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Bacterial Microflora in Cultivated and Uncultivated Rice Fields and Their Effects on Rice Fungal Pathogens *In vitro*

Emelita P. Solante and Tomizo Ohguchi*
The United Graduate School of Agricultural Sciences,
*Laboratory of Physiological Plant Pathology, Faculty of Agriculture,
Ehime University, 3-5-7 Tarumi, Matsuyama 790-8566, Japan

Abstract: The population densities of the bacterial microflora in the soil and rice seed, samples collected from the cultivated (plowed) and uncultivated (unplowed) rice fields previously planted with hairy vetch, Chinese milk vetch (Cmv.) and clover were assessed during 1997 and 1998. The antagonistic effects of the bacterial isolates against different rice pathogens were also studied *in vitro*. The population density in soil of the cultivated field differed considerably from uncultivated one. The THB (Total Heterotrophic Bacteria) and *Bacillus* populations were higher in the cultivated field than the uncultivated one regardless of the crop planted previously. The THB population was lower in 1997 than in 1998. The trend was reverse for *Bacillus*. *Pseudomonas* had a high population in the uncultivated field in 1997 while it was high in the cultivated field during 1998. Rice seeds collected from cultivated fields harbored a high population of THB, *Pseudomonas* and *Bacillus*. However, bacterial populations were higher in 1997 than in 1998. In the soil, *Bacillus* was higher than *Pseudomonas* but the trend was reverse in the seed. Out of the 630 and 82 isolates from soil and seed samples, 130 and 45 isolates showed antagonistic activity against different rice pathogens *in vitro*, respectively.

Key words: Antagonistic activity, *Bacillus, Pseudomonas*, Total Heterotrophic Bacteria, cultivated and uncultivated fields

Introduction

The soil contains the rich microflora in which most resident antagonists are found. It is one of the sources of candidate biological control agents necessary to control almost all root pathogens (Baker and Cook, 1974) and the occurrence of disease merely shows that some necessary environmental condition is lacking. Depending upon the physical, chemical and biological qualities of soils, field soils are either suppressive or conducive (Scher and Baker, 1980). Several agronomic practices affect the physical, chemical and biological properties of the soil. These practices in turn may affect directly or indirectly the population of the soil microflora.

Soil cultivation is one of the agronomic practices that had a profound effect on the physical as well as the biological properties of the soil. Cultivated soils were found to stimulate microbial activity as shown by a higher census and an increase in the rate of soil respiration (Paul and Clark, 1989). Such stimulation results from the improvement of the physical properties of the soil as affected by tillage. The methods of tillage and of handling crop residue also affect the temperature and water potential of the soil (Baker and Cook, 1974), a practical means of changing the environment and perhaps nudging the biological balance in desired direction.

The microbes in no-till and plowed soil have been studied (Leong 1986) and the effect of cultivation on the infectivity of a microbial propagule is also known (Glenn and Sivasithamparam, 1990; Rovira, 1986; Weller *et al*., 1986). However, the bacterial microflora associated in cultivated and uncultivated rice ecosystem has not been explored. The antagonistic effects of these bacteria isolated from the soil and rice seeds against rice fungal pathogens have also not been established.

This experiment was conducted to assess the population density of the bacterial microflora in soil and rice seeds collected from cultivated and uncultivated rice fields previously planted with various legume crops. The antagonistic effects of these bacteria against important rice fungal pathogens were also investigated *in vitro*.

Materials and Methods

Collection of samples: Soil and seed samples were collected from cultivated (plowed) and uncultivated (unplowed) rice field of the Ehime University Farm which were previously planted with clover, hairy vetch and Chinese milk vetch. Five soil samples of approximately 500 g each were randomly collected from every plot for six sampling periods (July, August and October of 1997 and August, September and November of 1998). These samples were placed in plastic bags fastened with rubber bands and kept in a cold room for isolation. Seeds grown in the same fields were also collected one week before harvesting. Five panicles per plot were randomly collected and placed in plastic bags.

