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Associated Nitrogen Fixation in the Rhizosphere of Rice in Saline and Reclaimed Saline Paddy Soil

1. Enumeration of aerobic heterotrophic bacteria associated in histosphere of grasses and rice

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干拓地土壤의 水稻根圈에서 協生窒素固定에 關한 研究

第1報 水稻 및 自然生雜草 根組織內 協生窒素固定菌의 分離同定

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Summary

The aerobic heterotrophic bacteria in the histosphere associated with grasses (Gramineae, Caryphyllaceae, Crucifereae) and rice cultivars in saline and reclaimed saline paddy soils were varied with species and rice cultivars.

The fraction of aerobic heterotrophic N_2-fixing bacteria to the total aerobic heterotrophic bacteria were averaged to eighteen percent in the histosphere of grasses and rice.

Acetylene reducing activity of these bacteria were ranged from 1 to 24 n mole/tube/hr. Most of the bacteria strains were predominated of hydrogen utilizing bacteria. The majority of these bacteria were closed to *Pseudomonas, Azospirillum, Klebsiella* and *Agrobacter*.

Introduction

The reclaimed saline paddy soils in coastal area of Korea are usually of low fertility.[5] Organic matter content and cation exchange capacity are comparatively low with coarse silty texture.[5] Since salt concentration is high with much of sodium ion showing high pH value, the excessive sodium ion gives unbalanced condition in soil solution in the rhizosphere and raises physico-chemical and physiological problems during the rice growing period.

The consecutive leaching to eliminate excess salts could result in deterioration of coagulated soil aggregation and loss of nutrient materials such as soluble organic matter, silica and minerals.[5] Use of calcium materials to improve chemical composition and coagulation preventing dispersion of fine particles during the leaching process and application of raw straws and compost might also change dynamics in soil plant system including life cycle of soil microorganisms.

Among many problems occured in the rhizosphere of a reclaimed saline paddy soils, low ammonium nitrogen content and low nitrogen efficiency draw much attention in view of nutritional evaluation of a soil to

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rice plants.[8] Soil analysis recommends heavy application of nitrogen fertilizer at dose of 120 to 180kg/ha for normal paddy.[7] Some farmers apply as much as 300 to 350kg/10a. Main reasons of this heavy application are due to high salt concentration depressing nitrogen uptake by plants and to low urease activity with poor nitrogen transformation in soil. The urease activity of a normal soil has been known as 300 ppm of soil but that of saline paddy soils has been reported as low as 20 ppm.[8] Therefore, frequent application with small doses during the growing stage is known as an effective way to increase nitrogen uptake efficiency and yield of rice.

The use of natural sources of organic materials and of nitrogen fixing microorganisms is a potential measure. In rice cultivation, practical interest is emphasized on associative nitrogen fixers, *Klebsiella, Azospirillum, Agrobacter*, and *Pseudomonas*, which inhabited on the root surface of grasses and rice plants making use of organic substances exudated from the roots or living in the root tissues.[3] Limited investigation, however, has been carried out on the histosphere of rice and grasses and little attention has been paid to saline paddy soils for increasing nitrogen fixing activity.

In a saline soil with high salt concentration, some grasses can actively grow even though exchangeable ammonium nitrogen exists only in order of several part per million as concentration. Possible sources of nitrogen would be irrigation water or biological fixation. Several kinds of nitrogen fixing bacteria have been identified in the histosphere of grasses, but little has been known about rice plant histosphere in reclaimed saline soils. This investigation was focussed on identification of the associative nitrogen fixing bacteria in histosphere of grasses living in saline and saline paddy soils, and to utilizer the nitrogen fixing capabilities in the histosphere of rice plants.

Mateirals and Methods

Plant sampling and Soil analysis

Fig. 1 shows the smpling sites of plants and soils. *Sagina maxima, Puccinellia chinnampoensis* and the *Oryza sativa* were collected from the Gwanghwa island (I), *Zoysia sinica, Miscanthus sacchariflorus* were collected from the sari (II), *Plantago depressa, Imperata cylindrica* were taken from the Taean (III) and Seosan (IV). *Ischaemum anthephoroides* and *Plantago lanceolata* were collected from Buan (V) and Cerastium *caespitosum var lanthes, Oryza sativa* and *Poa acroleuca* were collected from Muan (VI).

