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KAZUHIKO MIURA AND TOMIO YOSHIDA

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Microflora of the rice root zone in submerged Maahas clay soil

KAZUHIKO MIURA and TOMIO YOSHIDA
The International Rice Research Institute,
Los Baños, Laguna

Bacteria were the dominant microflora found in the rice rhizosphere of submerged Maahas clay soil. They increased rapidly soon after submergence. When organic matter was added to the soil, large increases in the bacterial population occurred in the rhizosphere during the early growth stages of the rice plant. Many of the bacteria in the rice rhizosphere were denitrifiers, acid-producers, or sulfate-reducers. The fungi were not numerous nor did the population change greatly. We found no evidence that significant numbers of micro-organisms exist inside the roots of the rice plant.

The rhizosphere is the zone in which the roots are directly associated with soil. It is generally considered as the root zone. The root zone consists of the root tissues, the rhizosphere soil, and the soil adjacent to the rhizosphere. Here various and numerous microorganisms have saprophytic, parasitic, or symbiotic relationships with plants.

Hiltner (1) first applied the term "rhizosphere" to the layer of soil whose microflora are affected by the plant root. Many workers after him have proposed definitions of the

rhizosphere. Tesic (2) divided the rhizosphere into three zones: 1) the rhizoplane—the root surface, 2) the rhizosphere—the soil adhering to the root, and 3) the edaphosphere—the soil farther away from the root. On the other hand Timonin (3) proposed subdividing the rhizosphere into three fractions: rhizosphere, rhizoplane, and histosphere. He referred to histosphere as the root tissues colonized by "bacteriorhizae" occuring inside the plant cells or by organisms growing in the free space between the root cells. Just as fungi, known as mycorrhizae, are symbiotically associated with the roots of plants, bacteria also live in close connection with plant root in a site such as mucigel (4).

Few studies involving microbiological surveys of the rice root zone under submerged conditions have been done (5, 6, 7). Kawata, Ishihara, and Iizuka (6) found that some microorganisms were closely associated with rice roots grown in a field. They identified the fungal isolates *Fusarium* sp. and yeast. The *Fusarium* isolates were pathogenic to rice plants. *Pythium* species also are known to be pathogenic to rice in flooded soil (8, 9). Sakai and Yoshida (10, 11) reported that unfavorable soil conditions and a very weak pathogen of *Pythium* sp. cause the rice seedling disease "murenae".

Wu (7) reported that the application of organic matter, such as rice straw and green manure, to flooded soil is harmful to the growth of rice, probably because the soil becomes highly reduced. The biological decomposition of organic matter results in phytotoxic by-products, and may harbor certain microorganisms.

In some tropical areas, farmers incorporate large amounts of rice straw and residues into rice paddy soils. In others, the weeds that grow luxuriantly during fallow periods are incorporated into the paddy before transplanting. Addition of such organic matter to paddy soil may enhance microbial infection of the root tissue and result in the early retardation of the growth of the rice plant.

This paper reports a study undertaken to examine the microbial transformation in the rice rhizosphere, particularly transformation affected by organic materials applied to flooded soils.

Materials and methods

Soil samples. Air-dried Maahas clay soil from the IRRI experimental farm (pH, 6.6; organic matter, 2.0%; total nitrogen, 0.13%) was used throughout the experiments. Ten kilograms of air-dried Maahas clay soil were placed in porcelain pots and submerged with tap water. Fifty grams of organic matter was mixed with the soil. *Sesbania* sp., dried and ground into a powder, and cellulose powder were used as the source of organic materials at a level of 0.5 percent (dry weight basis). Soil prepared without addition of organic matter served as the control. Each treatment was replicated three times.

Seeds of *Oryza sativa* cv. IR8 were soaked in 2 percent formaldehyde solution for 30 minutes and grown for 2 weeks in a nursery culture solution (12). The culture solution was replaced with a fresh solution every other day. Sixty 2-week-old seedlings were transplanted in each pot in the greenhouse.

At each sampling, rice roots with soil attached to them were placed in 500-ml Erlenmeyer flasks. To obtain a rhizosphere suspension, the soil clinging to the roots was removed by dipping the roots in sterile water in another Erlenmeyer flask under aseptic conditions. The flask containing the suspension was shaken for 15 min to get a uniform suspension for use in microbial counting.