Isolation of bacteria: The dilution plate technique was used in isolating the associated bacteria from the soil and seed samples. The oven-dried 25 g soil from cultivated and uncultivated plots were determined. Ten grams of soil (based on oven-dry weight) were placed in 250-ml flasks containing 90 ml of distilled water. Serial dilution was prepared until 10^{-5} dilution. One-tenth ml of 10^{-4} and 10^{-5} dilutions was pipetted to solidified potato sucrose agar (PSA) or King's Medium B (KMB) plates for plating. The plates were incubated for 48 hr at 28°C after which bacterial colonies were enumerated by the use of an electronic colony counter. Bacterial colonies were grouped as *Bacillus, Pseudomonas* and Total Heterotrophic Bacteria (THB). Bacterial groupings were based on the cultural growth in PSA for *Bacillus* and in KMB for *Pseudomonas*. THB included all colonies belonging to different groups and were assessed on PSA plates.

The same method was used in the isolation of bacteria from seed samples. One gram of air-dried seed sample from a composite sample per plot was added to 9 ml sterile distilled water thoroughly shaken for 5 min with the use of a vortex stirrer. The diluted samples (0.1 ml) from 10^{-4} and 10^{-5} was spread on two PSA and KMB plates. Enumeration of bacterial colonies was the same as in the soil samples. Individual colonies of bacteria isolated from the soil and rice seeds in the first sampling were randomly picked and transferred to PSA slants for use in the *in vitro* tests.

***In vitro* test:** Bacteria isolated from the soil and seed samples were bioassayed for their antagonistic activity against different rice pathogens such as *Pyricularia oryzae, Rhizoctonia solani, Helminthosporium oryzae* and *Nigrospora* sp. using the dual-agar culture test. These isolates were individually tested for their ability to inhibit mycelial growth of the pathogen.

Three strains of *P. oryzae* (007 kita 1, 003 ken 54-04 and 003 ken 54-20) were used in the screening. Spore suspension of a sporulating culture of *P. oryzae* was prepared and mixed to melted PDA cooled to about 45°C and plated (Solante and Mew, 1994). After 12 hrs of incubation, four bacterial isolates were spotted on the edge of the agar plate. The fungus-bacteria dual culture was replicated three times and incubated at 28°C. Inhibition zones were measured after 5 days of incubation. The same method was used for the test against *H. oryzae.*

Two strains of *Rhizoctonia* were used in the test such as Toyama isolate and R 9701. Four bacterial isolates were spotted on the edge of the agar plates and an agar disk of *R. solani* was placed in the center (Mew and Rosales, 1986). The culture was replicated three times and incubated at 28°C. After 5 days of incubation, inhibition zones were measured from the edge of the mycelium to the margin of each bacterial colony. The same method was also used for the test against *Nigrospora.*

Results

Bacterial population in soil: The population densities of THB and *Bacillus* were higher in cultivated fields than the uncultivated one every year regardless of the crop planted previously (Fig. 1 and 2). THB population was lower in 1997 than in 1998. The trend was reverse in the case of *Bacillus.* The highest population of THB was 84.9×10^5 cfu/g soil collected from plots previously planted with clover in the cultivated field in the first sampling of 1998 (Fig. 1). A different trend was observed in the case of *Pseudomonas*. The *Pseudomonas* population was higher in 1998 than in 1997. In 1997, samples from uncultivated fields had a higher population of *Pseudomonas* than from the cultivated ones (Fig. 3). The reverse trend was observed in 1998, with a higher *Pseudomonas* population in cultivated fields than in uncultivated ones. In 1997, there was a trend of *Pseudomonas* population increase from the first to the third sampling while a decreasing trend was observed in 1998. Generally, *Pseudomonas* population was relatively lower than *Bacillus* in both cultivated and uncultivated fields.

Bacterial population in rice seeds: The population densities of THB, *Pseudomonas* and *Bacillus* were higher in rice seeds collected from cultivated fields than in uncultivated ones in all samples (Fig. 4).The population of THB, *Bacillus* and *Pseudomonas* were higher in rice seeds in 1997 than collected in 1998. In 1997, rice seeds of cultivated plots previously planted with hairy vetch and clover, harbored high populations of THB, *Pseudomonas* and *Bacillus*. The uncultivated fields showed different trends between the years. The population of *Pseudomonas* was always relatively higher than *Bacillus* both in cultivated and uncultivated fields. The highest population of *Pseudomonas* and *Bacillus* were 126.7×10^5 Cfu/g seed and 16×10^5 cfu/g seed, respectively in 1997. In 1998, 31.5×10^5 cfu/g seed was the highest population of *Pseudomonas*, while it was 4.6×10^5 Cfu/g seed for *Bacillus*.

