# Effect of organic matter amendment on the rhizosphere microbial community and root-infecting pathogens of aerobic rice variety Apo

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The effect of organic matter (OM) amendment on *Meloidogyne graminicola* and *Pythium* sp. infection of aerobic rice was determined in field experiments conducted during the 2009–2010 dry seasons. OM was added as *in situ*-composted rice straw. *M. graminicola* occurrence was unaffected but percent root galling was reduced. *Pythium* occurrence was lower in treated than in control plots. Microbial diversity was higher in treated plots during active plant growth. This increase in microbial diversity correlated with the observed decrease in disease symptoms. OM amendment is proposed as an alternative way of managing *M. graminicola* and *Pythium* infection in aerobic rice fields.

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**KEYWORDS**

aerobic rice, *Pythium*, root knot nematode, soil sickness

## INTRODUCTION

Water is important for socio-economic development as well as for maintaining healthy ecosystems. Unfortunately, 1.2 billion or approximately one-fifth of the world's population are currently experiencing physical water scarcity and even more are approaching this situation as many face economic water shortage (UN-Water 2007). Furthermore, climate change is estimated to account for about 20% of the expected increase in global water scarcity (World Water Assessment Programme 2009), thus aggravating current trends. Abiotic environmental stresses such as water scarcity are major constraints to global food security as agriculture accounts for more than 70% of the world's total water use (UN-Water 2007). Rice production is subject to these environmental changes and reduced availability of agricultural water is seen in nearly all rice-producing countries (Arnell 1999; Vorosmarty et al. 2000). In order to reduce water inputs and increase water use efficiency, rice can be grown like an irrigated upland crop such as maize and wheat, now termed aerobic rice system.

In aerobic rice systems, improved upland rice varieties (i.e., drought tolerant and nutrient-responsive) are grown in non-flooded aerobic soil. Irrigation is applied only up to field capacity after it has reached a certain lower threshold (Bouman 2001). Seepage and percolation are largely diminished, and water evaporation is lessened since there is no standing water. Field application efficiencies of irrigation water in this system range from 60–70% in flash/furrow irrigation to 80–90% in sprinkler/drip irrigation (Bouman 2001). Water savings can be as much as 51% compared with flooded fields but with a consequent yield reduction of 22–32% lower than those under flooded conditions (Bouman et al. 2005), mainly due to water stress effects under conditions when nitrogen is not limiting (Belder et al. 2005). Continuous aerobic rice cultivation, however, has caused progressive decline in yield and crop quality, both characteristic of developing soil sickness (George et al. 2002; Grodzinsky 2006; Peng et al. 2006; Ventura and Watanabe 1978).

Nie et al. (2007b; 2008; 2009a; 2009b) have conducted a series of studies on alleviating problem soil caused by continuous aerobic rice monocropping. These studies have concentrated on nutrient deficiencies but evidence also points to involvement of biotic factors. Initial observations indicated increased activity of root pathogens such as *Pythium* sp. and root knot nematodes (RKN) such as *Meloidogyne graminicola* (Kreye et al. 2009a; 2009b). Although other potentially pathogenic fungi such as *Rhizoctonia*-like, *Fusarium*, and *Sclerotium*-like fungi were also isolated, pathogenicity tests conducted later showed that these were not pathogenic, or only slightly so, to aerobic rice variety Apo. *Pythium* sp. and *Meloidogyne*

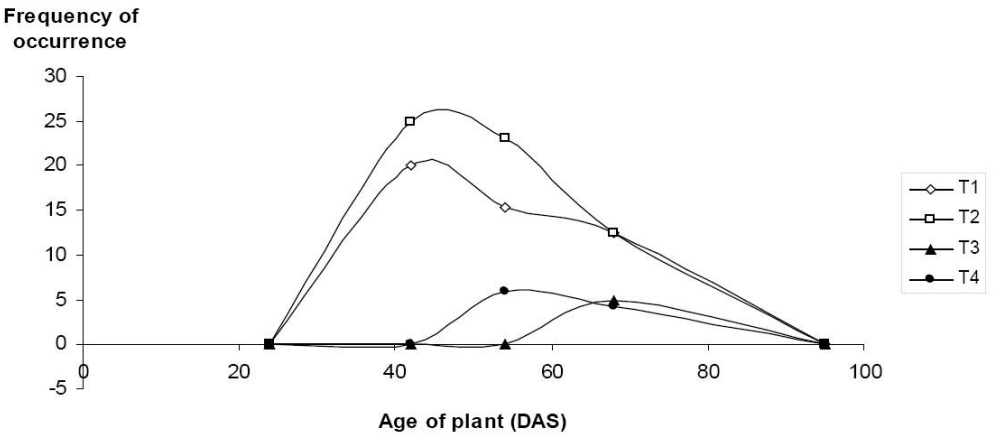


**Figure 1.** Frequency of occurrence of *Pythium* sp. isolation from plant roots at different ages in control (T1), with fungicide (T2), with compost (T3), and with compost + *Trichoderma* (T4).