To study the effect of ammonium sulfate and green manure on the microbial transformation of submerged soil, the soil was submerged in distilled water for 3 weeks in a porcelain pot in the laboratory. The presubmerged soil was treated with 0.5 percent (dry weight basis) green manure or with 400 ppm of ammonium sulfate, or with both. Soil prepared without organic matter served as the control.

Ten-gram samples of each of the treated soils were placed in 50-ml beakers. Distilled water was added to a depth of 3 cm to keep the soil submerged. The samples were incubated in the dark at 30 C under anaerobic conditions with nitrogen gas according to the method of Parker (13). After 1, 2, 3, and 5 weeks of incubation, the samples were taken from the jar and analyzed for microbial population by the plate-count and the dilution frequency methods.

Root samples. Except for some modifications, the procedure used for preparing the root samples was essentially the same as that reported by Ishizawa et al. (14) and by Timonin (3). Soil particles and organic debris were washed from the roots under running tap water. Two grams of the fresh roots were immersed in 100 ml sterile water in a 500-ml Erlenmeyer flask. The flask was gently shaken for 5 min. The roots were washed several times, with fresh sterile water. Then the roots were soaked in 0.1 percent mercuric chloride solution for 1 min and rinsed with sterile water several times.

The roots were placed on sterile paper to dry and were cut with sterilized blades into sections several millimeters long. The root sections were blended at high speed with sterile water in a semi-micro Waring Blendor (Eberbach Corporation) for 2 min. The microorganisms in the resulting suspensions of macerated root tissues were counted.

In another experiment we measured a number of microflora and the organic-acid producing activity of bacteria in roots grown in Maahas clay soil and in roots grown in an Akiochi soil (pH, 4.9; organic matter, 3.2%; total nitrogen, 0.19%) collected from Bo-Eoun Gun, Korea.

To check the validity of the root-tissue sampling method, a serial washing technique (15) was used. Pieces of roots were placed in water and freed of soil and debris. The roots were then cut into sections 3 cm long, and 10 of these pieces were each placed in a 30 ml phial containing 20 ml sterile water. The phials were shaken for 1 min for 20 times. After each shaking a number of bacteria in the phials were counted, then the water was replaced with another 20 ml of fresh, sterile water. Finally the root pieces were macerated with the sharp edges of broken glass rods and suspended in sterile water. The numbers of microbes in the suspensions were counted.

Microbial analysis. One milliliter of the suspension of soil or macerated roots at each dilution was placed in petri dishes or test tubes containing media for each microflora. The number of aerobic bacteria, including facultative aerobic bacteria, was determined by the plate-count method with a medium that was similar to "the soil extract medium" of Bunt and Rovira (16). It contained 0.4 g K_2HPO_4, 0.5 g $(NH_4)_2HPO_4$, 0.05 g $MgSO_4.7H_2O$,

0.1 g $MgCl_2$, 0.01 g $FeCl_3$, 0.1 g $CaCl_2$, 1.0 g peptone, 1.0 g yeast extract, 0.01 g 2,3,5-triphenyl tetrazolium chloride, 15 g agar, 250 ml soil extract, and 750 ml distilled water. The pH was adjusted to 7.4. The medium was steamed for 30 min, then filtered and autoclaved for 20 min at 115 C. The inoculated agar plates were incubated at 30 C. The tetrazolium compound colored the colonies and made them easy to see on the agar plates.

The medium used for the anaerobic bacteria was the same as that used for aerobic bacteria except that it had 0.5 g thioglycolate, 0.002 g methylene blue, and no 2,3,5-triphenyl tetrazolium chloride. The medium was steamed for 30 min and filtered; 10 ml was poured into 15- x 125-mm test tubes and autoclaved for 20 min at 115 C. The inoculated test tubes were incubated at 30 C under anaerobic conditions with nitrogen gas by the Parker's method.

