1} \text{ of soil)} = \frac{(B-V) \times N \times E}{\text{Dry weight of soil}}$$

Where,

B = volume in ml of acid needed to titrate the NaOH in the control bottle

V = Volume in ml of acid needed to titrate the NaOH in the sample

N = Normality of the acid

E = Equivalent weight of CO₂

Two-way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) were performed for gas emissions and CLPP studies to determine difference between the treatments using Statistical Package for Social Sciences (SPSS) software (ver. 17, SPSS, Inc, Chicago, IL, USA).

RESULTS

Effect of different fertilizer treatment on different root attributes

Roots play the primary role in the uptake and translocation of nutrients and balance the ions and water balance in the plant. Root development is directly related to yield and quality of the crop with its tolerant to different stress like salinity, sodicity and water stress etc. Treatment with NGMBF showed a significant increase in the fresh root weight (7.4g) which was equally followed by NPK and BF (4.4g) treatment, minimum root weight was observed in SC (Table 1). Similarly maximum dry root weight was observed in NGMBF treatments (15.4g) which was followed by NPK (9.4 g) and BF (8.5g) treatments. The highest root length was also observed in NGMBF treatments (18.1cm). NPK treatment roots grown to the second maximum length (16.2cm) which was followed by BF treated

Table 1
Effect of nutrient management practices on different parameters of roots of the rice

Treatments	Fresh root weight (g)	Root Volume (cm ³ plant ⁻¹)	Root dry weight (g plant ⁻¹)	Root Length (cm plant ⁻¹)	Specific root length
SC	2.0	5.3	4.56	13.9	3.18
SU	3.3	7.7	7.19*	14.4	2.04
NPK	4.4*	11.4*	9.46*	16.2	1.86
NGMBF	7.4*	12.0*	15.54*	18.1*	1.17
BF	4.4	9.4*	8.59*	15.0	1.79
SE (m) ±	0.65	1.9	1.19	1.73	0.44
CD (P = 0.05)	1.41	4.04	2.59	3.77	0.96

roots (15.0 cm). Root volume (cm³/plant) was largest for NGMBF treated roots (12).

Effect of different fertilizer treatment on yield and yielding attributes in paddy

A significant increase plant height was noted in plants treated with NGMBF (112cm), which was followed by NPK (111.2 cm) and SU (110.47 cm). Similarly, no. of tillers (6.5), no. of spikelets (12.5) along with grain (60.2 q ha⁻¹) and straw yield (113.6 q ha⁻¹) was maximum in NGMBF treatments (Table 2). Panicle length was approximately same for NGMBF and BF treatments (28.3 cm) whereas control plants showed the least (3.4cm). Treatment with BF showed the second highest yielding attributes in no. of tillers (4.8),

no. of spikelets (12.3), grain yield (56.7). An interesting observation from the data is the second highest grain yield was recorded in BF treatment (56.6 q ha⁻¹).

CH₄ emission after soil incubation

CH₄ emission from soil as a result of different fertilizer treatments showed a different pattern. Evolution of CH₄ from rice field soil was highest (0.39 μ mole/g of soil) at day3 of incubation with NPK treatment in fully submerged condition as well as in the partially submerged condition (0.36 μ mole/g of soil) in the same treatment. The emission of CH₄ was decreased in BF (16.71%) when compared with NPK (27.10%) in partially and fully submerged condition on day 3 of soil incubation respectively.

Table 2
Effect of nutrient management practices on yield and yield attributes of the rice crop

Treatments	Plant height (cm)	Number of tillers	Length of panicles (cm)	Number of spikelets	Per plant weight (gm)		Grain yield (q ha ⁻¹)	Straw yield (q ha ⁻¹)
					Grain	Straw		
SC	92.0	3.4	23.8	9.5	11.4	20.0	35.89	85.93
SU	110.0*	3.6	27.3*	12.3*	15.5	29.3	45.59*	101.79*
NPK	111.2*	4.7*	27.5*	12.3*	19.8*	52.7*	55.31*	111.27*
NGMBF	112.0*	6.5*	28.3*	12.5*	27.1*	50.5*	60.22*	113.63*
BF	109.3*	4.8*	28.0*	12.3*	20.0*	38.6*	56.67*	93.86
SE (m) ±	2.69	0.51	1.60	1.12	2.53	6.70	1.22	4.00
CD (P=0.05)	5.86	1.11	3.48	2.44	5.52	14.6	2.66	8.71

