*Research article*

# Diversity of the Actinomycetes Community Colonising Rice Straw Residues in Cultured Soil Undergoing Various Crop Rotation Systems in the Mekong Delta of Vietnam

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**Abstract** Actinomycetes are involved in important environmental processes such as the decomposition of organic matter. In this study, we examined the impact of crop rotation on the actinomycetes community colonizing rice straw residues in soil over 3 field seasons by means of Denaturing Gradient Gel Electrophoresis (DGGE) fingerprinting analysis of actinomycetes 16S rRNA gene fragments amplified from field-incubated rice straw residues and analysis of actinomycetes 16S rRNA gene clone libraries from selected samples. The studied yearly crop rotation systems were rice-rice-rice (CRS1), rice-rice-baby corn (CRS2), rice-rice-mungbean (CRS3) and baby corn-rice-mungbean (CRS4), applied on different experimental plots of the same field location. Litter bags containing rice stems were inserted into the soil and recollected at different time points for comparison of the structure of the actinomycetes community colonizing the rice straw. The actinomycetes community was significantly different in composition in the baby corn-rice-mungbean rotation system (CRS4) compared to those in the 3 other systems during the growth of the first crop and second crop. In contrast, during the cultivation of the third crop, actinomycetes communities were significantly different in the rice-rice-rice (CRS1) system compared to those in the 3 other systems. The analysis of the 16S rRNA gene libraries constructed from selected samples of rotation systems CRS1 and CRS4 during growth of the first two crops confirmed the DGGE results. The diversity of actinomycetes tended to be highest in the CRS4 system and lowest in the CRS1 system.

**Keywords** Actinomycetes community, diversity, crop rotation, Mekong delta

## INTRODUCTION

Actinomycetes, phylogenetically defined as a number of taxa within the high-GC subdivision of the gram-positive phylum (Embley et al., 1994), represent a group of relatively abundant and metabolically diverse bacteria in soils (Labeda and Shearer, 1990; McCarthy and Williams, 1992; Holmalahti et al., 1994). They are involved in important processes in a wide range of habitats (Williams et al., 1984), such as the decomposition of organic materials in soil, including lignin and other recalcitrant polymers, and in the degradation of agricultural and urban wastes (Crawford, 1988; McCarthy, 1987).

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The composition of a soil microbial community can be affected by various factors such as soil characteristics, environmental conditions, plant growth and crop management strategies (Curl and Truelove, 1986). Numerous studies have compared microbial communities among different ecosystems such as agricultural soils versus soils from forest or grassland systems (Ovreas and Torsvik, 1998; Waldrop et al., 2000; Yao et al., 2000), among soils undergoing different long-term cropping and management regimes (Zelles et al., 1992, 1995; Bossio et al., 1998; Drijber et al., 2000), and among soils cultivated by different plant species (Grayston et al., 1998; Siciliano et al., 1998; Ibekwe and Kennedy, 1999; Miethling et al., 2000; Marschner et al., 2001). However, there are few studies documenting the effects of crop rotation systems on specific soil microbial communities (Lupwayi et al., 1998). Especially, the effect of crop rotation on soil actinomycetes has been poorly studied despite the important role of this group of micro-organisms in degradation of organic matter and nutrient cycling.

Rice is the most important crop in the Mekong Delta of Vietnam. The total area cultivated with rice in that region occupies nearly 4 million hectares per year. The introduction of high yielding rice varieties and intensive rice cultivation has resulted into numerous constraints, such as high plant disease pressure, the extensive use of fertilizers and pesticides and soil degradation. In order to reduce chemical inputs, to improve profitability, and to increase sustainability, new cropping systems and management practices for rice production in the Mekong Delta are being assessed. In this context, we have initiated different field experiments in which we assessed the effect of crop rotation on rice productivity and soil health. As a part of this study, we examined whether the type of crop rotation affected microbial communities playing a role in soil functioning. In this paper, we assessed the effect of crop rotation system on the actinomycetes community colonizing and degrading rice straw residues in soil which had been historically cultivated for continuous rice production for more than 10 years.