The number of denitrifiers was determined by the most probable number method with the medium described by Alexander (17). Solutions A and B were mixed, and the pH of the resulting solution was adjusted to 7.0 to 7.2. Solution A contained 1.0 g KNO_3, 1.0 g asparagine, 5.0 ml 1-percent alcoholic solution of bromthymol blue, and 500 ml distilled water. Solution B contained 8.5 g sodium citrate, 1.0 g KH_2PO_4, 1.0 g $MgSO_4.7H_2O$, 0.2 g $CaCl_2.6H_2O$, 0.05 g $FeCl_3.6H_2O$, and 500 ml distilled water. Ten milliliters of the liquid medium was poured into 15-x 125-mm test tubes containing Durham's tubes to detect gas formation in the inoculated medium. The medium was autoclaved at 121 C for 15 min. Inoculated tubes were incubated at 30 C and observed for active gassing and change in color to blue. Five test tubes from each dilution were inoculated. The number of sulfate reducers was examined by the most probable number method with the medium devised by Abd-El Malek and Rizk (18). The medium contained 3.5 g sodium lactate, 1.0 g NH_4Cl, 0.5 g K_2HPO_4, 2.0 g $MgSO_4.7H_2O$, 0.5 g Na_2SO_4, 0.1 g $CaCl_2$, a trace of Mohr's salt, and 1.0 liter distilled water. The pH was 7. Seven milliliters of the medium were placed in 15- x 25-mm test tubes, each containing an iron nail 3 cm long, 0.3 to 0.4 cm thick. The test tubes were autoclaved at 121 C for 15 min. Inoculated tubes were incubated at 30 C. Every dilution was further

diluted by 10 times. Fungi were counted through use of a corn meal agar medium: 20 g of corn meal powder mixed in 1.0 liter of distilled water, steamed for 1 hr, and filtered. Twenty grams of agar was added to make a total volume of 1.0 liter. The medium was autoclaved at 115 C for 20 min. Streptomycin sulfate was added at 300 ppm just before the medium was used. The pH was between 5.0 and 6.0.

To detect the presence of fungi belonging to the genus *Fusarium* and *Pythium* in macerated root tissue, two media were used. The medium for *Fusarium* contained 7.8 g potato dextrose agar (Difco), 17 g agar in 1.0 liter water, 1 g pentachloronitrobenzene, and 300 ppm of streptomycin sulfate, with the pH adjusted to 4.0. The medium for *Pythium*—the corn meal agar medium or Czapeck-Dox agar medium—contained 2.0 g $NaNO_3$, 1.0 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4.7H_2O$, 5.0 g yeast extract, 10 g glucose, and 15 g agar in 1.0 liter distilled water, with the pH adjusted to 4.2. The agar plates were incubated at room temperature (about 20 C) in the laboratory.

The hyphal growth of fungi in flooded soil was studied by the contact slide method (19). Fourteen glass slides for microscopy were buried in submerged Maahas clay soil in 1-liter beakers. Six IR8 seedlings, grown in nursery culture solution for 2 weeks after germination, were transplanted in the soil. Another set of 14 slides was buried in the soil in other beakers and five seedlings were transplanted in the soil in each beaker. After 5 or 10 days the slides were carefully removed from the flooded soil and dried over a water bath. Large dried soil particles were removed from the slides by gently tapping the slides on the bench top. The slides were rinsed with water, immersed in acetic acid for 3 min, and then immersed in the stain solution of phenolic rose bengal for 10 to 20 min. The stained slides were examined under the microscope. Aerobic and anaerobic bacteria, fungi, actinomycetes (20), denitrifying bacteria, sulfate-reducing bacteria (21), and nitrifying bacteria (22) were counted in the root samples. The acid-producing bacteria in the rhizoplane were counted by the method of Louw and Webley (23). The organic-acid production by the bacteria was also examined. Seven

grams of freshly prepared root samples taken from Maahas clay soil and the Korean soil 6 weeks after transplanting were incubated with a 150-ml soil-extract solution with and without glucose in 250-ml Erlenmeyer flasks under aerobic and anaerobic conditions at 30 C on a rotary shaker. After 1, 2, and 3 days of incubation, 10 ml of the solution was extracted with 10 ml ether in a separatory funnel. Ten microliters of ether fraction was analyzed by gas chromatography for the lower volatile fatty acids (24).

Results

At 2, at 11, at 17, at 40 days after transplanting the numbers of each microflora, i.e. aerobic bacteria, anaerobic bacteria, denitrifiers, and fungi in the rhizosphere soil were counted. The number of fungi decreased within 2 days after submergence after which it did not change significantly, suggesting that most of the fungi were inactive in the soil (Fig. 1).