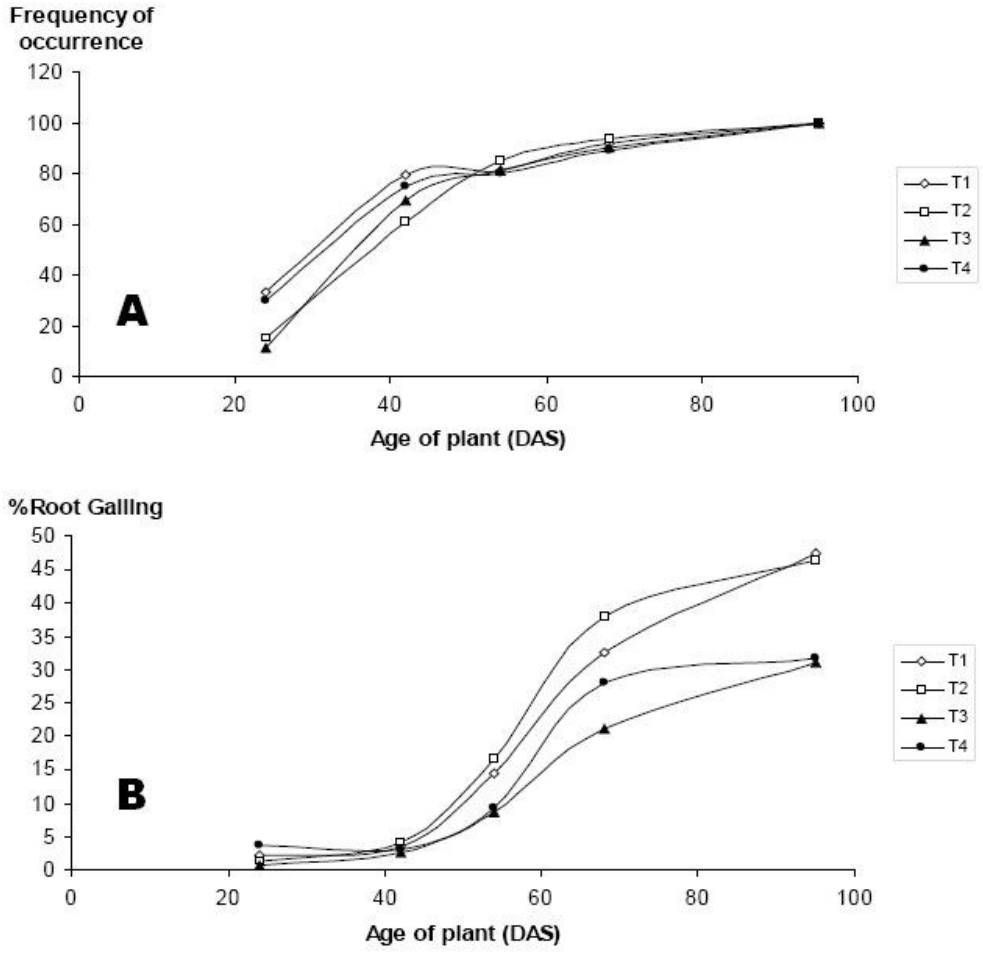


**Figure 2.** Population dynamics of RKN in roots of aerobic rice variety Apo planted in IRRI Field B912 in control (T1), with fungicide (T2), with compost (T3), and with compost + *Trichoderma* (T4) plots. Frequency of occurrence is shown in (A) while average % root galling in infested roots is shown in (B).

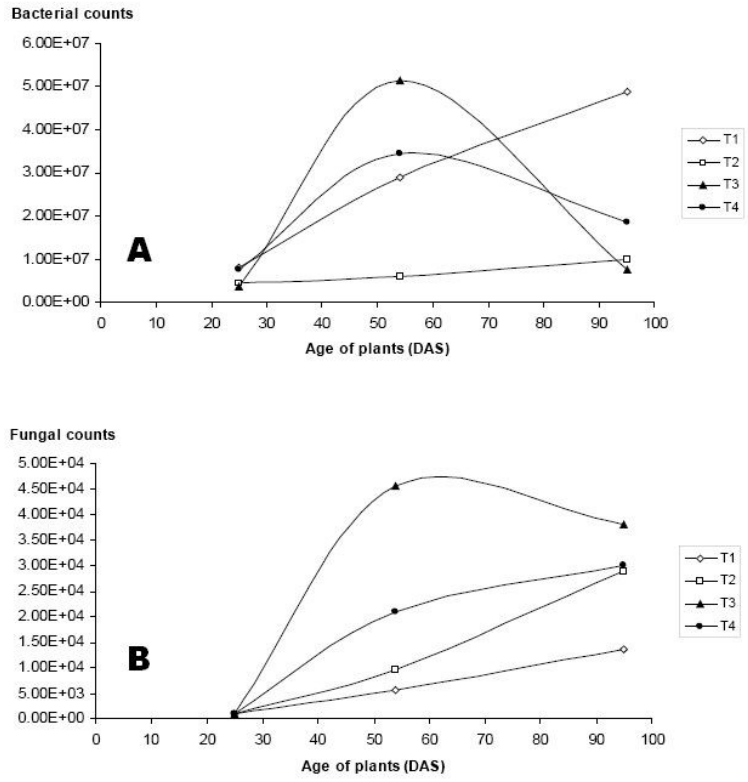


**Figure 3.** Bacterial (A) and fungal (B) counts of rice variety Apo rhizosphere soil in control (T1), with fungicide (T2), with compost (T3), and with compost + *Trichoderma* (T4).

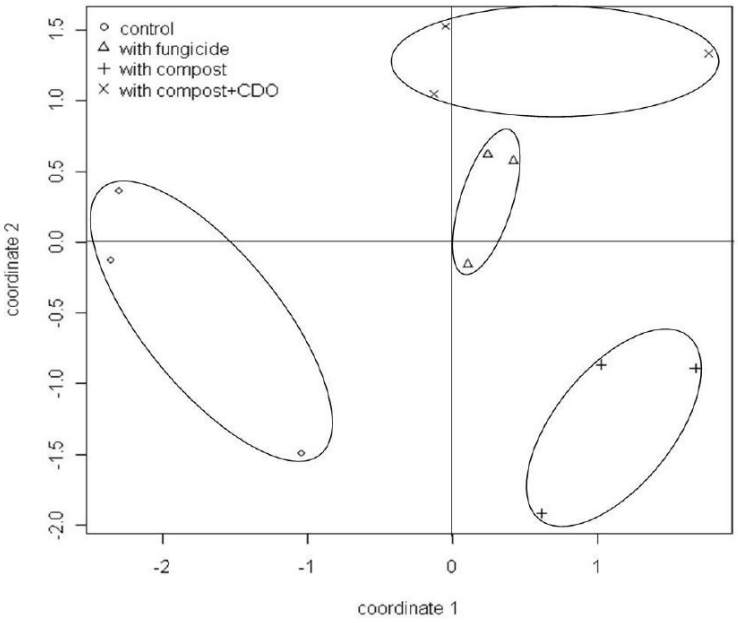


**Figure 4.** Multi dimensional scaling of carbon utilization profiles of aerobic rice variety Apo rhizosphere soil microorganisms using BIOLOG® EcoPlates™. Carbon utilization profiles were obtained from readings after 48 hours incubation of rhizosphere microorganisms from 95 DAS rice roots.

*graminicola* were then identified as the biotic factors most likely to contribute to the yield decline observed in aerobic rice fields (Kreye et al. 2009b; Pinili et al. 2009; Van Buyten et al. 2013).

Proper management of soil organic matter may not only help in nutrient and water retention, and maintenance of good soil structure, which are all important in alleviating the effects of water scarcity, but it may also help in mitigating soil sickness caused by build-up of *Pythium* and RKN due to continuous aerobic rice monocropping. Several studies have shown the disease-suppressive properties of soils rich in organic matter or of soil organic matter amendments against *Pythium* and RKN (Bailey and Lazarovits 2003; Everts et al. 2006; Lumsden et al. 1987; McKellar and Nelson 2003). Studies have often suggested the role of microorganisms in the bio-control effect of composts and other organic matter amendments. This study therefore aims to determine the effect of organic matter amendment on the occurrence of *Pythium* sp. and RKN in an aerobic rice system and relate this to the observed rhizosphere microbial community diversity.