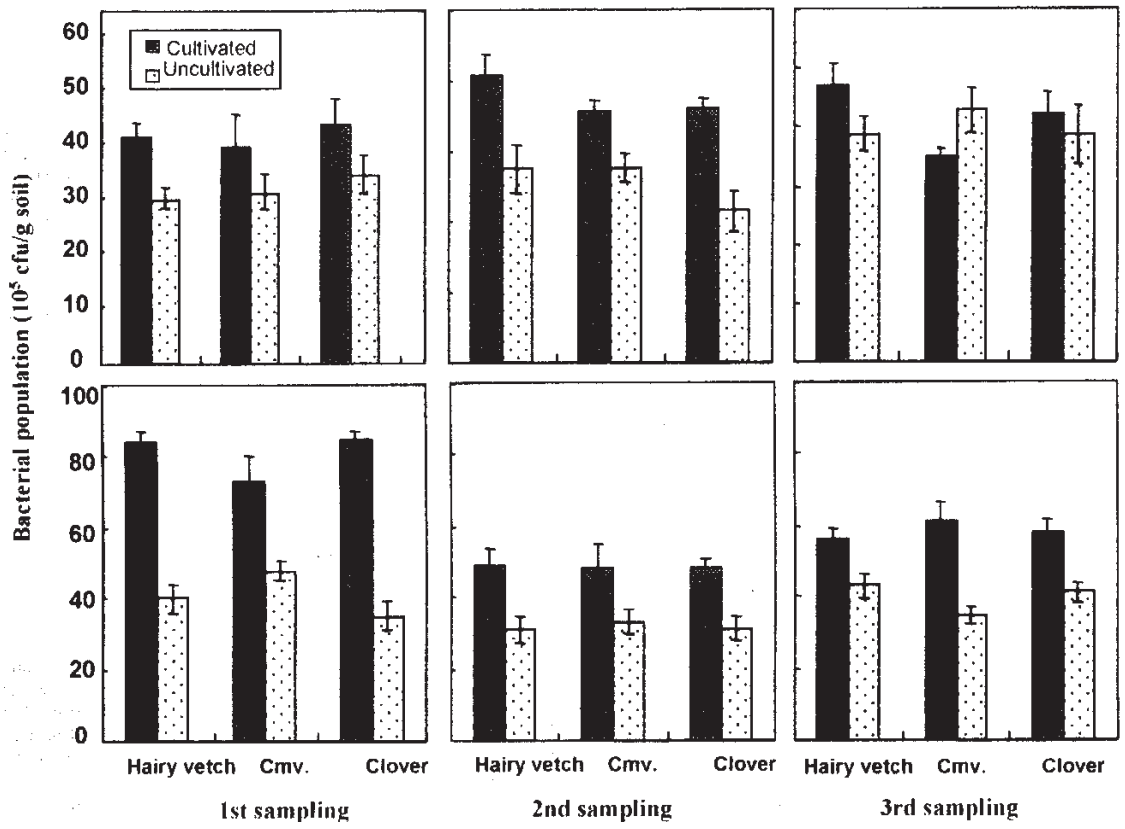

Fig. 1: Population of the Total Heterotrophic Bacteria (THB) of soil samples collected from cultivated and uncultivated rice fields at Ehime University Farm planted previously with various legume crops for two years. Bars are standard deviations of five replications