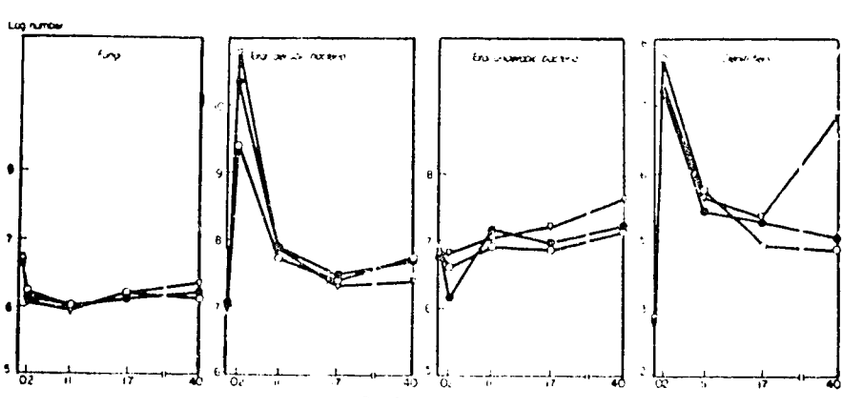

Fig. 1. Number of microorganisms per gram of dry soil from the rhizosphere of rice growing in paddy soil with or without amendment: soil without amendment (●), soil with 0.5% cellulose (○), soil with 0.5% green manure (▽).

No hyphal growth of fungi occurred on the glass slides buried in the submerged Maahas clay for 10 days. But a thin hyphal structure of actinomycetes was observed on the slides

under the microscope. The other slides buried in Maahas clay soil in which several rice seedlings were transplanted showed traces of root growth but only a negligible amount of hyphal growth.

The population of aerobic bacteria, including facultative anaerobic bacteria, in the rhizosphere of each treated soil markedly increased during the 2 days after transplanting. In pots with green manure and in pots with cellulose, the population of aerobic bacteria in the rhizosphere was several thousand times greater than the population in the initial soil samples. The control soil had 200 times more aerobic bacteria than the initial soil samples. The number of aerobic bacteria decreased, however, to about 10 million per gram of dry soil at 11 days after transplanting and remained steady thereafter.

In contrast with aerobic bacteria, the anaerobic bacteria did not increase rapidly during the early growth stages of the plant. But they increased gradually until 40 days after transplanting. The green-manure treatment had a significantly more total anaerobic bacteria in the rhizosphere than the cellulose treatment. The number of denitrifiers in the rhizo-

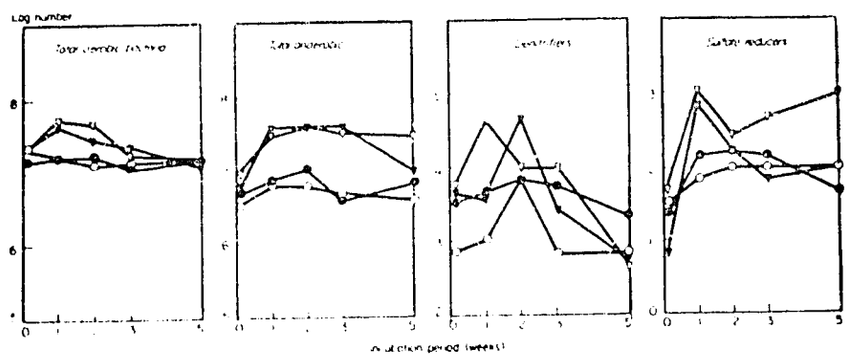

Fig. 2. Number of microorganisms per gram dry soil of flooded Maahas with or without amendment: soil with no amendment (●), soil with 400 ppm ammonium sulphate (○), soil with 0.5% green manure (▼), soil with 400 ppm ammonium sulfate and 0.5% green manure (▽).

sphere also increased soon after transplanting, then gradually decreased, except in the green-manure treatment, 40 days after transplanting.

Figure 2 shows the microbial changes in soil under anaerobic conditions in the laboratory. Anaerobic soil with green manure generally contained more bacteria than anaerobic without amendment, with ammonium sulfate, or with both ammonium sulfate and green manure. The population of aerobic bacteria was significantly higher in soil with green manure than in soil without green manure for 3 weeks after the start of incubation. The population of anaerobic bacteria was significantly higher in the soil with green manure than in untreated soil. The denitrifiers reached peak population after 2 weeks of incubation. When ammonium sulfate was added, this peak occurred after 1 week of incubation. An addition of ammonium sulfate did not significantly increase any microbial population. One week after the start of incubation the sulfate reducers had higher population in the soil with green manure than in the soil without green manure. Ammonium sulfate increased the number of sulfate reducers only in the presence of green manure; the soil with green manure and ammonium sulfate had the highest population of sulfate reducers among the soil treatments.