## METHODOLOGY

### Set-up of field experiment and sampling approach

The experimental field used in this study is located in Cay Lay district, Tien Giang province, Vietnam. The field was designed as a complete randomized block of experimental plots undergoing 4 different crop rotation systems (CRS) with 3 replicate plots per system since the year 2001. Each plot covered an experimental area of 90 $m^2$ (6 by 15m). The 4 applied rotation systems were (1) CRS1: rice (Crop I) - rice (Crop II) - rice (Crop III), (2) CRS2: rice (Crop I) - rice (Crop II) - baby corn (Crop III), (3) CRS3: rice (Crop I) - rice (Crop II) - mungbean (Crop III) and (4) CRS4: baby corn (Crop I) - rice (Crop II) - mungbean (Crop III).


| Crop I | Crop II | Crop III | |
|---|---|---|---|
| Rice | Rice | Rice | CRS1 |
| Rice | Rice | Baby corn | CRS2 |
| Rice | Rice | Mungbean | CRS3 |
| Baby corn | Rice | Mungbean | CRS4 |

**Fig. 1 Time schedule showing the recovery of litter bags from the Cay Lay field experiment**
*Arrow indicated with 1 refers to litter bags inserted before the start of the experiment. Arrows indicated with 2, 5 & 8 refer to sampling of litter bags after 14 days of cultivation of crops I, II and III, respectively. Arrows indicated with 3 & 6 refer to sampling of litter bags after 50 days of cultivation of crops I and II, respectively. Arrows indicated with 4, 7 & 9 refer to sampling of litter bags at harvest of crops I, II and III, respectively. The cropping calendars of three cropping patterns were as follow: crop I (between 20/6/2006 and 1/10/2006), crop II (between 1/11/2006 and 10/2/2007) and crop III (between 30/2/2007 and 10/6/2007).*

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Litter bags (nylon material with a pore size of 200 ηm) were filled with 5 g of dried rice straw residues and buried, prior to seeding of the first crop, into the soil at a depth of around 10 cm on 20 June 2006.Before inserting, the bags with rice straw were sterilized at 121$^{o}$C for 20 minutes. The litter bags were periodically recovered from the soil for 16S rRNA gene based DGGE analysis of the actinomycetes community colonizing rice straw according to the time schedule shown in Fig 1. At each time point, 3 litter bags were recovered from each plot.

**DNA extraction and DGGE analysis**

DNA was extracted from the rice straw as described by Boon et al. (2000). 30µl of the extract was cleaned from humic acids by adding 2mg acid-washed PVPP (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and 30µl TE-buffer. The mix was vortexed and centrifuged at 11,000 rpm during 5 min. The supernatant was recovered and subjected to PCR amplification of the target 16S rRNA gene. To amplify actinomycetes specific 16S rRNA gene fragments, a nested PCR approach was used in which in the first PCR the actinomycete specific forward primer F243 (Heuer et al., 1997) was used together with the bacterial reverse primer R1378 (Heuer et al., 1997). The product of this PCR was used as template in a second PCR with primers F984GC (Nubel et al., 1996) and R1378. PCR reactions were performed in a Mastercycler apparatus (Eppendorf; Hamburg, Germany), according to van Dillewijn et al. (2002). DGGE of amplified actinomycetes 16S rRNA gene fragments was performed on an Ingeny phor U-2 system (Leiden, The Netherlands). 30 µl portions of the PCR product were loaded onto an 8% (w/v) polyacrylamide gel with a denaturing gradient ranging from 35% denaturant to 65% denaturant in Tris–acetate–EDTA (TAE) buffer. Electrophoresis was performed for 15 h at 60°C and 120 V. The gels were stained for 30 min with 1xSYBR Gold (Molecular Probes, Leiden, and The Netherlands) and photographed on a UV transilluminator with a GeneLink camera system (SYNGENE, Cambridge, UK). Gelcompar II version 3.5 (Applied Math's, Sint-Martens-Latern, Belgium) was used for UPGMA cluster analysis of DGGE fingerprints. Dendrograms were constructed by using the Pearson correlation index for each pair of lanes within a gel and cluster analysis by the unweighted pair group method using arithmetic averages.