The rice plants grown in farmers paddy with fertilizered condition were sampled. Grasses grown in the upland nearby the paddy without fertilization were collected from the sites I, II, V and VI.

Rice plant roots were collected about 40 days after harvest. On the other hand, *Impereta cylindrica, Plantago depressa* were naturally grown in a tidal flat saline soil. All the grasses were sampled together with rhizosphere soil. The sample of the grasses and rhizos-

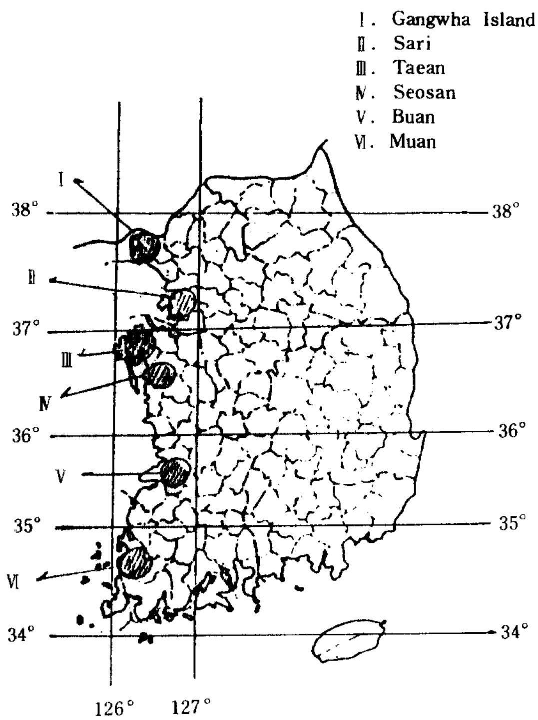

Fig. 1. The map of sampling sites of reclaimed saline soils and tidal flat saline soils.

phere soil was kept together in a polyethylene sampling sac.

Rhizosphere soil and rice roots were separated from grasses in laboratory. Separated rhizosphere soil was dried under shadow.

The pH (H_2O) was measured with Towa A-5 pH meter using glass and reference electrodes, the organic matter content was analyzed by the Turin's method and the total nitrogen by the Bremner's method, cation exchange capacity by the Chapman's method, available phosphorus by the Bray and Kurtz method, respectively. The cation compositions were determined by emission method using atomic absorption spectrometer (Instrumental Laboratory Inc., Model 65-1).

According to chemical analysis of sampled tidal flat saline and newly reclaimed saline paddy soils were characterized with high pH value, low organic matter contents with very low total nitrogen. The cation exchange capacity was range to 5.5-8.1, and very high total salt concentration. Specially, the composition of exchangeable cation, sodium, and magnesium ions were much more higher than potassium and calcium ion. The ratio of $K/\sqrt{Ca+Mg+Na}$ was abnormally lower than that of normal paddy soil.