## METHODOLOGY

### Field Management, Design, and Sampling

Field experiments were conducted at a 42 m × 54 m lot located at block 912 (B912) in the International Rice Research Institute (IRRI) experimental farm at Los Baños, Laguna, Philippines (14°9′ N, 121°16′ E). During the 2009 dry season (DS), the whole field was equally divided into 32 plots measuring 6 m × 10 m each. Only half of B912 was used during 2010 DS which was divided into 16 plots measuring 6 m × 5 m each.

Direct seeding (2009 and 2010 DS) and transplanting (2009 DS only) were done in rows of 20 cm × 20 cm spacing with three seeds or seedlings per hill. Improved indica upland rice variety Apo NSIC Rc9 (UPLRi5 × IR12979-24-1; formerly IR55423-01) was used as test crop at 20 kg $ha^{-1}$ seeding rate because of its relatively good performance under aerobic conditions (Lafitte et al. 2002).

Irrigation was once or twice a week whenever drying of the soil surface was observed, which corresponded to a soil moisture tension at depth of 15 cm of -15 to -25 kPa. Soil water content was not monitored and controlled rigorously, but frequent watering using flood irrigation delivering up to 2–4 cm of water ensured that the plants did not experience drought stress even when no standing water was kept in the field throughout the experiments, except for brief periods during irrigation.

During the 2009 DS, fertilizer application was at 90

kg N per hectare split into three (30-30-30). Application of N fertilizer (ammonium sulfate) was at 12–16 days after sowing (DAS), 28–32 DAS at mid-tillering (MT) and 43–47 DAS at panicle initiation (PI). No P and K fertilizers were added since previous soil analyses showed that it is not needed as there is enough P and K in the soil. During the 2010 DS, Site-Specific Nutrient Management (SSNM) protocol with the aid of a leaf color chart (LCC) was carried out with instructions from the Nutrient Manager for Rice Philippines Version 1.11 (http://webapps.irri.org/ssnm; now upgraded to Version 2.0). Using the guidelines from this program, 120 kg $ha^{-1}$ N, 30 kg $ha^{-1}$ P, 29 kg $ha^{-1}$ K, and 25 kg $ha^{-1}$ Zn was applied to the field. Ammonium sulfate (N) application was split into three (30:45:45 kg $ha^{-1}$) given at early (12–16 DAS), at MT, and at PI according to the leaf N status as reflected in the LCC readings. P, K, and Zn were applied at an early stage. No insecticides were applied and weeds were removed manually.

The field was divided into plots for the each of the four treatments, namely – T1: Control, T2: with fungicide (metalaxyl applied as seed coating at a rate of 140 mg per kg seeds), T3: with *in situ* composted rice straw, and T4: with in situ composted rice straw and *Trichoderma ghanense* CDO inoculant (applied as seed coating at a rate of 12.5 g per kg seeds). Each treatment had 4–5 replicates (1 plot = 1 replicate) arranged in RCBD. *In situ* composting was done as follows: six weeks before planting, cut rice straw was added to the field at a rate of 4 t $ha^{-1}$, soaked in water with NPK (14-14-14) added at a rate of 10 kg $ha^{-1}$ and allowed to decompose for five weeks. At the end of the decomposition period, rice straw compost was plowed under for incorporation into the soil one week prior to planting.

Each of the plots was divided into border area (2 rows on all sides excluded from sampling), sampling area, and harvest area (6 $m^2$ at the center). A total of five hills (three seedlings per hill) were uprooted along an imaginary zigzag sampling line within the sampling area. The sampling lines were rotated each sampling period to ensure that the whole plot was adequately sampled. The plants were brought to the lab for assessment of plant height and root health, taking note of galling and discolorations, and isolation of putative *Pythium* sp. from discolored roots. RKN were recorded as % galled roots and discolorations were recorded as % discolored roots. Frequency of occurrence of *Pythium* and RKN were computed based on the number of samples positive for either RKN or *Pythium*. Yield was measured from 6 $m^2$ harvest area located at the center of each plot and reported at 14% moisture content.

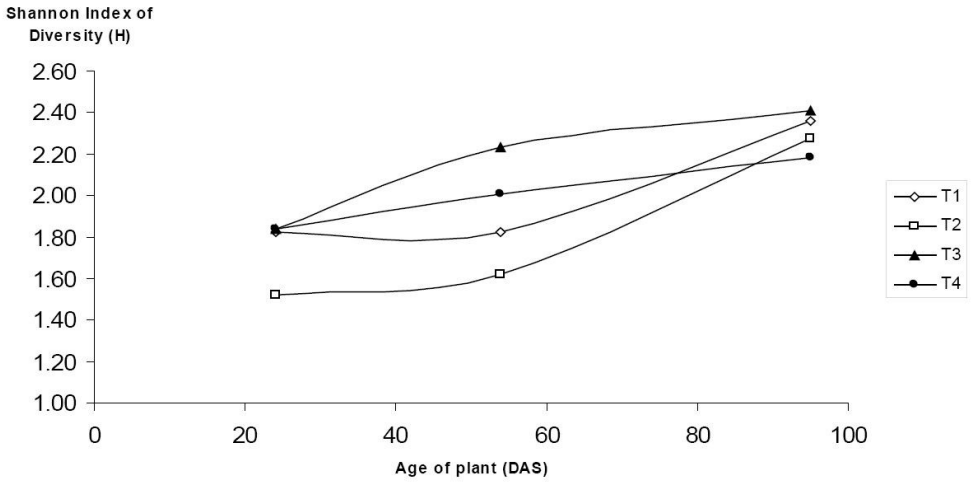


**Figure 5.** Changes in bacterial diversity with plant age in the different treatments based on DGGE. T1–Control; T2–with fungicide; T3–with compost; T4–with compost + *Trichoderma*.

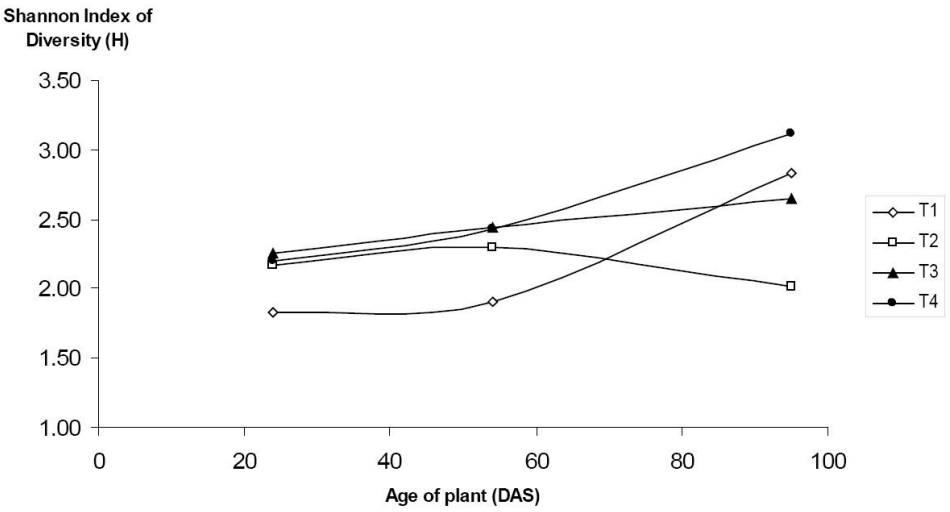


**Figure 6.** Changes in fungal diversity with plant age in the different treatments based on DGGE. T1–Control; T2–with fungicide; T3–with compost; T4–with compost + *Trichoderma*.