The microflora in tissues of plant roots in pots that received the same soil treatments at 0, 7, 14, 25, and 35 days after transplanting were studied. There were at most several hundred of fungi per gram of fresh root throughout the experiment, except in nursery seedling roots which on the day of transplanting contained 4.8×10^3 fungal propagules per gram of fresh root. None of these fungal species were *Pythium* sp. and *Fusarium* sp., however. Table 1 shows the total number of aerobic bacteria and anaerobic bacteria. A week after transplanting there were more aerobic bacteria in the macerated root tissues grown in the soil treated with green manure and in the soil treated with cellulose than in the control soil. The number of anaerobic bacteria in roots was consistently higher in the soil treated with green manure than in the other soil treatments.

The validity of the root tissue sampling method was examined with a serial washing technique used on several differ-

Table 1. Number of microorganisms (per g fresh roots) in macerates of rice roots grown in flooded Maahas clay soil with and without amendment: I, soil without any amendment; II, soil with 0.5% cellulose; III, soil with 0.5% green manure. The values are the means with their corresponding fiducial limits at P = 0.05.

Sampling time (days after transplanting)	Treatment I	II	III
	Total aerobic bacteria (10^6)		
0	*	*	*
7	10 ± 8	260 ± 115	250 ± 55
14	23 ± 21	50 ± 38	65 ± 27
25	2 ± 1	9.2 ± 3	5 ± 1
35	26 ± 6	4.1 ± 1	5 ± 1
	Total anaerobic bacteria (10^5)		
0	10 ± 6	10 ± 6	10 ± 6
7	83 ± 31	660 ± 220	3110 ± 1730
14	95 ± 62	88 ± 43	1900 ± 770
25	120 ± 24	86 ± 24	310 ± 36
35	170 ± 74	130 ± 2	740 ± 210

* not determined

Table 2. Number of total aerobic bacteria that were released from the surface of the roots and remained in disinfected root tissue after 20 washings (number of bacteria per 1-cm long root).

Growth stage	Portion of roots	Washing 1st	3rd	7th	12th	20th	Macerate of roots
Nursery	Apexes	numerous	690	210	66	0	0
Tillering	Apexes	numerous	32	4	4	0	0
	Basal parts	numerous	94	230	4	0	0

ent parts of the root system. The total number of aerobic bacteria released from the root surface decreased with repeated serial washings (Table 2). Numerous microorganisms were released from the roots at the first washing, but the macerated roots did not show the presence of microorganisms. The same result was obtained in another experiment in which roots of seedlings 2 weeks after transplanting were serially washed three times, soaked in 0.1 percent mercuric chloride, rinsed with sterilized water, and macerated with the edge of a glass rod for the bacterial count. The data in Table 1, therefore, indicate the presence of bacteria in the surface of root but not in the root tissue.

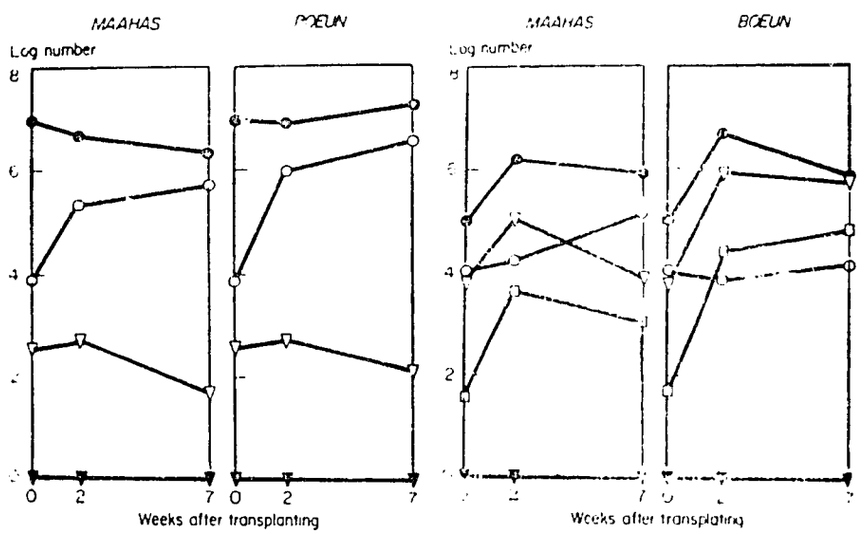

Fig. 3. (Left). Number of microorganisms in rice (IR8) root (histosphere) in two soils per gram of fresh roots: total aerobic bacteria (●), total anaerobic bacteria (○), fungi (▽), actinomycetes (▼).