The effect of different fertilizer treatments on the emission of methane was estimated till day90 in both partially and fully submerged conditions. The highest emission of methane was recorded at day3 in both partially and fully submerged condition with NPK treatments. The second highest emission was noted in SU treatment on the same day in both conditions. The least emission of methane was recorded with BF treatment. CH₄ emission significantly increased in NPK > SU > NGMBF > SC > BF treatments in both partial as well as fully submerged conditions (Fig. 1a and 1b).

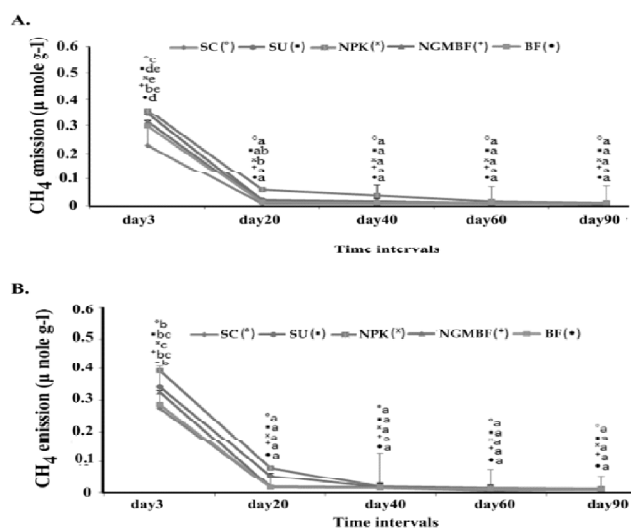


Figure 1: Effect of different fertilization practices on CH₄ emission from rice field soil incubated in a) partially submerged condition. b. submerged condition

CO₂ emission after soil incubation

The cumulative CO₂ emission showed significant correlations with incubation time for both partially and fully submerged moisture levels (Fig. 2a-b). The emission of CO₂ increased under submerged condition and reached the maximum value of 40.43 µ mole/g of soil in NPK at day 90 of incubation. Our results indicated that CO₂ emission gradually increased with incubation period. Lowest CO₂ emission (1.0 µ mole/g of soil) in BF in partially submerged condition was recorded at day 3 of incubation. Throughout incubation study, the highest

CO₂ emission was recorded from NPK in submerged condition. Significant difference was observed in CO₂ emission from NPK and BF in both moisture conditions. CO₂ emission was decreased by 67.81 and 19.92% in BF than NPK in 60% moisture and submerged condition of 90th day of soil incubation respectively. There was no significant difference in the CO₂ emission from the soils between SU and NPK. Results revealed that mixing of green manure and biofertilizer with soil along with moisture levels significantly affected CH₄ and CO₂ emission.

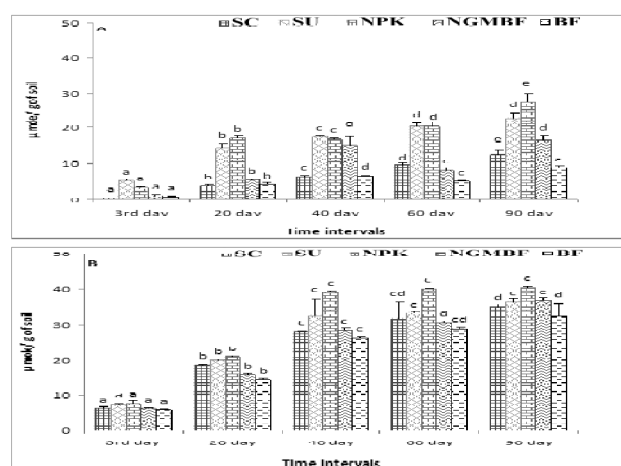


Figure 2. Effect of different fertilization practices on CO₂ emission from rice field soil incubation in a. partially submerged condition b. submerged condition

Community-level physiological profiles of microbes under different soil treatments