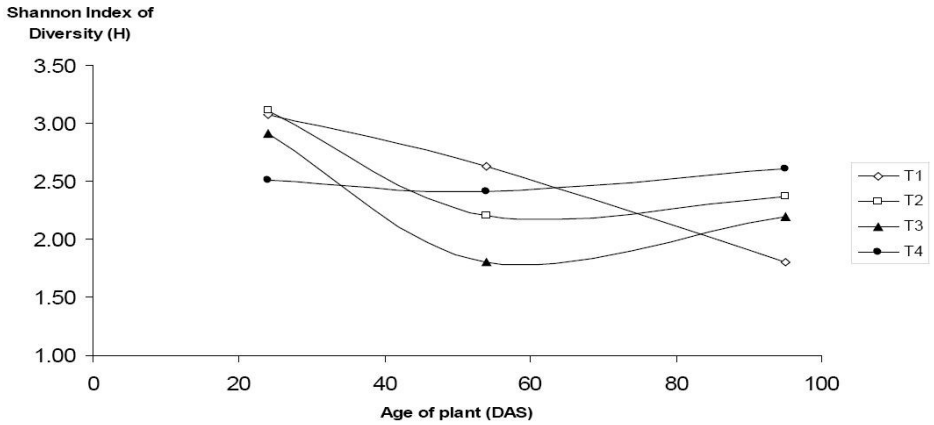


**Figure 7.** Changes in archaeal diversity with plant age in the different treatments based on DGGE. T1–Control; T2–with fungicide; T3–with compost; T4–with compost + *Trichoderma*

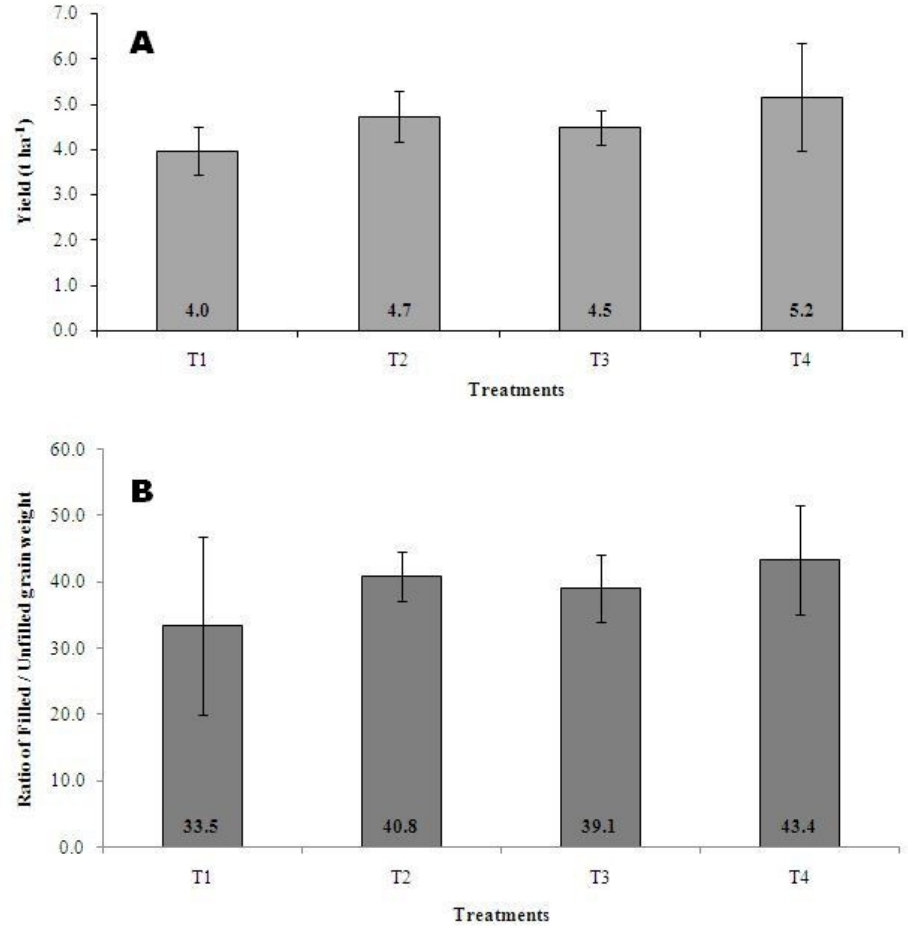


**Figure 8.** Yield (A) and ratio of filled to unfilled grain weight (B) of rice variety Apo under different treatments. T1–control; T2–with fungicide; T3–with compost; T4–with compost +*Trichoderma*.

Isolation of *Pythium* sp. from root samples was done using a tissue planting technique. Roots were thoroughly washed in running water to remove soil and other debris. Root sections with chocolate brown necrotic lesions were excised into 5-mm sections. These were surface sterilized with 5% sodium hypochlorite for 1 minute. Sterilized root sections were washed in three changes of sterile distilled water and then blotted dry using sterile paper towels. Root sections were plated onto 1.5% water agar and incubated at 28°C for 2–4 days. Actively growing hyphal tips were transferred to Potato Dextrose Agar (PDA) plates and incubated at 28°C for 2 days. Isolates were observed under the microscope for putative identification of *Pythium* spp. Cultures were maintained as agar blocks in sterile distilled water kept at room temperature.

**Aerobic Plate Count**

Ten grams of rhizosphere soil from each of the treatments were collected for serial dilution and subsequent plating on agar media as follows: Plate Count Agar (PCA) for total aerobic heterotrophic bacteria and PDA with streptomycin for fungi. Plates were incubated at 28°C for 1–2 days. Colony forming units per gram dry weight of rhizosphere soil were computed from the colony counts.