**Cloning and sequencing of 16S rRNA gene fragments**

PCR products were cloned into pCR®2.1-TOPO®, using the TOPO TA Cloning kit (Invitrogen, Merelbeke, Belgium) as described by the manufacturer. To check transformants for the incorporation of the actinomycetes 16S rRNA gene, a nested PCR was performed consisting of a first PCR with M13f and M13r primers as described by Invitrogen followed by a second PCR with bacterial primers GC-984F and 1378R. DGGE fingerprints from fragments amplified from the clones were compared with the actinomycetes 16S rRNA gene DGGE fingerprints obtained from environmental DNA and appropriate clones were chosen for sequence analysis. The PCR products obtained from the clones were purified with the PCR purification kit (Promega) as described by Promega and subjected to DNA sequencing reactions performed with the QuickStart DNA sequencing kit (Beckman) and analysed on an automatic sequencer (CEQTM8000, Beckman Coulter, Fullerton, CA, USA). Resulting partial 16S rRNA gene sequences (about 400 bp) were analyzed by BLASTN search (Altschul et al., 1997).

**RESULTS AND DISCUSSION**

**DGGE analysis of actinomycetes community colonizing rice straw incubated under various crop rotation systems**

Fig. 2 shows the actinomycetes 16S rRNA gene DGGE profiles obtained from rice straw residues incubated under the different crop rotation systems at 14 days of cultivation, at 50 days of cultivation and at harvest of crop I, crop II and crop III while Fig. 3 shows the corresponding

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UPGMA cluster analysis. The DGGE profiles revealed that the composition of the actinomycetes community in colonizing rice straw residues was strongly affected by crop rotation. The clearest difference was observed between plots undergoing system CRS4 on the one hand and the 3 other systems on the other hand during growth of crop I and II, and between CRS1 plots on the one hand and the other 3 systems during growth of crop III.

During growth of crop I, at 14 days of cultivation, at 50 days of cultivation and at harvest, the profiles of the replicates of systems CRS1, CRS2 and CRS3 clustered as a separate group from the profiles of replicates of system CRS4 in the UPGMA clustering analysis (Fig. 3A). Profiles from replicate plots of systems CRS1, CRS2 and CRS3 were highly similar with a similar level among the 3 systems of 48.2, 42 and 76% for samples taken at 14 days of cultivation, at 50 days of cultivation and at harvest, respectively. In contrast, profiles from system CRS4 were 36.5%, 31 and 72.8% similar to profiles originating from the other 3 rotation systems at 14 days, at 50 days of cultivation and at harvest, respectively.

During growth of crop II, which was rice in all systems, at 14 days of cultivation and at 50 days of cultivation, profiles for systems CRS1, CRS2 and CRS3 were still more similar to each other than to profiles obtained for system CRS4, i.e., the profiles of CRS1, CRS2 and CRS3 were about 70.4 % and 71.5% similar to the profiles of CRS4 at 14 days and 50 days of cultivation, respectively, while the values of similarity were 87 and 87.5% among the profiles of CRS1, CRS2 and CRS3 at day 14 and day 50, respectively. However, at harvest of crop II, the profiles clustered into two main groups (Fig. 3B). One group consisted of the profiles of CRS1 and CRS2 and the other group of profiles of CRS3 and CRS4. The similarity between the group containing CRS1 and CRS2 and the group containing CRS3 and CRS4 was 59.5% while the similarity of profiles within the two groups was 69% and 66.9%, respectively.

During cultivation of crop III, at day 14 of crop cultivation and at harvest, the actinomycetes community profiles clustered into two main groups, i.e., a first group consisting of profiles from systems CRS2, CRS3 and CRS4 and a second group consisting of profiles derived from system CRS1 (Fig. 3C). Profiles from CRS1 were 42.1% and 34.5% similar to profiles originating from the other rotation systems at day 14 and at harvest, respectively. The similarity among the CRS2, CRS3 and CRS4 profiles was 50.9% at day 14 day and 45.0% at harvest.