Fig. 4. (Right). Number of functional groups of bacteria in rice (IR8) root (histosphere) in two soils per gram of fresh roots: acid-producing aerobic bacteria (○), acid-producing anaerobic bacteria (▽), denitrifying bacteria (●), H_2S producing bacteria (□), nitrifying bacteria (▼).

Figures 3 and 4 show the number of microflora in the rice histosphere samples grown in Maahas clay and in the Korean "Akiochi" soil. Bacterial flora were most abundant, fungi were scarce, and no actinomycetes were found (Fig. 3). A large number of aerobic bacteria were present throughout the study, the number of anaerobic bacteria increased after transplanting, particularly in the Korean soil. Many bacteria in the roots were acid-producers or denitrifiers (Fig. 4). No nitrifying bacteria were found. More sulfide-producing bacteria were found in rice roots in the Korean soil than in Maahas clay soil.

The experiment that measured the acid-producing activity of rhizosphere bacteria showed that very little of the volatile fatty acids was produced under aerobic conditions without glucose. Under anaerobic conditions with glucose, however, two kinds of volatile fatty acids, acetic and butyric, were detected (Table 3). More butyric acid was found than acetic acid, and more of both were found in roots in Korean soil

Table 3. Amounts of organic acid produced by excised rice roots*.

Incubation time (hr)	Organic acid	Rice grown in Maahas clay soil	Rice grown in Boeun soil
		nmole/100 g fresh material	
24	Butyric	0.1	0.1
	Acetic	0.0	0.0
48	Butyric	0.4	1.3
	Acetic	0.2	0.5
72	Butyric	1.7	3.9
	Acetic	0.4	0.5

* Rice plant: 6-week-old Peta in pot; Incubation: Anaerobic conditions under N_2 gas atmosphere.

than in roots in Maahas clay soil. Bacteria that produce organic acids inhabit the rice root and ferment carbohydrate to organic acids when the root environment becomes favorable for anaerobic metabolism.

Discussion

An anaerobic condition is a characteristic feature of paddy soils. Takai, Koyama, and Kamura (25) reported that anaerobiosis developed in submerged soil whose Eh decreased below zero within several days after submergence and that this correlated with the increases in population and activity of anaerobic bacteria. Our study showed that in the soils with green manure or cellulose the total number of anaerobic bacteria increased and the total number of aerobic bacteria also increased slightly, probably because the facultative aerobic bacteria increased in population. Applying organic material to the submerged soil also increased the bacterial population in the rice root zone for 1 to 2 weeks after flooding. Kumada (26) demonstrated that the rice roots are oxidative enough to give oxygen to luminescent bacteria. Mitsui (27) found that the oxygen was excreted from the rice root. This oxidative function of roots may stimulate the aerobic bacteria in the rice root zone.

Venkatesan and Rangaswami (28) reported that the cultivation of the rice crop greatly stimulated the population of bacteria in a field paddy soil during the early growth stages of the plant, and that of actinomycetes, and to some extent that of the fungi, in the plant's later growth stages.

Wu (7) reported that rice roots responded to organic amendments with a proliferation of aerobic and anaerobic tillering stage. Chen (5) also reported that microbes living on rice roots are greatly increased by the addition of organic matter, such as green manure.

In our experiments, fungal population showed almost no change. With the contact slide method no significant amount of hyphal growth was observed in the root zone. Thus the rice root may not influence the growth of the microflora in the root zone although the microflora may be present very near the roots. The bacteria are the major microflora in the rice root zone in submerged soils.

Timonin (3) described a method of getting a histosphere fraction of root samples. The histosphere is a site for microbes in the root tissue. This method, however, does not

adequately remove microorganisms attached to the roots. We found that sometimes even more than 20 repeated washings were required to remove them from the roots.

The results obtained with the serial washing technique showed that the bacteria counted in the root samples were not inside the roots but probably those firmly attached to the root surface or rhizoplane. The rhizoplane was defined by Clark (20) as the ecological niche or habitat provided for microorganisms by the root surface.

A large population of bacteria that produce organic acid in the rice rhizosphere should not be overlooked in the study of physiological disorders of rice. If organic acids are toxic to the rice plant and retard plant growth, the toxins would show the most direct effect in the rhizosphere. The injury to rice roots by organic acids has been reported as one cause of "Akiochi" which usually occurs in ill-drained paddy soil (29).

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