**Community-Level Physiological Profiling (CLPP)**

Microbial cells were extracted from the rhizosphere soil samples so that differential utilization of the carbon sources in the BIOLOG® EcoPlates™ could be assessed. Methods for the extraction of microbial cells from the samples were according to the protocol of Calbrix et al. (2005) with modifications. Five grams of rhizosphere soil were mixed with 45 mL of phosphate buffered saline (PBS) in a plastic conical tube. The mixture was vigorously shaken manually for 5 minutes. The mixture was then

centrifuged at 129 × g (~1300 rpm) for 5 minutes to clarify the suspension. The soil suspension was then diluted to $10^{-2}$ with sterile PBS in a final volume of 20 mL for each microplate corresponding to approximately $10^5$ cultivable microorganisms based on previous plate counts. This amount delivered around 1500 CFU per well on the EcoPlate™.

The diluted soil suspension was inoculated into the EcoPlate™ containing 3 replicates of 31 Carbon sources at a rate of 150 µL per well using a multi-channel pipettor. The plates were then incubated at 28°C for 2 days. The $OD_{590}$ was read after 12, 24, and 48 hours using the MicroLog BIOLOG® microplate reader.

Average well color development (AWCD) for plates was calculated as the mean of the corrected absorbance values for all 93 response wells per reading time. AWCD for each plate was computed at each time the plate was read. Since the actual rate of color development changes with time, the incubation time necessary to reach a midpoint in AWCD was used to compare relative rates of color production among samples. The Shannon indices of diversity and evenness were computed and compared for each sample. The formulae for each index are given in Table 1 as presented by Tam et al. (2001). Differences in patterns of carbon utilization were determined through multi-dimensional scaling (MDS) using the statistical package R (http://cran.r-project.org/).

**Denaturing Gradient Gel Electrophoresis (DGGE) of Rhizosphere Microbial DNA**

Rhizosphere soil was collected from roots of rice variety Apo samples. Soil community DNA was extracted using the experienced user protocol for MoBio PowerSoilTM DNA Isolation Kit (Mo Bio Laboratories Inc., USA). Isolated DNA was used for DGGE.

All polymerase chain reaction (PCR) were executed in a G-Storm GS482 thermal cycler (G-Storm Ltd., Surrey, UK) using a nested PCR approach. For the bacterial and archaeal communities, primers for the 16S rRNA gene were used, and for the fungal community, primers for 18S rRNA gene were used (Table 2). GC-clamps at the 5'end of primers are as described by Muyzer et al. (1993). Each reaction (20 µL for the first set of PCR primers and 50 µL for the nested primers) contained 1× PCR buffer, 0.2 µM each of the forward and reverse primers, 250 µM of dNTPs, 1 unit Taq polymerase, and 10 ng/µL of DNA template.

The thermal cycling scheme for the two sets of bacterial primers consist of initial denaturation of 5 minutes at 94°C then 35 cycles of 1 min at 94°C, 1 min at 53°C, and 2 min at 72°C (1 min at 72°C for the nested PCR) followed by a final chase at 72°C for 5 minutes. For the two sets of archaeal primers, the thermal cycling scheme reported by Øvreås et al. (1997) for the 1st primer set and Ohene-Adjei et al. (2007) for the 2nd primer set was followed. The thermal cycling scheme reported by Van Elsas et al. (2000) was followed for the two sets of fungal primers.

DGGE analyses were carried out using the DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, California, USA). Thirty microliters of nested PCR product were mixed with 15 µL of 2× loading dye and loaded on a DGGE gel. Gels, 1 mm thick and 16 × 16 cm, consisted of

**Table 1.** Formulae for Shannon Diversity and Shannon Evenness used in community level physiological profiling using BIOLOG® EcoPlate™. (adapted from Tam et al. 2001)

| INDEX | FORMULA | DEFINITIONS |
|---|---|---|
| Shannon diversity | $H' = \sum p_i \ln p_i$ | $p_i$ = proportional color development of the $i^{th}$ well over total color development of all wells of a plate |
| Shannon evenness | $E = \frac{H'}{\ln S}$ | S = number of wells with positive color development |

**Table 2.** Primer sets used for nested PCR-DGGE of rice variety Apo rhizosphere DNA.

| MICROBIAL GROUP | 1ST PRIMER SET | 2ND PRIMER SET |
|---|---|---|
| **Bacteria** | **8f** (AGA GTT TGA TCC TGG CTC AG)<br>**1492r** (GGT TAC CTT GTT ACG ACT T) | **341f-GC** (GC clamp + CCT ACG GGA GGC AGC AG)<br>**926r** (CCG TCA ATT CCT TTR AGT TT) |
| **Archaea** | **PRA46f** (YTA AGC CAT GCR AGT)<br>**PREA1100r** (YGG GTC TCG CTC GTT RCC) | **Arc344f-GC** (GC clamp + ACG GGG YGC AGC AGG CGC GA)<br>**Arc915r** (GTG CTC CCC CGC CAA TTC CT) |
| **Fungi** | **EF4f** (GGA AGG GRT GTA TTT ATT AG)<br>**F5r** (GTA AAA GTC CTG GTT CCC) | **NS2f** (GGC TGC TGG CAC CAG ACT TGC)<br>**F5r-GC** (GC + F5r) |

polyacrylamide (37.5:1 acrylamide/bis) and a 40-70% increasing denaturant gradient (35-60% for archaea and 35-65% for fungi), where 100% denaturant was defined as 7M urea and 40% (v/v) formamide. Gels were poured with the Model 475 Gradient Delivery System (Bio-Rad) and allowed to polymerize for 2 hours. Loaded gels were run at constant temperature of 60°C at 100V for the first 10 minutes then at 60V for 15 hours in 1× TAE. After the runs, gels were removed from the glass supports and stained with ethidium bromide prior to UV transillumination and documentation. Banding patterns of DGGE profiles were analyzed using the QuantityOne™ software. After background subtraction, banding patterns were matched and digitized. The intensity and position of individual bands were transferred to spreadsheets. Band intensity was expressed as the relative proportion of band trace over the total band traces in one lane. The Shannon diversity index was computed for describing the structural diversity of the microbial community and was calculated as $H' = -\Sigma PilnP_i$, where $P_i$ is calculated as $P_i=n_i/N$, where $n_i$ is the band trace and N is the sum of traces of all bands in the profile (Hoshino and Matsumoto 2007). Similarities of bands were expressed as Dice Coefficient computed in Quantity One™.

## RESULTS AND DISCUSSION

### Dynamics of *Pythium* in the Rhizosphere

Figure 1 illustrates the population dynamics of *Pythium* sp. in the roots of rice variety Apo. Frequency of occurrence was lower in plots with organic matter amendment (T3 and T4). A Chi square test (Table 3) showed the association of *Pythium* isolation with absence of organic matter amendment in treatments.