**Analysis of 16S rRNA gene clone libraries**

16S rRNA gene clone libraries were constructed from two representative samples of the CRS1 and CRS4 system, i.e., a sample taken at 50 days of cultivation of crop I and a sample taken at 50 days of cultivation of crop II. For each library 15 to 20 clones were analyzed by DNA sequence analysis. Figure 4 shows the phylogenetic distribution of the 16S rRNA gene sequences within each library.

In the sample taken at 50 days of cultivation of crop I in system CRS1, most of the 16S rRNA sequences matched with 16S rRNA gene sequences of actinomycetes especially with the family Nocardioidaceae (22.3%) and uncultured actinomycetes (33.3%).Unexpectedly, other clones carried sequences which were not related to 16S rRNA gene sequences of actinomycetes but rather to sequences associated with the phyla verrucomicrobia and fimircutes (44.4%). In contrast, in the corresponding samples taken from the CRS4 rotation system, almost all sequences were associated with the actinomycetes (84.75%) while only a minority was non-actinomycetes sequences such as sequences associated with the phylum verrucomicrobia (15.3%). Interestingly, the actinomycetes present in the CRS4 system were apparently more diverse than those in the CRS1 system, showing the presence of four different families of actinomycetes, i.e., Microbacteriaceae, Promicromonosporaceae, Nocardioidaceae, and Mycobacteriaceaee. The family Microbacteriaceae constituted the most abundant family (53.8%).

Diversity in the actinomycetes community was also found at day 50 of cultivation of crop II. Moreover, although in both systems, rice was the cultivated crop, the samples from the two systems apparently contained two different actinomycetes communities. The community in the CRS4 system was more diverse and included members of five actinomycetes different families, i.e., Streptomycetaceae, Catenulisporaceae, Nocardioidaceae, Mycobacteriaceae and Promicromonosporaceae while the CRS1 sample contained members of two families, i.e.,

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Microbacteriaceae and Mycobacteriaceae. The family Streptomycetaceae was dominant (43.2%) in the CRS4 system while in the CRS1 system, the Microbacteriaceae family was dominant (46.7%). In both systems, the actinomycetes were still the most highly represented sequences (53.3% in CRS1 and 88.3% in CRS4) but also non- actinomycetes 16S rRNA gene sequences were found (46.7% in CRS1 and 14.2% in CRS4), which were associated with the groups of proteobacteria, verricomicrobia and firmicutes. Xuan et al (2007) found that the family Microbacteriaceae was present in rice straw decomposing in soil of two experimental sites in the Mekong Delta while Hesham et al (2006) showed Streptomyces as the dominant actinomycetes genus in rice straw decomposition although also members of the genera Nocardiopsis, Micromonospora and Nocardioides were present.

Three main conclusions can be taken from our study. First, the actinomycetes community seems to be dynamic in function of time, both during the growth of a particular crop and of different crops. This can be explained by changes in substrate (i.e., the rice straw) composition during decomposition (Lynch and Harper, 1985; Aulakh et al., 1991).