Microbial community structure in soil samples was assessed by using Biolog Eco and MT plates to monitor changes in microbial diversity. Principal component analysis (PCA) of carbon source utilization pattern showed close clustering among treatments SU, NPK and NGMBF, whereas control SC and BF were placed separately from each other and form the cluster BF at 78.58 and 8.09% on factor 1 and 2 axis (Fig. 3). A significant difference in diversity was observed using Shannon diversity Index and evenness, Simpson diversity Index, McIntosh diversity Index and McIntosh evenness. There were significant differences among SU, NGMBF and BF

Table 3
Diversity and Evenness index calculated based on substrate utilization by rice field soil microflora

Diversity index	SC	SU	NPK	NGMBF	BF
Shannon Diversity Index	4.445±0.018 ^b	4.377±0.02 ^a	4.457±0.016 ^b	4.441±0.023 ^b	4.547±0.007 ^c
Shannon Evenness Index	0.960±0.004 ^{ab}	0.952±0.001 ^a	0.965±0.002 ^b	0.961±0.005 ^{ab}	0.984±0.002 ^c
McIntosh Diversity Index	0.984±0.001 ^b	0.979±0.002 ^a	0.984±0.001 ^b	0.983±0.001 ^{ab}	0.992±0.000 ^c
Simpson Diversity Index	0.997±0.000 ^b	0.996±0.000 ^a	0.997±0.000 ^b	0.997±0.000 ^{ab}	0.998±0.000 ^c
McIntosh Evenness index	0.986±0.002 ^{ab}	0.982±0.001 ^a	0.987±0.001 ^b	0.985±0.002 ^{ab}	0.994±0.000 ^c

Different letters showing significant difference at $p = 0.05$ using Waller Duncan test

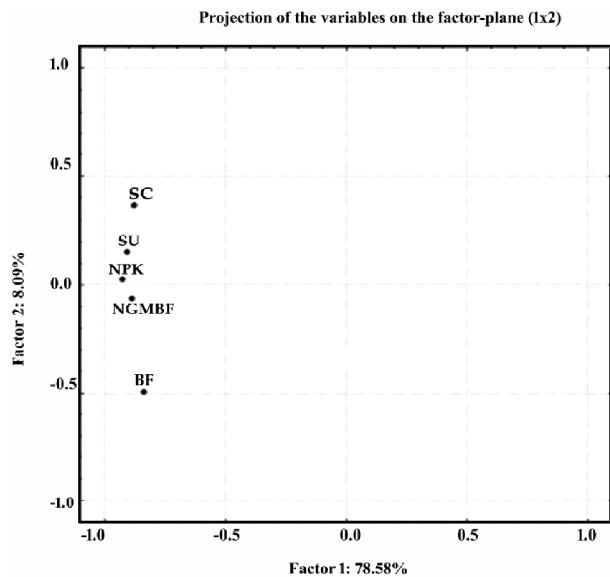


Figure 3: Principal Component analysis based on carbon source utilization by microflora of rice field soils

on basis of Shannon diversity Index and evenness, Simpson diversity Index, McIntosh diversity Index and McIntosh evenness (Table 3) whereas no significant differences were observed in SC and NPK on the basis of all the diversity indices.

The interpretation of clustering obtained from Principal component analysis based on carbon source utilization pattern by the microflora of rice field soil indicated that amendment with different fertilizers causes significant changes in the microbial community. As compared with SC, the maximum change was recorded in BF which was followed by NGMBF, SU and NPK respectively. In another

experiment, substrate utilization pattern was observed in Biolog MT plates, NGMBF and BF efficiently utilized urea, lignin, pectin, chitin cellulose, phytic acid, ferulic acid, sodium meta silicate and silicic acid which was followed by SU, NPK and SC (Fig. S1). It was also observed that polymers as carbon substrates were efficiently utilized by SU followed by BF when compared with SC (Fig. S2).