### Dynamics of RKN in the Rhizosphere

Figure 2 illustrates the population dynamics of RKN in the roots of rice variety Apo. While no significant difference in frequency of occurrence was observed between treatments, percent root galling was significantly ($P<0.05$) lower in plots with organic matter amendment (T3 and T4) at 95 DAS.

### Bacterial and Fungal Plate Counts

Bacterial and fungal plate counts revealed varying patterns of increase and decrease in population levels of both groups. Plots with organic matter amendment displayed larger and earlier increase in population levels compared with T1 and T2 as shown in Figure 3. Typical colonies of *Bacillus*, *Pseudomonas*, *actinomycetes*, *Trichoderma*, and *Penicillium* species were also observed on the plates, although their counts were not determined.

**Table 3.** Percentage of *Pythium*-infected plant samples coming from plots without organic matter amendment (T1 and T2) and plots with organic matter (OM) amendment (T3 and T4).

| TREATMENT | PERCENTAGE OF *PYTHIUM*-INFECTED PLANTS FROM TREATMENTS DURING 2009 AND 2010 DRY SEASON CROPPINGS | |
|---|---|---|
| | 2009 DS | 2010 DS |
| Without OM amendment (T1 and T2) | 65 | 83 |
| With OM amendment (T3 and T4) | 35 | 17 |
| Chi Square test *P* value* | 0.0027 | $4.1116 \times 10^{-11}$ |

*hypothesis of independence is rejected; isolation of *Pythium* is associated with OM amendment

**Table 4.** Shannon diversity (H') of aerobic rice rhizosphere microorganisms under different treatments based on carbon utilization patterns determined from BIOLOG® EcoPlates™.

| TREATMENTS | RHIZOSPHERE MICROBIAL DIVERSITY (H') AT DIFFERENT PLANT AGES (DAS) | | |
|---|---|---|---|
| | 25 | 54 | 95 |
| **T1, Control** | 2.83 | 3.22 | 3.21 |
| **T2, with fungicide** | 2.71 | 3.18 | 3.20 |
| **T3, with compost** | 2.87 | 3.17 | 3.10 |
| **T4, with compost + *Trichoderma*** | 2.85 | 3.11 | 3.10 |

### Functional Diversity of Rhizosphere Microbial Community (based on CLPP)

Functional diversity as determined from BIOLOG® EcoPlate™ carbon utilization profiles showed similar diversities for all treatments (Table 4). An increase in diversity was observed in each treatment only between seedling (24 DAS) and maximum tillering (54 DAS) stages but not between maximum tillering and heading (95 DAS) stages. However, patterns of carbon utilization became increasingly different as the plants matured as revealed in multidimensional scaling analysis (Figure 4). At

seedling stage, the carbon utilization profiles were not clearly different as the points revealed a scattered plot (data not shown). But as the plants reached heading stage, differences between treatments became clearer with the control set-ups clustering to one side and all the other treatments forming their own groups on the other side.

**Rhizosphere Bacterial Dynamics (based on DGGE)**

Figure 5 illustrates the changes in rhizosphere bacterial diversity with plant age in each treatment. In the control set-up (T1), almost no increase in diversity was observed from seedling stage to maximum tillering stage. However, a 2-fold increase in species richness increased diversity at heading stage. In the fungicide set-up (T2), a slight increase in species richness and diversity was observed as the plant matured but the increase was not as large as that of the control. In the compost set-up (T3), a larger increase in richness and diversity was observed between seedling and maximum tillering stage than that observed in T1 and T2. A 50% increase in species richness from maximum tillering to heading stage likewise increased diversity greater than in any other treatment. In the compost with *Trichoderma* set-up (T4), increase in species richness and diversity was similar to the trend observed in T3 but at a slower rate. The banding profiles of both T4 and T2 were most similar to T3 banding pattern at heading. For all treatments, banding patterns were very similar (76–84% similar to T1) at seedling stage, gradually becoming more different from T1 at maximum tillering with 52–70% similarity, then diverging farther at heading stage with only 14–33% similarity with T1.

**Rhizosphere Fungal Dynamics (based on DGGE)**

As illustrated in Figure 6, the general tendency in rhizosphere fungal diversity is increasing trends from seedling to heading stage, except for T2 (fungicide-treated). In T2, the relatively low diversity at seedling stage changed very little during plant growth and even decreased towards plant maturity. While all other treatments displayed increased fungal diversity, T2 had a slight decrease as the plant reached heading stage. In T1, like in bacterial dynamics, only a slight increase in diversity was observed from seedling to tillering but a 2-fold increase in species richness was observed from tillering to heading. A progressive steady increase in diversity was seen in T3 from seedling to heading. In T4, like T1, a 2-fold increase in species richness led to an increase in diversity at heading stage. T1 bands persisted from seedling to heading stage in nearly all treatments. At seedling stage, banding patterns of treatments (T2, T3, and T4) were 44–61% similar to the control (T1). At tillering stage, similarities with the control decreased to 36–41%. At heading stage, however, when rhizosphere fungal community in the control set-up exhibited a large increase in diversity, similarity coefficients rose to 50–65%.

**Rhizosphere Archaeal Dynamics (based on DGGE)**

Changes in diversity in the rhizosphere archaeal community are different from bacterial and fungal diversity trends in that instead of an increase in diversity as the plant matured, the opposite was observed (Figure 7). A progressive decline in diversity was observed in T1 from seedling to heading stage. Similar trends were observed in T2 and T3 with a 2-fold decline in species richness. However, instead of a continuous decline at heading stage, a slight increase in diversity was observed. In T4, however, archaeal diversity was more or less stable – relatively low at the start, slightly decreasing at tillering, then again slightly increasing at heading stage. At the end, T4 had the highest rhizosphere archaeal diversity among the four treatments. Archaeal banding patterns of treatments were 31–44% similar to the control. At tillering stage, similarities with the control decreased to 27–34%. At heading stage, similarity coefficients increased to 53–60%.

**Correlations Between Parameters**

For *Pythium*, the critical stage of pathogen and disease control was observed at the maximum tillering stage. *Pythium* frequency of occurrence at 54 DAS in treatments with compost decreased and the decrease linearly correlated with a reduction in root discoloration ($r=0.91$, $P<0.05$), which is moderately negatively correlated with plant height ($r=-0.53$, $P<0.05$). The Chi square test also indicated that *Pythium* isolation was associated with presence/absence of compost in treatments. *Pythium* frequency of occurrence at 54 DAS was also strongly negatively correlated with log bacterial plate count ($r=-0.91$, $P<0.05$) and with bacterial diversity based on DGGE profiles ($r=-0.99$, $P<0.05$), both at 54 DAS. Fungal plate counts and diversity obtained from PCR-DGGE profiles were not significantly correlated with *Pythium* frequency of occurrence (f). Although fungal plate counts were not significantly correlated with diversity obtained from DGGE, the high correlation coefficient ($r=-0.843$, $df=3$) may indicate that, with more experimental data, a significant value may be arrived at. However, when data from T2 were excluded from the analysis, highly significant correlation values were calculated ($r=-0.9998$, $df=2$, $P<0.05$) between *Pythium* frequency and log fungal plate count. The hypothesis is that fungicide treatment could have disturbed the balance of fungal components in the rhizosphere such that even if a relatively higher plate count was observed for T2 than the control, the fungal species present were not inhibitory to the pathogen. This could indicate the effect of the chemical on non-target species.