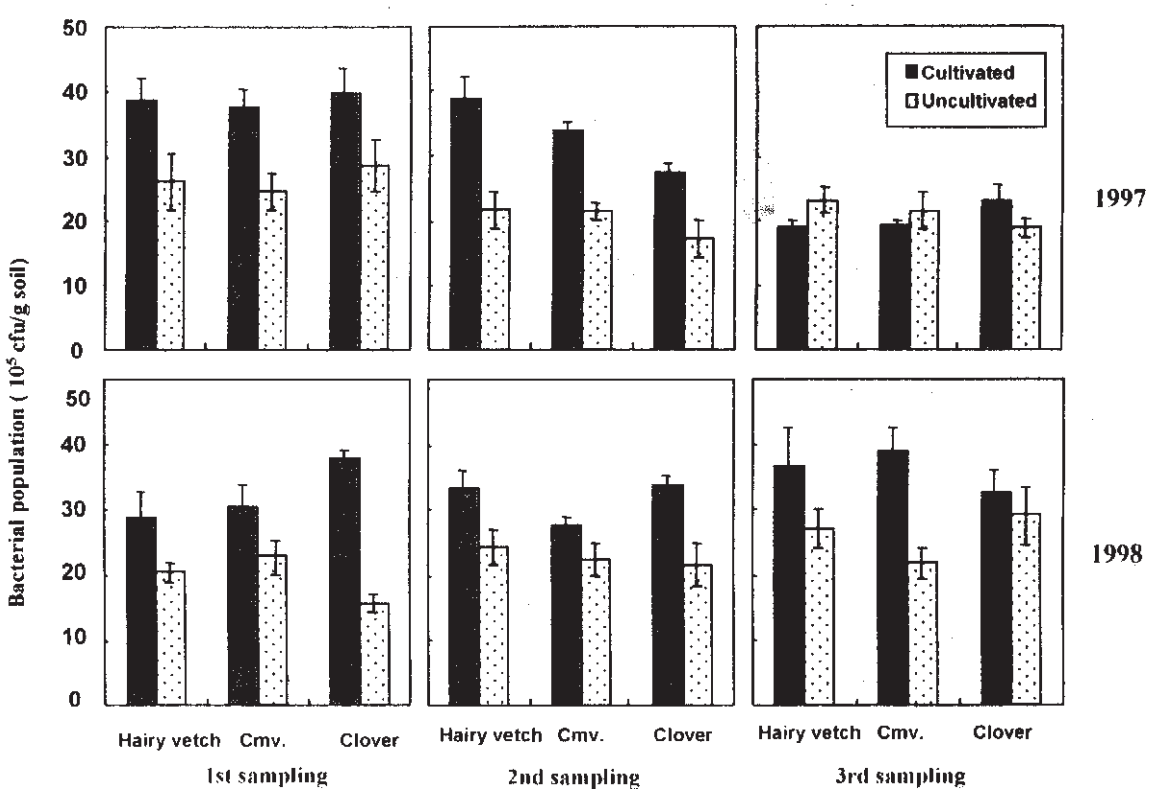

Fig. 2: *Bacillus* population of soil samples collected from cultivated and uncultivated rice fields at Ehime University Farm planted previously with various legume crops for two years. Bars are standard deviations of five replications