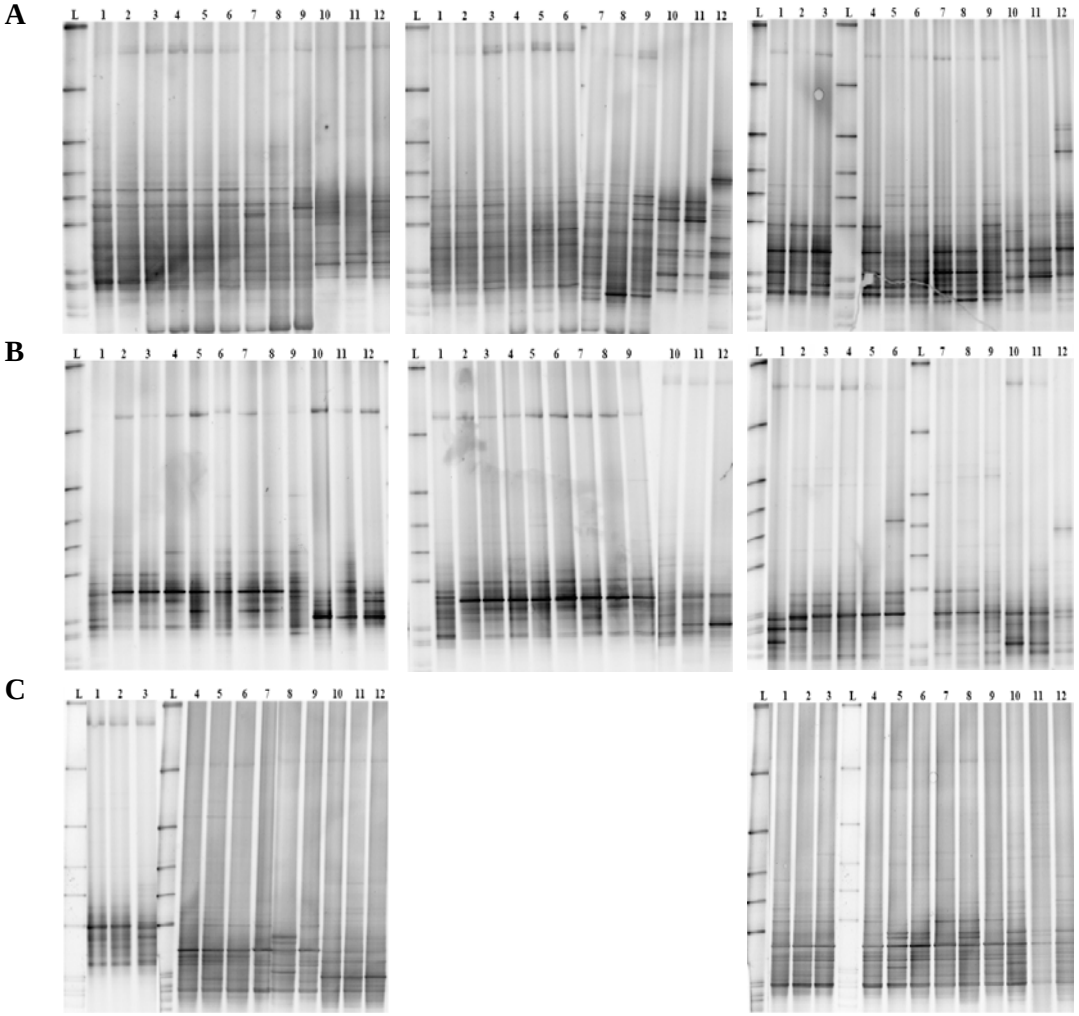


**Fig. 2 16S rRNA gene DGGE fingerprints of the actinomycetes community colonizing rice straw residues in 3 replicate plots undergoing the studied crop rotation systems at 14 days (left), 50 days (middle) and harvest (right) of crops I (A), II (B) and III (C)**
*Lanes 1-3: replicate plots undergoing system CRS1; lanes 4-6: replicate plots undergoing system CRS2; lanes 7-9: replicate plots undergoing system CRS3; lanes 10-12: replicate plots undergoing system CRS4. Lane L: reference bacterial 16S rRNA gene ladder.*

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Alternatively, the different environmental conditions implemented when different crops are cultivated can be a reason. Different crop species might produce different types of root exudates which supply nutrients to the microbial community (Miethling et al., 2000; Smalla et al., 2001; Marschner et al., 2001).