For RKN, frequency of occurrence was not affected by treatments but % root galling was significantly reduced in the presence of additional organic matter (T3 and T4). This, however, was not accompanied by any increase in yield, probably owing to the tolerance of variety Apo to RKN and *Pythium* as determined from other previous studies. The % root galling at 95 DAS, when strong correlations with stunting and

root discolorations were previously observed, was not correlated with any microbial diversity index. However, % root galling at 54 and 68 DAS were strongly negatively linearly correlated with rhizosphere bacterial diversity determined from DGGE profiles with r=-0.96 (P<0.05) at 54 DAS and r=-0.99 (P<0.05) at 68 DAS. Fungal plate counts and diversity (from DGGE profiles) were not significantly correlated with % root galling at 54 and 68 DAS. However, the high correlation coefficients (r=-0.860 and r=-0.848, respectively) between fungal plate counts and % root galling at these plant stages may indicate that with more experimental data, a significant correlation may be observed. However, when data from T2 were excluded from the analysis, as was done for *Pythium* f and log fungal plate count, significant correlation values were computed for % root galling vs. log fungal plate count (r=-0.972, df=2, P<0.05) and for % root galling vs. fungal H' computed from DGGE (r=-0.994, df=2, P<0.05). Again, the hypothesis is that fungicide treatment could have disturbed the balance of fungal components in the rhizosphere such that even if a relatively higher fungal plate count and diversity were observed for T2 than the control, the fungal species present were not inhibitory to RKN.

For both *Pythium* and RKN, archaeal diversity based on DGGE profiles showed no linear correlation with frequency of occurrence and % root galling, respectively. Diversity indices computed from BIOLOG® EcoPlates™ were not useful parameters to distinguish between treatments and to correlate with the observed reduction in root disease symptoms. The carbon utilization profiles, however, do indicate variations in microbial activity between treatments even though functional diversities were the same.

**Yield Data**

Figure 8 shows the yield of rice variety Apo from the different treatments. No significant differences in yield as well as in ratio of filled to unfilled grain weights were observed between treatments despite significant reductions in pathogen levels. This could be due to the tolerance of rice variety Apo to these two pathogens as determined from results of previous experiments.

*Pythium* and RKN population levels in rice roots were affected by organic matter amendment. Correlation analysis indicated the connection between suppressive effects and bacterial and fungal diversity. It has been generally accepted that the primary basis for the suppressive effect of organic amendments such as composts to soil-borne pathogens is the antagonistic activity of microorganisms (Hoitink and Fahy 1986). Organic matter amendment stimulates the growth of a suppressive consortium of microorganisms that act against soil-borne pathogens through competition, parasitism, antibiosis, degradation of pathogen germination stimulants, or a combination of these (Bailey and Lazarovits 2003; Hoitink and Fahy 1986; McKellar and Nelson 2003). The reason composts seem to work better than inoculants of single species of antagonist is due to the combined action of a variety of organisms that the compost can nutritionally support (Hoitink and Fahy 1986). A synergistic effect is possible especially since these organisms have developed together as autochthonous residents of the soil.

In this study, possible antagonists observed in the rhizosphere soil microbial plate counts were *Bacillus*, *Pseudomonas*, *actinomycetes*, *Trichoderma*, and *Penicillium* species. Although their potential antagonistic activities have not been assessed, these organisms have been repeatedly reported to contribute to the activity of suppressive soils. In a study by Boehm et al. (1993), organic matter decomposition level significantly influenced the composition of bacterial species in the rhizosphere and that *Pseudomonas* spp. and taxa suppressive to *Pythium* damping-off predominated in suppressive mixes. Although potential antagonists were isolated from both suppressive and conducive mixes, majority were isolated from the former. In the present study, actinomycetes and pseudomonads were more commonly observed in T3 and T4 samples (data not shown).

From the graphs of pathogen population dynamics (Figures 1 and 2) and their correlation with diversity indices, it was inferred that the critical point for disease control was around 54 DAS or during the period of active plant growth. This was clear for *Pythium* sp. For RKN, however, because correlations were not evident at the point when % root galling declined (95 DAS), there seems to be a carry-over of suppressive effects from 54 DAS to 95 DAS. It is reasonable to infer that the suppressive effect of increased microbial diversity would be evident at the pre-reproductive stage of the plant. It has been known that microbial numbers closely follow patterns of root exudation and that root exudation is greatest during periods of active root growth, which occurs before the reproductive stage (Brimecombe et al. 2007). In the rice plant, this corresponds to the tillering stage. Since bacterial diversity in the control and fungicide-treated plots did not increase until after 54 DAS, the suppressive effect of high microbial diversity may not have been in place. On the other hand, organic matter-amended plots, probably because of additional nutrients, exhibited earlier increases in microbial diversity, which were able to exert their suppressive effects on the pathogens during a time of rapid pathogen growth. So for RKN, high microbial diversity during its log phase of growth served to slow down population increase hence decreasing final % root galling estimates.

*Trichoderma* application did not seem to have an effect on pathogen levels but EcoPlate™ and DGGE profiles reveal that there were effects on microbial diversity even if the end result on pathogen suppression was the same. *Trichoderma* spp. are known to benefit the host plant through growth promotion, bio-control of disease-causing organisms, and protection from various environmental stresses (Harman et al. 2004a). Species of *Trichoderma* have been widely used for these various purposes (Harman 2006; Harman et al. 2008; Howell 2003; Shoresh and

Harman 2008a; 2008b). Their effectivity, however, would largely depend on how well they compete with resident soil microflora. *Trichoderma* CDO was applied only as a seed coat and its effectivity might be limited in the field especially since composting one and a half months before planting would already have increased levels of indigenous microflora, which are more adapted to the prevailing soil conditions. Native *Trichoderma* spp. were present in the rhizosphere soil and these most probably have a competitive advantage over the inoculant. Alternatively, because the inoculant was applied as a seed coat it had better access to the radicle as the seed germinates so it may likewise have a competitive advantage over resident microflora. One possible effect is the significant ($P<0.05$) increase in leaf greenness (based on LCC readings) observed at 45 DAS in T4 plots. Leaf greenness has been reported as one of the effects of this inoculant (V.C. Cuevas, personal communication). This increase in leaf greenness has been attributed to increased activity of photosynthesis-associated proteins (Shoresh and Harman 2008a). This implies that the inoculant may have exerted its effect, but that the overall effect on the pathogens was not significantly different from the effect of compost-induced resident microbial activity.