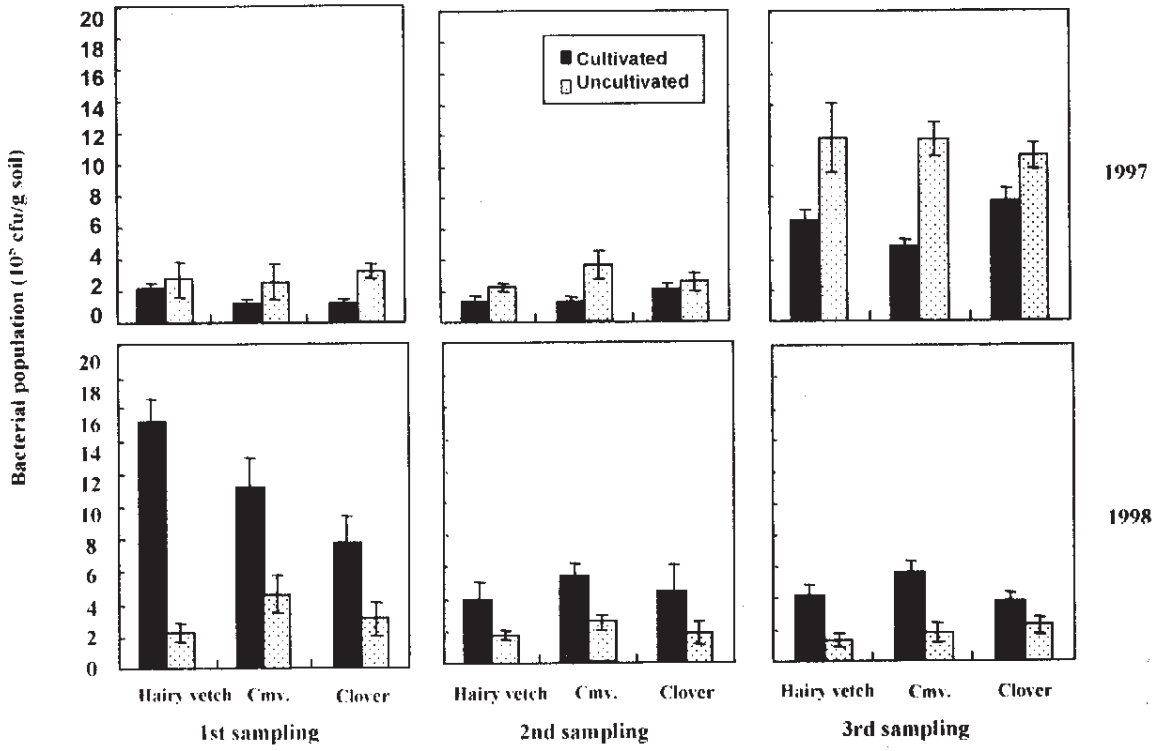

Fig.3: *Pseudomonas* population of soil samples collected from cultivated and uncultivated rice fields at Ehime University Farm planted previously with various legume crops for two years. Bars are standard deviations of five replications

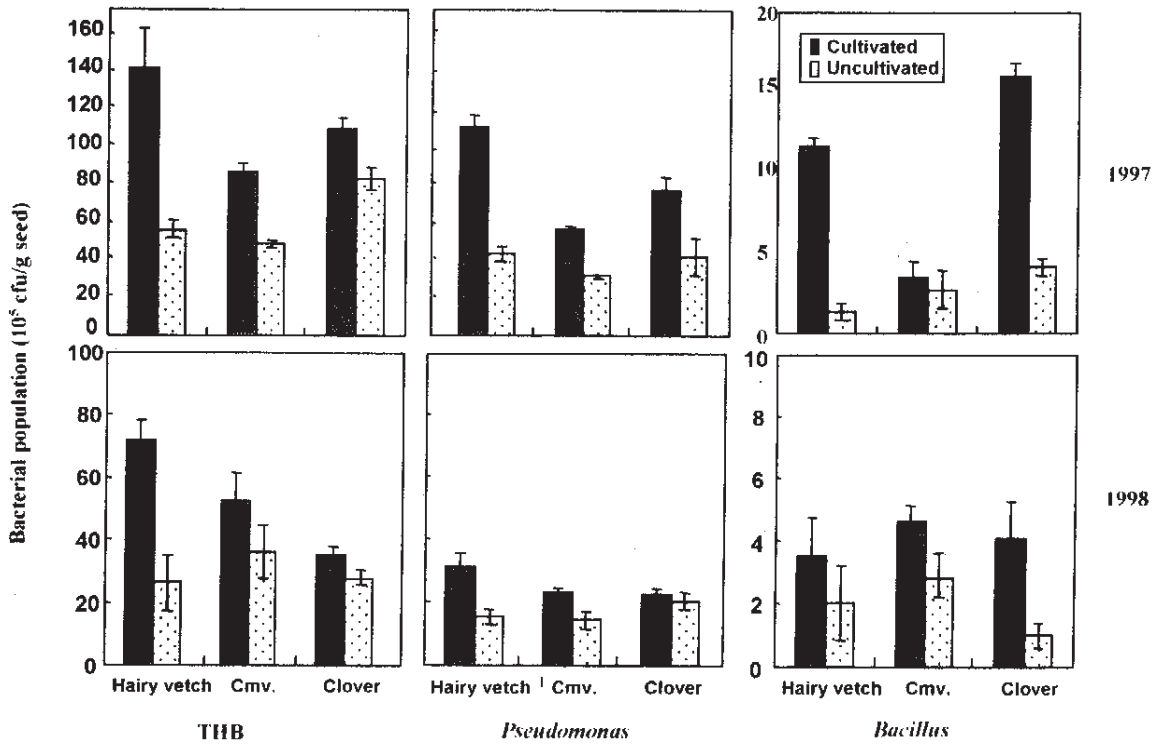

Fig. 4: Bacterial population of rice seeds collected from cultivated and uncultivated rice fields at Ehime University Farm planted previously with various legume crops for two years. Bars are standard deviations of five replications