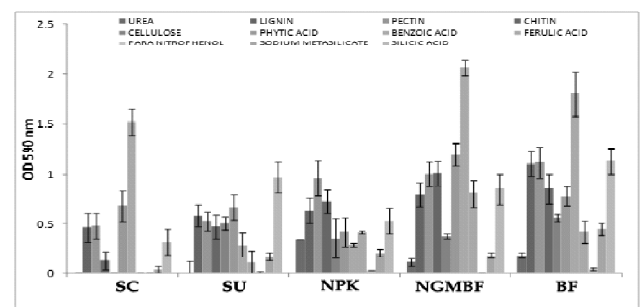
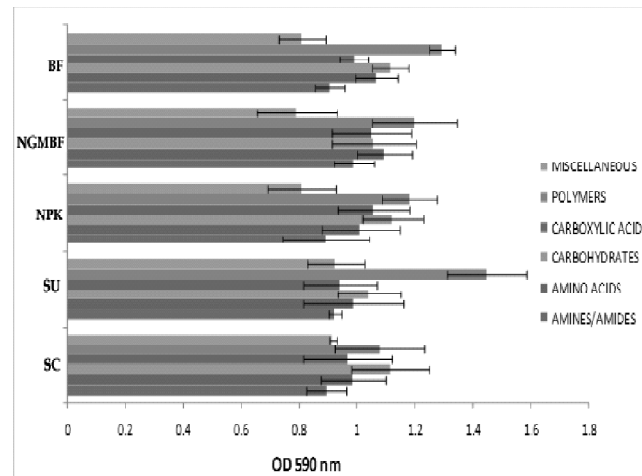


Figure 4: Dehydrogenase activity in soil with different fertilizer treatment

Soil dehydrogenase activity

The soil dehydrogenase activity provides correlative information on the biological and microbial activity in soil (Prakash *et al.* 2010). Dehydrogenase activity per gram soil was used to evaluate microbial metabolic activity. Dehydrogenase activity in the treatments under combined fertilization was significantly higher when compared to the control. Among all, treatments with BF exhibited highest dehydrogenase activity when compared to the remaining fertilizer treatments (Fig. 4).

Fourier transform infrared spectroscopy of Humic acid and Fulvic acid

Humic acid and fulvic acid are the degradation products of soil organic matter which enhance plant growth (Oyewole 2012). The relative intensities of FTIR absorption bands of the HAs of the five treatments SC (Spectrum No. 1192), SU (Spectrum No. 1189), NPK (Spectrum No. 1190), NGMBF (Spectrum No. 1191), and BF (Spectrum No. 1193) did not differ much in wave number range, 4000-2000 cm^{-1} (Fig. 5). The qualitative information of the functional groups of the HAs was elucidated by assigning the peaks, bands, and shoulders of FTIR spectra. FTIR spectra of HAs extracted from the soil of the five treatments showed some marked differences in the wave number range from 450 to 2000 cm^{-1} . FTIR spectra of SC, NPK, NGMBF, and BF treatments showed an apparent peak at 673 cm^{-1} (C-Cl stretching of acid chlorides), but SU did not produce any peak at the same region, indicating the absence of acid chlorides in HAs in imbalanced fertilized soil (Fig. 4). Band at 1009 cm^{-1} which represent carboxylic acids, esters, ethers and C-O stretch of alcohol was present only in NPK. A wide and specific spectrum observed at 2143 cm^{-1} with $\text{C}\equiv\text{C}$ stretching of alkynes was found in BF only. This functional group of humic acid enhanced the fertility of the soil.

Medium spectra at 1219 cm^{-1} with C-N stretch bonding of aliphatic amines, a medium absorption

band at 1634-1637 cm^{-1} with N-H bend of 1° amine group, a sharp and broad absorption band at 3436 cm^{-1} with O-H stretching and H-bonded bond of alcohols and phenols are present in all rice soil treatments. This showed that these functional groups of humic acids are common in rice soil irrespective of the treatment.

Fulvic acid is nature's way of "chelating" metallic minerals, turning them into readily absorbable bio-available forms that hold nutrients of the plant for long. FTIR absorption bands of the FA from all five treatments represents a strong spectra at 673-669 cm^{-1} with stretching of acid chlorides, strong bond of aromatics at 772 cm^{-1} , a medium C-N stretching of aliphatic amines at 1219 cm^{-1} , a medium N-H bending of 1° amine group at 1634-1637 cm^{-1} , sharp and broad O-H stretching and H-bonded bonding of alcohols and phenols. These groups were commonly present in all treatments (Spectrum No. SC 1198; SU 1195; NPK 1196; NGMBF 1197; BF 1194) of soil.