It was expected that T2 plots planted with metalaxyl-treated seeds would show decreased frequencies of *Pythium* isolation, but this was not the case. In fact, higher *Pythium* frequencies of occurrence were observed from this treatment. Metalaxyl has been used for control of diseases caused by *Pythium* but several studies have also detected variability in its sensitivity to the chemical, which was thought to be responsible for the failure in disease control in certain locations (Dorrance et al. 2004). This emphasizes the importance of alternative methods of disease control other than repeated use of a chemical, which eventually leads to proliferation of resistant types and therefore reduced levels of disease control in the field. Fungicide-treated plots have also shown lower levels of bacterial and fungal diversity (based on plating and DGGE) compared with +OM plots, which may account for the reduced level of disease control. However, higher diversity is observed in T2 than in the control plots. This shows that microbial diversity alone may not give an indication of disease-suppressiveness since it is the composition of the microbial community that would determine this effect.

Functional diversity (based on BIOLOG® EcoPlates™) was similar for all treatments and only slightly increased from seedling to heading stage. However, diversity based on DGGE clearly showed differences. This may imply redundancy of functions as seen in other studies on soil microorganisms (Torsvik and Øvreås 2002; Yin et al. 2000). Likewise, Konopka et al. (1998) have argued that CLPP is insensitive to changes in population structure due to metabolic redundancy. However, Garland (1999) also states that, despite microbial functional redundancy, CLPP can still discriminate changes in community profiles if the data is continuous (i.e. measured through time). In this study, shifts in population structure have been detected as changes in carbon utilization patterns through time in the different treatments. The shifts in composition of microbial communities were detected but it could not be related to changes in the abundance of specific taxa or functional groups. Also, functional diversity may have been the same for all treatments because BIOLOG® EcoPlates™ could have selected for only a minor part of the microbial community that were capable of utilizing readily available simple substrates, thus reflecting only the activity of culturable organisms and missing a lot of information on activity of non-culturable organisms, which are estimated to contribute more to diversity. This culture bias has been reported before (Garland 1999). On the other hand, DGGE has the capacity, at least theoretically, to capture the total community including non-culturable organisms. Hill et al. (2000) have enumerated several important points to consider when using CLPP for microbial community analysis. The point most relevant to this study is that the substrates found in the EcoPlate™ wells may not be necessarily ecologically relevant and most likely do not reflect the diversity of substrates in the environment of interest. In a study by Campbell et al. (1997), plant root exudate compounds were used as carbon sources to discriminate between rhizosphere microbial communities of nine sites. They reported that root exudates were more discriminatory than BIOLOG® plates in differentiating the samples tested. Another point to consider why functional diversity differences are not reflected in BIOLOG® plates is the possibility that differences may not lie in overall ability of the community to utilize different carbon sources but rather on some other physiological process or activity not captured by analysis using BIOLOG® EcoPlates™.

Denaturing gradient gel electrophoresis is a powerful tool for assessing microbial community structure. It is a culture-independent approach, hence non-culturable organisms can be detected and culture-bias is not an issue. In this study, relatively high species richness (up to 27 bands; compared with less than 10 colony types found in plate counts) was seen from just a fraction of a gram of soil. But like other methods, DGGE also has its limitations. DNA isolation from soil may be biased in that some organisms are more amenable to DNA extraction than others, and some substances in soil may also inhibit DNA isolation and subsequent PCR amplification. The MoBio PowerSoil DNA Isolation Kit used in this study has a mechanism for removing humic substances/brown color that is said to be effective in removing PCR inhibitors. The resulting DNA is of high purity allowing for more successful PCR amplification. Another limitation in DGGE is the problem of PCR bias and non-specific amplification. This study employs a nested PCR approach, which could also potentially amplify PCR bias, however when direct amplification using the nested primers (the primer set with GC clamps) was done, no product could be amplified; hence the nested PCR method was used. The primers used have been reported to be specific for the target organisms. However, this specificity was not anymore verified in this study. There may also be a problem with differential amplification of sequences depending on G+C content. Usually, organisms with higher G+C content are not as easy to amplify owing to the

greater difficulty in denaturation of strands while organisms with lower G+C are easier to denature. Touchdown PCR protocols were used in order to help overcome this tendency. But, like any other method, DGGE cannot give a complete picture of microbial diversity because of method biases and other methodological limitations. But at least, it gives a wider picture compared with culture-based methods.

The results indicate that although functional diversity based on CLPP is the same, Carbon source utilization patterns are different and the distinctions are clearer in older plants. On the other hand, differences in genetic diversity based on DGGE are clearly seen and, like in CLPP, DGGE profiles become more dissimilar as the plant matures.

Despite the utility of culture-independent approaches to microbial community analysis, there remains a need for pure cultures of microorganisms to better understand the roles they perform in the ecosystem. Studies on microbial communities must, therefore, rely on a combination of both culture-dependent and culture-independent approaches.

## SUMMARY AND CONCLUSIONS

Root pathogen dynamics in roots of aerobic rice variety Apo was affected by organic matter amendment and the basis of the effect seems to be related with increased bacterial and fungal diversity in the rice rhizosphere. *Pythium* frequency of occurrence and % root galling (caused by RKN) were both reduced in organic matter-amended plots and the critical control point seems to be at 54 DAS, which corresponds to the maximum tillering to panicle initiation stage in rice variety Apo, or during periods of active plant growth. Although reduction of percentage root galling was statistically significant, frequency of occurrence was still 100% and percentage root galling was still high at 30%. Further reduction of RKN levels requires the use of other strategies such as crop rotation with non-host plants and cultivation of resistant varieties. The use of chemical inhibitors may be counterproductive as it has deleterious effects on non-target organisms as seen in metalaxyl-treated plots in this study.

Developing disease-suppressive soils through organic matter amendments takes time and the effects are slow, but the benefits are incremental and accumulate across successive years, improving soil health and contributing to ecosystem functions. The effects seen in this study may be small but continuous amendment with organic matter may redound to larger benefits in the future.

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## CONFLICTS OF INTEREST

The authors declare no conflict of interest.

## CONTRIBUTIONS OF INDIVIDUAL AUTHORS

CGBB contributed to the conceptualization of the study and did the experimentation, data gathering and analysis, and writing of the manuscript. CMVC and VCC contributed to the conceptualization of the study, analysis of data and editing of the manuscript.

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