Table 1: Antagonistic activity of bacterial isolates associated with soil samples collected from cultivated and uncultivated rice fields against different rice pathogens *in vitro*

Test Pathogens	Antagonists		Inhibition Zones[a]	
	Number	%[b]	Max	Min
Pyricularia oryzae (007 kita 1)	97	14.7	42.0	3.0
Pyricularia oryzae (003 ken 54-20)	84	12.7	36.0	3.0
Pyricularia oryzae (003 ken 54-04)	72	10.9	40.0	4.0
Rhizoctonia solani (R 9701)	81	12.2	40.0	7.0
Rhizoctonia solani (Toyama)	73	11.0	42.0	6.0
Nigrospora sp.	70	10.6	38.0	5.7
Helminthosporium oryzae (KU-13)	75	11.3	34.0	5.0

[a]Average of the three replicate plates in the dual-agar culture test
[b]Based on the total number of isolates

Table 2: Antagonistic activity of bacterial isolates associated with seed samples collected from cultivated and uncultivated rice fields against different rice pathogens *in vitro*

Test Pathogens	Antagonists		Inhibition Zones[a]	
	Number	%[b]	Max	Min
Pyricularia oryzae (007 kita 1)	43	52.4	20.3	4.0
Pyricularia oryzae (003 ken 54-20)	21	50.0	37.7	3.0
Pyricularia oryzae (003 ken 54-04)	33	40.2	25.7	3.0
Rhizoctonia solani (R 9701)	22	26.8	33.3	4.7
Rhizoctonia solani (Toyama isolate)	26	31.7	30.7	9.0
Nigrospora sp.	25	30.5	32.0	7.3
Helminthosporium oryzae (KU-13)	20	24.4	20.7	6.7

[a]Average of the three replicate plates in the dual-agar culture test
[b]Based on the total number of isolates

Antagonistic activity of the bacterial isolates *in vitro:* The antagonistic activity of the bacterial isolates of cultivated and uncultivated rice field soils differed among the test pathogens and within their strains (Table 1). This was exhibited by the difference in the number of antagonists per pathogen as well as the inhibition zones formed by the antagonists. Six hundred thirty (630) bacteria were isolated from the soil samples. Out of this, 130 isolates were found to be antagonistic against different rice pathogens. Ninety-one antagonists out of 455 isolates and 39 out of 175 isolates were obtained from the cultivated and uncultivated fields, respectively. The *in vitro* test revealed that the greatest number of antagonists showed an inhibitory effect against *P. oryzae* (strain 007 kita 1) while *Nigrospora* sp. had the least number of antagonists. Among the strains of *P. oryzae*, 007 kita 1 had the highest number of antagonists while 003 ken 54-20 had the least. The biggest inhibition zone was 42.0 mm exhibited by isolates effective against *P. oryzae* (strain 007 kita 1) and *R. solani* (Toyama isolate). Moreover, the smallest inhibition zone was 3.0 mm.

The same trend was observed in terms of the antagonistic activity of bacterial isolates from seed samples (Table 2). From rice seeds, 82 bacteria were isolated. *In vitro* test revealed that 45 isolates (54.87%) were effective against rice pathogens exhibited by the presence of inhibition zones. Out of 45 antagonists, 33 were obtained from seeds of the cultivated field using 50 isolates. On the other hand, 12 antagonists were obtained from uncultivated

field out of 32 isolates. *P. oryzae* (strain 007 kita 1) had the highest number of antagonists (52.4%) of the total isolates (Table 2). However, *H. oryzae* had the least number of antagonists (24.4%). On the basis of inhibition zones, an isolate effective against *P. oryzae* (strain 003 ken 54-20) exhibited the biggest inhibition zone of 37.67 mm while the smallest was 3.0 mm of isolates inhibiting *P. oryzae* (strains 003 ken 54-20 and 54-04).