Medium spectra at 3019-3020 cm^{-1} wave number was present only in NGMBF (Spectrum No. 1197), and BF (Spectrum No. 1194) treatments with $\text{C}=\text{H}$ stretching of alkenes. Alkyne groups were present only in NGMBF and BF treatments. It shows that biofertilizer (BF) helps to maintain organic carbon pool. The spectra of NGMBF and BF were similar, both in shape and relative intensities of the peaks, indicating more or less similar composition. In the case of both treatments, peak at 3019-3020 cm^{-1} and 1523-1519 cm^{-1} absorption spectra region of the IR-spectrum differed significantly from the other treatments with N-O asymmetric stretch of nitro compounds. A wide band of the spectrum at 2143 cm^{-1} was detected in NGMBF with O-H stretching of carboxylic acids having high exchange capacity of cations and anions. A sharp band at 3685 cm^{-1} which belongs to the hydroxyl (Al-OH) group was observed only in BF.

DISCUSSION

Rice being the most important cereal crop in the world needs constant attention to increase its potential in fulfilling the growing human need for food. To achieve higher grain yield, indiscriminate use of nitrogen, phosphorus and potassium fertilizers has been witnessed in the last five decades. We performed an experiment where the effect of different fertilizer practices was recorded in the different growth parameters of rice plant. Among the five treatments, maximum growth was recorded in NGMBF treatment in the different root parameters. Similarly a marked increase in yielding parameters was also evident in NGMBF treatment. Biofertilizer plays a significant role in crop cultivation with reduced the chemical fertilizer use. In a similar experiment, three *Trichoderma*-enriched biofertilizers for mustard and tomato cultivation at field condition. Sole application of biofertilizers didn't show remarkable contribution but all *Trichoderma* enriched biofertilizers when supplemented with N fertilizer significantly boosted up the growth and yield of rice (Haque *et al.* 2012).

Apart from fulfilling human needs, rice fields also acts as an important source of the atmospheric CH₄ and CO₂. Many researchers are involved in measurements of CH₄ emissions from rice fields (Wang *et al.* 2000), mechanisms for CH₄ production, oxidation and transport, and factors influencing these processes (Yao *et al.* 1999), along with estimate of local and global CH₄ emissions, and development of mitigation option (Cai *et al.*, 2003). The emission of CH₄ resulting from microbial activity in rice field soil is dependent on the soil moisture content. Higher moisture concentration cuts off O₂ supply from the atmosphere, resulting in anaerobic fermentation by methanogens and releases CH₄ from submerged soils to the atmosphere through roots and stems of rice plants (Sass *et al.* 2002). Higher emission rate of CH₄ on early days and less emission of CH₄ after longer incubation is dependent upon anaerobic microenvironments, consumption and oxidation in

aerobic microenvironments, both of which can be found side by side in submerged condition of rice field soils (Ding *et al.* 2015). Rising CH₄ emission during initial days of incubation was due to the availability of plenty of substrate in the form of rice root exudates having acetate, which was gradually utilized by anaerobic microbes. Later on, anaerobic activity was decreased due to deficiency of substrate which resulted in less emission of CH₄. It has been reported that soil incubation period effects the emission of CH₄ that was suppressed after 4 days (Yao *et al.* 1999).

The anaerobic degradation of organic matter in soil involves hydrolysis of polymers, acid formation from an organic compound, acetate formation by hydrolytic microorganisms and fermentative bacteria. CH₄ formation takes place by methanogenesis using substrates as H₂/CO₂, acetate, simple methylated compounds or alcohols (Zhao *et al.* 2014). The species of *Trichoderma* are having a reputation of being an iron reducing agent. Applying *Trichoderma* to soil could increase levels of Fe²⁺ and siderophores, as well as increase Fe²⁺ and Fe³⁺ chelate reductase activity (Minderlein and Blodau 2010). When used as bio-fertilizer, they provided adequate amount of iron to soil which was derived from soil organic matter and acted as a bridge to reach iron from organic matters to soil by releasing Fe (III) ions which acts as an alternate carbon acceptor in the rice rhizosphere. The presence of alternative electron acceptors is reported to mitigate CH₄ production (Nautiyal *et al.* 2010). ¹³CH₄ concentrations were largely suppressed in the presence of FER (ferrihydrite), which served as electron acceptor (Ding *et al.* 2015). The absence of electron acceptors might be the reason for higher emission of CH₄ in chemically fertilizer treated soil. As reported in earlier study, CH₄ is produced by sequential reduction of O₂, nitrate, manganese, iron and sulphate, which serve as electron acceptors for oxidation of organic matter during soil incubation (Nautiyal *et al.* 2010). The carbon liberated by decomposition of organic