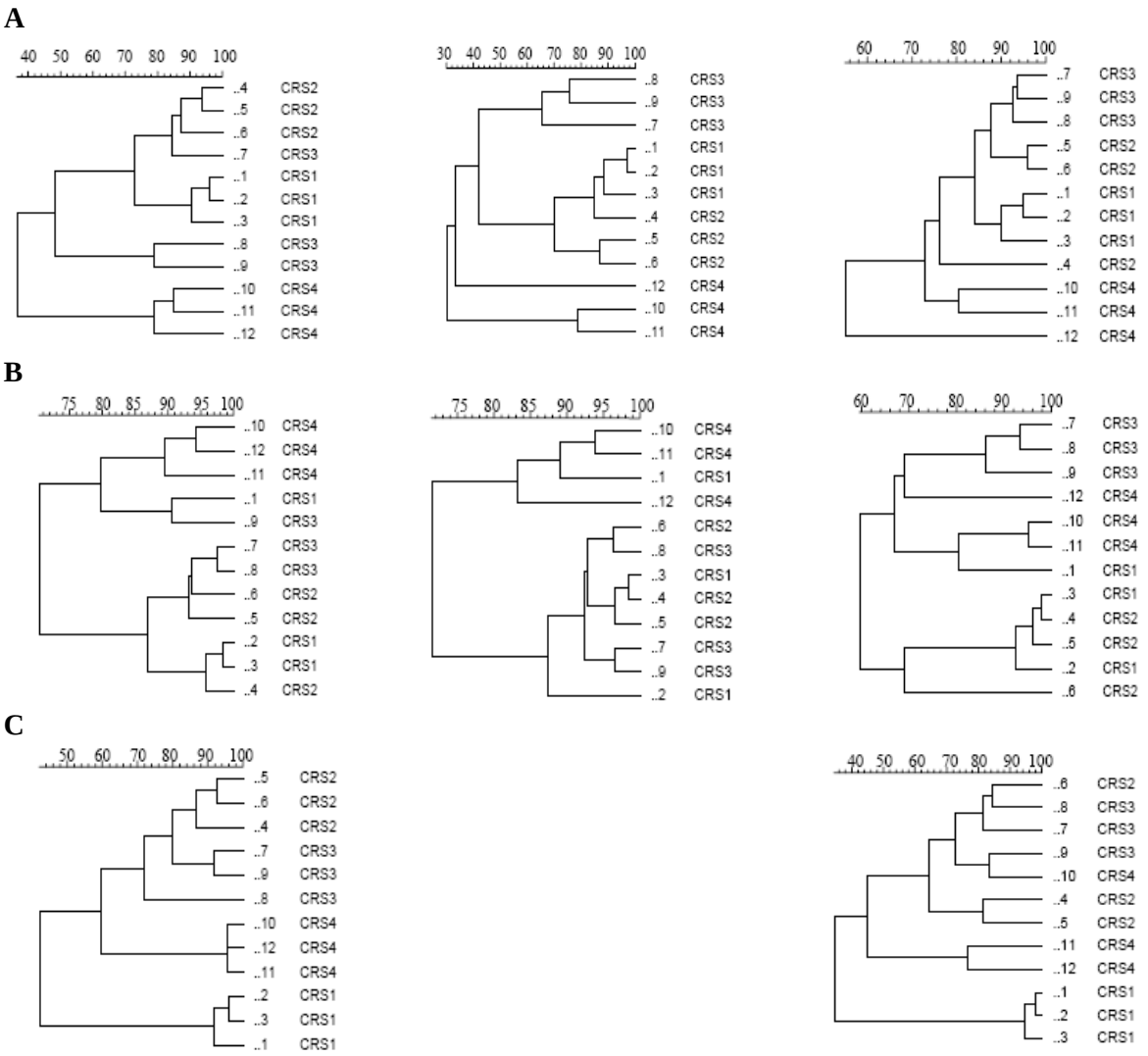


**Fig. 3 UPGMA clustering of actinomycetes 16S rRNA gene DGGE profiles recovered from rice straw residues in replicate plots undergoing the different crop rotation systems during cultivation of crops I (A), II (B) and III (C) at 14 days of incubation (left), 50 days of incubation (middle) and harvest (right), respectively**
*Indicated numbers correspond with lane numbers used in Fig. 3.*

Second, the main factor which drives actinomycetes community composition in the different crop rotation systems seems to be whether the soils were saturated and unsaturated with water, i.e., whether aerobic and anaerobic conditions were implemented. Indeed, during growth of crop I, profiles from systems CRS1, CRS2 and CRS3 clustered in one group different from profiles of systems CRS4. Crop I was rice for systems CRS1, CRS2 and CRS3 cultivated under saturated soil conditions while it was an upland crop for system CRS4 cultivated under unsaturated soil conditions.