Table 1. Chemical properties of rhizosphere soils with rice plant and grasses

Plant species	Soil condition	pH (1:5)	O · M (%)	Ava. P_2O_5 (ppm)	Exch. cat. (me/100g soil) K	Ca	Mg	Na	$\frac{K}{\sqrt{Ca+Mg+Na}}$	C.E.C (me/100g)	Total salt conc. (%)
Sagina maxima	Reclaimed saline paddy	6.8	1.1	172	0.32	4.1	2.14	6.09	0.09	8.1	0.10
Puccinellia chinnampoenis	〃	6.8	1.1	172	0.32	[illegible]	[illegible]	6.09	0.09	8.1	0.10
Oryza sativa	〃	7.5	1.2	106	0.28	4.0	5.78	7.39	0.07	8.0	0.28
Oryza sativa	〃	6.8	1.1	172	0.23	[illegible]	[illegible]	6.09	0.09	8.1	0.10
Zoysia sinica	Tidal flat saline soil	6.8	0.5	19	2.40	0.8	12.30	35.22	0.35	5.4	4.73
Miscanthus sacchariflorus	〃	8.3	0.5	19	2.40	0.8	12.30	35.22	0.35	5.4	4.73
Plantago depressa	〃	8.3	0.5	19	2.40	0.8	12.30	35.22	0.35	5.4	4.73
Imperata cylindrica	〃	8.3	0.5	19	2.40	0.8	12.30	35.22	0.35	5.4	4.73
Ischaemum anthephoroides	〃	8.3	0.5	19	2.40	0.8	12.22	35.22	0.09	5.4	4.73
Plantago lanceolata	Reclaimed saline paddy	8.1	0.6	53	1.78	[illegible]	7.89	18.70	0.34	9.6	2.91
Cerastium caespitosum var lanthes	〃	8.1	0.6	97	0.91	1.4	6.25	18.70	0.02	9.6	2.91
Oryza sativa	〃	7.9	0.4	25	1.19	1.1	5.10	13.48	0.27	5.5	1.05
Poa acroleuca	〃	7.9	0.4	25	1.19	1.1	5.10	13.48	0.27	5.5	1.05

Enumeration of aerobic heterotrophic N_2-fixing bacteria:

Roots of grasses and rice plants collected from saline paddy and saline soils were washed gently by running water to remove the bulk of rhizosphere and adhering soil, and then rinsed in sterilized water. Roots were cut into 1 cm length segments. Moisten roots surface was blotted gently with sterilized filter paper. One gram of fresh root segments was put into a sterilized 100 ml Erlenmyer flask containing 60 ml sterilized demineralizer water, and then the flask was shaked vigorously by hand about 5 min.

Four replicated samples were made. The roots segments were taken out from Erlenmyer flask and were put into sterilized 10 cm diameter motor with one ml sterilized water to be ground until juicy. One mililitre of succulant was taken for the most probable-number (MPN) technique using semi solid media. Another one mililiter of root succulant were inoculated into saline soil extract medium.

The medium consisted of KH_2PO_4 1.0 g; K_2HPO_4 2.2 g; $MgSO_4.7H_2O$ 0.2 g; NaCl 0.1 g; $CaCl_2$ 0.02 g; $FeCl_2 . 6H_2O$ 0.015 g; $(NH_4)_6 Mo_7O_{24} . 4H_2O$; 0.002 g; peptone 5.0 g; succinic acid 1.0 g; Fe-EDTA (1.6% in distilled water) 4 ml; saline soil extractant 400 ml and distilled water 600 ml; noble agar 20 g and pH was adjusted to 7.0.

Twenty mililiters of saline soil extractant agar were poured into a 10cm diameter petridish. After the petri dishes were incubated at 30°C for 24 hours, aerobic heterotrophic bacteria were counted one by one.

On the other hand, the aerobic heterotrophic bacteria were transfered into a 2.0cm diameter and 15cm length side armed test tube containing 15 ml tryptic soy broth (Difco)-1.5% noble agar (Difco) In the side armed test tube, ten percents of air were replaced by argon gas containing C_2H_2 2.0%, and CO_2 0.038% at the given pressures. Then test tube were closed and incubated at 30°C for 24 hr.

The C_2H_2-C_2H_4 assay:

A 1-ml gas sample was taken with a gas tight syringe through the stopper for analysis of C_2H_4 by gas chromatography (Shimadzu Gas Chromatograph GC-A). A 3mm x 2m long stainless steel column was used with 80-100 mesh Porapark-R at 45°C with He gas carrier. The flow rate was 45 ml/min. and a hydrogen-flame ionization detector was used.