matter is utilized by the BC, hence not enough available for the methanogens to utilize it as a substrate.

Decomposition of organic carbon is responsible for the emission of CO₂ and is directly proportional to the amount of soil carbon biochemical composition and rate of microbial oxidation. Highest production of CO₂ in NPK in submerged condition reflects the high decomposition rate in higher moisture condition during soil incubation. Increasing soil moisture affects soil respiration and raise CO₂ evolution. Applying water might release cellular material as a result of water stress or release of organic material via physical processes (Xiang et al., 2008). Higher moisture stimulates microbial activity and elevates emissions of CO₂ (Ryan et al., 2015).

Fig. 2a-b shows CO₂ evolution was increased with increasing longevity of incubation period. Low CO₂ evolution in day3 and high CO₂ evolution after day90 incubation of soil might be due to persistence of carbon for longer, because of low decomposition which favors protracted bacterial activity. Microbial activity in soil increased in the presence of higher moisture and chemical fertilizer, which induced fast decomposition, resulting in high CO₂ production. The cumulative CO₂-C evolution was increased with the increase of time (Hossain and Puteh, 2013). CO₂ emission increased with application of N fertilizer, probably due to urea hydrolysis (Zwieten et al., 2010). Microbial activity is often stimulated by the use of inorganic N fertilizer which might enhance the emission of CO₂ (Lopes et al., 2011).

Microbial communities have great potential for temporal or spatial change, and it represents a powerful tool for understanding community dynamics in both basic and applied ecological contexts. Community-level physiological profiling has been used extensively to study the microbial communities in various habitats (Dong et al. 2014). The functional diversity of microorganisms was

defined by the substrates used for energy metabolism. Our results indicated that microbial community under BF treatment had a higher efficiency of carbon metabolism, which decreased under unbalanced chemical fertilization condition. In addition, the metabolic efficiency is depending on the carbon conversion ability. The amended bio-fertilizer in the form of *T. reesei* is a C sequester (Mishra and Nautiyal, 2009) which helped in retaining soil carbon while the other fertilizers stimulate C losses as CO₂ through respiration. Application of *T. reesei* as BF tended to result in an overall higher substrate utilization activity, diversity and richness, whereas soil treated with chemical fertilizer tended to have lower substrate richness and diversity. SU, NPK and NGMBF treatments altered the pattern of potential carbon utilization and increased the Shannon and Simpson index values. Thus, when compared to BF and chemical fertilizer treatments, BF treatment showed higher values of AWCD, suggesting that treatments applying organic fertilizer had significantly increased soil biological activity and promoted a higher microbial number by providing different carbon resources. The carbon utilization richness in BF treatment was higher, compared to the control and the chemical fertilizer treated soil. We observed a collapse of microbial functional diversity in SU, NPK and NGMBF; however, SC and BF soil gave different pattern in PCA component (Fig. 3). Microbial activities and diversity are significantly reduced in the chemical fertilizer-managed soil. Organic manure has a significant impact on the microbial activity, compared with the mineral fertilizers it was shown in the principal component analysis (Dong et al. 2014).

We further analysed the dehydrogenase activity of soil microorganisms which is an indicator of biological activity in soil (Stevenson 1959). When compared with other chemical fertilizer treatment and control, we observed a higher dehydrogenase activity in BF treatments. The higher dehydrogenase activity in BF treatments could be attributed to the

fact that dehydrogenase generally promotes mineralization of organic matter (Prakash *et al.* 2010). The release of excess carbon may be utilized by the biocontrol and hence unavailable to the methanogens. In view of the rising levels of methane due to rice cultivation, the above study has demonstrated that amendments with BF have potential to significantly reduce the emission of CH₄ from soil. Our findings were further supported by higher soil dehydrogenase activity and principle component analysis of carbon utilization pattern which showed maximum changes in the microbial community structure in soil amended with BF.