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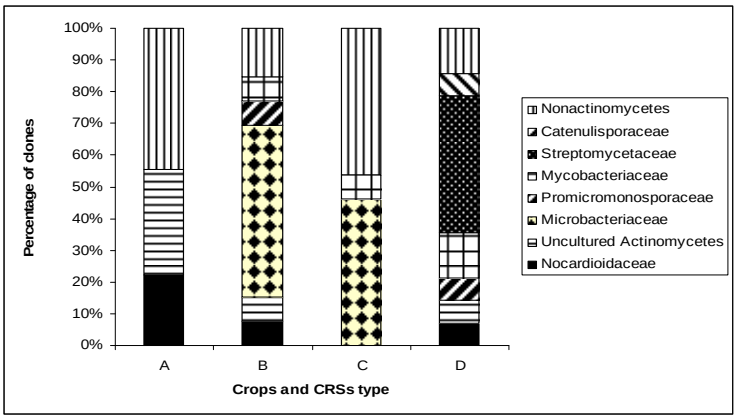


**Fig. 4 Fractions of recovered 16S rRNA gene sequences divided among different relevant bacterial families**

*The codes A and B indicate 16S rRNA gene libraries recovered from samples taken at 50 days of cultivation of crop I in rotation system CRS1 and CRS4, respectively. Codes C and D indicate 16S rRNA gene libraries recovered from samples taken at 50 days of cultivation of crop II in rotation systems CRS1 and CRS4, respectively.*

Previously, Reichardt et al. (2001) showed a negative correlation between the biomass of fungi, as primary microbial decomposers of crop residues, in soil and the soil-water content. Moreover, micro-site differences of aerobic or anaerobic conditions such as those influenced by the rhizosphere and residue decomposition were previously shown to affect the makeup of a soil microbial community (Lynch and Harper, 1985; Aulakh et al., 1991). However, no data exist on the effect of soil moisture content on Actinomycetes community structure.

As a third conclusion, differently structured actinomycetes communities were also observed between systems CRS4 on the one hand and systems CRS1, CRS2 and CRS3 on the other hand, during growth of crop II which was rice cultivated in water saturated soil in all systems. This was especially the case at day 14 of sampling while at day 50 and especially at harvest, major differences in community profiles disappeared. The differences in actinomycetes community profiles between systems CR4 and CRS1 during growth of crop II was also shown by the 16S rRNA gene library analysis. This might indicate that the type of rotation system affects the actinomycetes community profile despite similar cultivation conditions. It is well-accepted that crop rotations have an important effect on soil microbiology. Several studies indicated that crop rotation tend to result in a higher microbial diversity and/or biomass (Zelles et al., 1992, 1995; Drijber et al., 2000; Larkin , 2003; Collins et al., 1992; Kirchner et al., 1993; Olsson and Gerhardson, 1992). However, studies on the effect of crop rotation directed to the actinomycetes are rare. Martyniuk and Wagner (1978) found a higher number of actinomycetes in a rotation system including maize, oats, wheat and red clover than in continuous systems of maize or wheat. Similarly, Kirchner et al. (1993) found that the number of actinomycetes is significantly higher in soil undergoing maize-crimson clover rotation than in soil undergoing continuous maize cultivation. On the other hand, Collins et al. (1992) reported that actinomycetes biomass was significantly higher under monoculture wheat than under wheat-fallow rotation. On the other hand, it is possible that DNA of actinomycetes communities colonizing the rice straw during cultivation of crop I and which died off when anaerobic conditions where implemented, did remain under those conditions and resulted into amplification.

## CONCLUSIONS

Overall, our results show that the actinomycetes communities were affected by the crop rotation systems, but that the implemented environmental conditions rather than the rotation system as such affected the community composition. Further studies will identify more specific changes associated

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with particular rotations and relate these changes to potential effects on disease management, crop health, and crop productivity.

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