Discussion

This study revealed that the cultivated rice fields harbored higher population of bacterial microflora than uncultivated ones. This was reflected by higher bacterial counts of THB and *Bacillus* in soil samples from cultivated fields regardless of the previous legume crops planted. Likewise, THB, *Pseudomonas* and *Bacillus* population in seeds from cultivated fields were higher than in uncultivated ones. Tillage operation usually changes the physical structure of the soil resulting in an aggregated structure and loose condition. Such a condition increases soil aeration and infiltration of water and better exposure of the degradable materials (Foth and Turk, 1973; Paul and Clark, 1989). Improved soil conditions mentioned as a result of tillage were found to stimulate microbial activity, as shown by higher census counts and an increase in the rate of soil respiration (Paul and Clark, 1989).

The high population of THB in 1998 particularly in the cultivated field could be attributed to the increase in the population of *Pseudomonas* (Fig. 3) and other types of bacteria. This variation in the THB population could be due to the highly variable bacterial populations present in the samples. On the other hand, increased population of *Bacillus* in soils corresponded to the decreased levels of *Pseudomonas* (Fig. 2 and 3). The opposite trend was observed in seed samples, wherein an increase in *Pseudomonas* population was coupled by a decrease in the population of *Bacillus* (Fig. 4). A similar trend were reported by Aristizabal (1993) and Solante and Mew (1994) who worked on the bacterial population of plant surfaces of different rice cultivars and on rice blast lesions, respectively. The inversely proportional relationship in the population of *Bacillus* and *Pseudomonas* in soil and seed samples for both years led us to assume that antagonism exists between these two groups of bacteria. However, existence of antagonism was not verified in this study. Such phenomena could also be interpreted as a result of competition between the microorganisms occupying the same niche.

Pseudomonas population in 1997 was higher in samples from the uncultivated field than from the cultivated one. However, the opposite trend appeared to in 1998. A change was made in the cropping practice in the cultivated field using paper mulch to minimize the growth of weeds. This change might have had an indirect effect on the population of bacteria, particularly *Pseudomonas*, thus causing its population to increase in the cultivated field but this assumption deserves detailed study.

Rice seeds of the same field were found to harbor certain populations of bacteria. Soil microorganisms that suppress plant diseases were found to have evolved with plants and is a primary factor determining plant health (Schrth and Hancock, 1982). The population of bacteria in seed samples from the cultivated field was higher than in the uncultivated field as was in soil samples. However, the population of *Pseudomonas* was higher than *Bacillus,* which was the reverse case in the soil samples. It might be that *Pseudomonas* spp. are more competitive than *Bacillus* in the seeds or that the nutrients present in the seed surface favor the growth of *Pseudomonas*. However, the kind of nutrients present in rice seeds was not verified in this study. This result also indicates that the bacterial population in the soil does not determine the population of the bacteria in the aerial parts of the plants. The entirely different environmental conditions in the soil and plant surfaces also could be a plausible explanation for the difference in the bacterial population present in these samples.

In this study, a good number of antagonistic bacteria were isolated from soil and seed samples. However, the antagonistic activity of an antagonist differs from one pathogen to another and among the strains of the same pathogen. This could be due to the specificity of the antagonist. The study of Lewis and Fravel (1996) demonstrated the importance of specificity in biocontrol. Germination of isolate Sr-1 sclerotia of *Sclerotium rolfsii* was more affected by *Gliocladium virens* isolates than was germination of isolate Sr-3 sclerotia. Such differences in the effectivity of the antagonists among pathogens could be attributed to the morphological differences of the test pathogens.

In conclusion, the populations of THB, *Pseudomonas* and *Bacillus* differed between cultivated and uncultivated fields regardless of the legume crops planted previously. THB and *Bacillus* were higher in the cultivated field than the uncultivated one during the time of sampling. On the other hand, *Pseudomonas* had a high population in the uncultivated field in 1997 while it was high in the cultivated field in 1998. Rice seeds collected from cultivated fields harbored a high population of THB, *Pseudomonas* and *Bacillus.* In the soil, *Bacillus* was higher than *Pseudomonas* but the trend was reverse in the seeds. Out of the 630 and 82 isolates from the soil and seed samples, 130 and 45 isolates showed antagonistic activity against different rice pathogens *in vitro*, respectively.

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