Assay for different gas composition:

The 39 bacterial isolates were inoculated by semisolid tryptic soy agar medium in side armed test tubes in the gas consisted of 20% H_2, 5% CO_2, air 2% and 73% He. Series of duplicated side armed test tubes were filled with 5% CO_2, air 2%, the balanced He gas. The side armed test tubes were incubated at 30°C for 48 hours. After that, 10% of total gas were removed by a gas tight syringe through the rubber stopper and then 10% of C_2H_2-gas were refilled. The test tubes were incubated again for 24 hours. A 1 ml gas was analyzed by gas chromatography.

Results and Discussion

Numeration of aerobic heterotrophic N_2-fixing bacteria:

The acetylene reducing activities of 39 morphologically different bacterial species in histosphere of rice and grasses were shown in Table 1.

The 19 species of morphologically different bacterial species in histosphere of *sagina maxima* were separated. Among them, 10 species of acetylene reducing bacteria were isolated. The various species of bacteria were also compared for their ability to enhance N_2-fixing activity. The 7 bacterial species were showed activity over 10 n mole/ tube/hr. The bacteria isolated from the histosphere of rice and *Poa acrolenca* collected at Muan, the south west part of Korea, were comparatively various in kinds of histospere microorganisms. The population of aerobic heterotrophic N_2-fixing bacteria was also high.

Sagina mxima from Gwanghwa island, *Oryza sativa*, *Poa acroleuea* from Muan, showed more than nitrogen fixing bacteria, but the others did only one or two species of aerobic heterotrophic nitrogen fixing bacteria. The N_2-fixing activity of these bacteria, however, were very high.

Especially, aerobic heterotrophic N_2-fixing bacteria isolated from histosphere of *Cerastium caespitosum var ianthes* showed more than 20 n mole/tube/hr of acetylene reducing activity. In situ condition, there

Table 2. Population and Acethylene reducing activity of aerobic heterotrophic N_2-fixing bacteria from the histosphere of rice and grasses

Plant species	Number of identified bacteria[a]	Total NFB	NFB[-b] 10 mole	Strain no.
Sagina maxima	19	10	7	1－10
Puccinellia chinnampoenis	6	1	1	11－12
Oriza sativa[-1]	7	2	1	13－15
Oriza sativa[-2]	12	1	1	16
Zoysia sinica	15	1	1	18
Miscanthus sacchuriflorum	11	1	1	19
Plantago depressa	10	1	–	20
Imperate cylindrica	9	1	1	21
Ischaemum anthephoroides	7	1	1	22
Plantago lánceolata	14	2	2	23－26
Cerastium caespitosum var ianthes	16	1	1	27－34
Oryza sativa	14	6	6	29－34
Poa acrolenca	8	5	4	35－40

－a : morphologically different bacteria
－b : more than 10 umole/tube/hr

were very lower available nitrogen with very high salts concentration. In spite of this scarcity, this plant grew well and young plants contained about 2% of total nitrogen as dry matter bases. Implication was that aerobic heterotrophic N_2-fixing bacteria possibly supplied di-nitrogen to fixed nitrogen to their host plant. As aerobic heterotrophic N_2-fixing bacteria in histosphere of rice plant, even though N_2-fixation capability of one species is low, the numbers of aerobic heterotrophs and N_2-fixer were high, therefore, the cumulative N_2-fixing activity and the amount of fixed nitrogen were high. It seemed that in natural environment the nitrogen balance in plant tissue would be always equilibrated with the role of aerobic heterotrophic N_2-fixing bacteria in histosphere on the rhizoplane.

Assay for different gas composition:

The effects of gas composition on the growth and the abilities of acetylene reducing activity of bacteria isolated from the histosphere of rice and grasses were shown in Table 2.

Ten species of morphologically different aerobic heterotrophic N_2-fixing bacteria were isolated from the histosphere of *Sagina maxima*. Among them strain No. 2, 4^{-a}, 6^{-b} showed similar acetylene reducing activity in the gas composition with and without H_2 while strain No. 1, 5, 6^{-a}, 7, 8^{-a}, 8^{-b} and 9 were very high in the gas composition with hydrogen gas. The stain No. 3 showed high in the gas composition without hydrogen gas. On the other hand, strain No. 4 and 10 showed negligible acetylene reducing activity under gas composition either with or without hydrogen gas. Only one species of aerobic heterotrophic N_2-fixing bacteria was isolated from *Puccinellia chinnampoenis* in reclaimed saline paddy soil. This bacteria showed very negligible acetylene reducing activity under gas composition without hydrogen gas while high actylene reducing activity under gas composition of with hydrogen gas was recognized.