Nonappearance of acid chlorides functional group in SU soil sample indicated imbalance chemical fertilizer effect. Similar observation was reported by Naidja *et al.* (2002) during the study of diffraction analyses of organic matter in humin, humic acid, and fulvic acid fractions in soil. Application of organic amendments in soil not only increases the organic carbon in its different fractions but also has a series of effects on microbial proliferation and activity (Bastida *et al.* 2008). Probably continuous application of only N might have possibly dematerialized the acid chlorides functional groups. It has long been debated about the approach to study soil organic matter. The occurrence of humic substances in soil has been studied by many researchers. However, recent reports contradicts the occurrence of these substances (Dungait *et al.* 2012; Lehmann and Kleber 2015; Schmidt *et al.* 2011). Despite the new contradictory reports on occurrence of humic substances, we stick to the old concept to study the soil. Few reports suggest that humic substances are made by the action of microorganisms (Lehmann and Kleber 2015) however it is important to note that these substances exist in natural conditions for a long and plays a major role in maintaining the fertility of soil. Commercial HA are currently chemically extracted from peat and coal (Motta and Santana 2014). Since humic acid and fulvic acids are the representative of passive pool of

soil, we studied the effect of different fertilizer treatment using FTIR. The FTIR spectra of BF showed additional functional groups supplementary to a basic backbone structure, signifying a more versatile compound. Distinct bands were observed in the FTIR at 1534, 1458, 1419, and 1397 cm⁻¹ in the biofertilizer treatment spectra but were weaker in the other spectra. Intermediate absorption bands were clearly visible at 2921 and 2850 cm⁻¹ due to valent vibrations of side chain CH₃ and CH₂ groups in HAs molecules. Band of carboxylic acids, esters, ethers and C-O stretch of alcohol in NPK only, clearly revealed the effect of chemical fertilizer on HAs of soil (Preethi *et al.* 2013). Chemical treatments caused increased concentrations of ester, ether, phenolic, alcoholic, and carbohydrate functional groups in HAs compared with Compost and peat + 33% N treatments in soil (Chien *et al.* 2006). Absorption spectra obtained with N-O asymmetric stretch of nitro compounds in NGMBF and BF might be attributed to the presence of GM+ or balance amount of N surroundings provided by either chemically or through *T. reesei*.

A wide band of the spectrum at 2143 cm⁻¹ was detected in the fulvic acid analysis in NGMBF with O-H stretching of carboxylic acids having high exchange capacity of cations and anions due to balanced chemical fertilizer amendment. FTIR spectroscopy of fulvic acid explained bridging of OH groups in BF. This group associates the micro and macronutrients of soil. The interaction of hydroxyl groups and the hydrogen bonding with the oxygen takes place in the BF that reflected the existence of hydroxyl (Al-OH) group and Cu-OH hydroxyl groups. The same pattern of hydroxyl groups were found on the soil surface during soil layer analysis (Frost and van der Gaast 1997), where Michaelian (Michaelian 1985) observed that the band at 3685 cm⁻¹ was due to uncoupled CuOH hydroxyl groups. these findings suggest that sustainable management practices like amendments with *T. reesei* as biofertilizer in soil may acts as a substitute of

chemical fertilizers to mitigate CH₄ production while maintaining the crop productivity.

CONCLUSIONS

In view of the rising levels of green house gases due to rice cultivation, the above study has demonstrated that soil amendments with *T. reesei* had a potential to significantly reduce the emission of methane and carbon dioxide. Our findings were further supported by higher soil dehydrogenase activity and principle component analysis of carbon utilization pattern which showed maximum changes in the microbial community structure in soil amended with *T. reesei*. Coupling of macro and micronutrients groups due to the presence of humic and fluvic acid ease the availability of nutrition as a result of *Trichoderma* treatment. These findings suggest that sustainable management practices like *T. reesei* as biofertilizer in soil may acts as a substitute of chemical fertilizers to mitigate the methane and carbon dioxide production while maintaining the crop productivity.

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