Bacteria isolated from histosphere of rice in paddy soil with sufficient amount of fertilizer nitrogen showed only three species of aerobic heterotrophic N_2-fixers. Among them the strain No. 12 and 13 showed high acetylene reducing activity under gas composition with hydrogen gas while strain No. 14 showed negligible both with and without hydrogen gas. However, the strain No. 17 showed the highest acetylene reducing activity

Table 3. Effects of different composition of gas on the aerobic heterotrophic N_2-fixing bacteria from the histosphere of rice and grasses

Strain no.	Composition of gas			
	CO_2 5% + Air 2% + H_2 93%		H_2 20% + CO_2 5% + Air 2% + He 73%	
	ARA nmole/tube	ARA nmole/tube/hr	ARA nmole/tube	ARA nmole/tube/hr
1	134	5.6	382	15.9
2	166	6.9	171	7.2
3	224	9.3	1	–
4-a	127	5.3	182	7.6
4-b	9	–	67	–
5	2	–	353	14.7
6-a	130	5.4	315	13.1
6-b	227	9.5	247	10.3
7	4	–	131	5.5
8-a	–	–	190	7.9
8-b	166	6.9	281	11.8
9	3	–	247	10.3
10	4	–	2	–
12	2	–	398	16.6
13	2	–	168	7.0
14	1	–	8	–
17	39	–	572	23.8
18	230	9.6	72	–
19	91	–	364	15.2
20	4	–	128	5.3
21	219	9.2	500	18.8
22	361	15.0	168	7.0
24	548	22.8	2	–
25	235	9.8	226	9.4
26	179	7.5	107	5.0
28	203	8.5	482	20.1
29	171	7.1	325	13.5
30	83	–	521	21.7
31	430	17.9	357	14.9
32	118	5.0	320	13.3
33	3	–	478	19.9
34	78	–	395	16.6
35	8	–	370	15.4
36-a	163	6.8	302	12.6
36-b	51	–	302	12.6
37	7	–	220	9.2
39	473	19.7	155	6.4

–a, –b : Same Nambered but different bacteria

among the 39 species. Only one species of aerobic heterotrophic N_2-fixing bacteria was isolated from histosphere of *Zoysia sinica*, *Miscanthus sacchuriflorum*, *Plantago depressa*, *Imperate cylindrica* and *Ischaemum anthephoroides*. The strain No. 18 showed high acetylene reducing activity under the condition with hydrogen gas while very low acetylene reducing activity under condition without hydrogen gas. In case of the strain No. 22, high acetylene reducing activity obtained under gas composition without hydrogen gas. In the case of *Plantago lanceolata*, two species of aerobic heterotrophic N_2-fixing bacteria were isolated. The strain No. 24 showed the highest acetylene reducing activity under the condition without hydrogen gas while revealed negligible acetylene reducing activity under the condition with hydrogen gas. The strain No. 25 and 26 showed very similar acetylene reducing activity under condition of with, without hydrogen gas.

Aerobic heterotrophic N_2-fixing bacteria isolated from *Cerastium caespitosum var ianthes* (No. 28) showed high acetylene reducing activity under the condition with hydrogen gas. Six strains of aerobic heterotrophic N_2 fixing bacteria were isolated from the histosphere of rice plants with normally applied fertilizer nitrogen on reclaimed saline paddy soil in the south western part of Korea. The most of bacteria among the isolated N_2-fixing bacteria showed very high acetylene reducing activity under the condition with hydrogen gas. The strain No. 31 showed high acetylene reducing activity under the condition with and without hydrogen gas.

Five aerobic heterotrophic N_2-fixing bacteria were isolated from *Poa acroleuca* which were grown in the reclaimed saline paddy with rice plants. Four species resulted more than 10 n mole/tube/hr. The strain No. 35, 36^{-b} and 37 showed high acetylene reducing activity under the condition with hydrogen gas while negligible acetylene reducing activity under the condition without hydrogen gas.

Under the experimental condition, the aerobic heterotrophic N_2-fixing activity associated with rice and grasses grown in saline and reclaimed saline paddy soil were varied with plant species. Six to nine species of aerobic heterotrophic bacteria were isolated. Among the bacterial strain, *Sagina maxima* was the highest aerobic heterotrophic bacteria and population of aerobic heterotrophic N_2-fixing bacteria were associated in histophsere, but only one or two species of aerobic heterotrophic N_2-fixing bacteria were isolated from the histosphere of other grasses. But the highest acetylene reducing activity was obtained in histosphere of rice. Specially, rice plants grown in reclaimed saline paddy soil in the south western parts, high population of aerobic heterotrophic N_2-fixing bacteria was identified.

Among the 39 morphologically different species of aerobic heterotrophic bacteria isolated from the histosphere or rice and grasses, five strains (No. 3, 18, 22, 28, 39) of acetylene reducing activity were high in condition without hydrogen gas but the most of bacteria grew well and high acetylene reducing activity was obtained under the condition with hydrogen gas. It seemed that those bacteria were unable to use hydrogen gas to provide energy and reducing equivalent for growth. And also their chemolithotrophy was suggested only from the capability of the growth under hydrogen gas mixture.

The population of aerobic heterotrophic bacteria associated in plant histosphere were high in graminaceous plant than others.

Gowda and Watanabe confirmed chemolighotrophic H_2-dependent N_2-fixation of several bacteria in and on histosphere/rhizoplane by N^{15}. H_2-supported N_2-fixation was also found when these bacteria were grown in association with rice seedlings.

Barraquio and Watanabe studied the population of aerobic heterotrophic N_2-fixing bacteria associated with rice root. They found that in addition to Spirillum-like bacteria in and on wetland rice roots. The population of these bacteria were ranged several to twenty percent to the total aerobic heterotrophic bacteria in roots.

References

1. Barraguio, W.L., Ladha, J.K. and I. Watanabe. 1983. Isolation and identification of N_2-fixing Pseudomonas associated with wetland rice. Can. J. Microbiol. Vol. 29, No. 8, p. 867-873.
2. Follet, R.H., Murphy, L.S. and R.L. Donahue. 981. Fertilizer and Soil Amendents. Prentice-Hall, Inc. p. 424-452.
3. Gowda, T.K.S. and I. Watanabe. 1983. Autoradiographic technique for screening chemolithotrophic hydrogen-utilizing bacteria. Can. J. Microbiol. Vol. 29. No. 9, p. 1081-1086.

4. ______________________ 1985. Stimulating effect of hydrogen on N_2-fixation in association with field grown wetland rice. 1985. Can. J. Microbiol. Vol. 31, No. 6, p. 503-507.

5. Jeong, Y.G. 1984. The influence of electric conductivity and inorganic salts on the growth of rice in saline soil. Ph. D thesis. p. 14-30.

7. Lee, C.H. 1986. Studies on determination of N-fertilizer rates for increasing rice yield in paddy soil. Research reports of O.R.D. Vol. 28, No. 2. p. 6-21.

6. Ladha, J.K., Barraquio, W.L. and I. Watanabe. 1982. Immunological technique to identify Azospirillum associated with wetland rice. Can. J. Microbiol. Vol. 28, No. 5, p. 478-485.

8. Lee, S.K., Kim, Y.S., Hwang, S.W., Park, J.K. and Y.S. Chang. 1985. Effect of rice straw and gypsum on the changes of Urease, nitrate reductase and nitrite reductase activities in saline paddy soil. J. Korean Soc. Soil Sci. and Fert. Vol. 18, No. 1 p. 105-110.