한국농화학회지 Vol. 29, No. 2, June 1986
J. Korean Agricultural Chemical Society

Dissimilatory Nitrate Reduction Characteristics of Indigenous Soybean *Rhizobia* Distributed in Korean Soils

Young Ju-Choi, Yong-Lark Choi, Han-Dae Yun, Jin-Chang Ryu*
Sang-Kyu Lee* and Moo-Je Cho

Department of Agricultural Chemistry, College of Agriculture, Gyeongsang National University, Chinju 620, *Institute of Agricultural Science, Suwon 170, Korea
(Received Feb. 19, 1986)

한국에 분포되어 있는 토착대두 근류균의 질산 환원 특성

최영주 · 최용락 · 윤한대 · 유진창* · 이상규* · 조무제

경상대학교 농과대학 농화학과, *농촌진흥청 농업기술연구소

요 약

연속적으로 대두를 재배한 토양과 신개간지 토양에서 재배한 대두 품종 단엽의 근류로부터 토착대두 근류균 87종을 분리하고 이들을 생장속도에 따라 *Bradyrhizobium japonicum* (slow-grower) 및 *Rhizobium fredii* (fast-grower)로 분류한 결과 전자가 55종, 후자가 32종이었다. 이두그룹의 대두 근류균을 질산염 환원특성에 따라 denitrifying fast-grower(F-1), nitrate respiring fast-grower(F-Ⅱ), denitrifying slow-grower(S-Ⅰ), 및 nitrate respiring slow-grower (S-Ⅱ)로 세분 되었으며, 분리균주 총 87종중 S-Ⅰ, S-Ⅱ, F-Ⅰ 및 F-Ⅱ의 수는 각각 10, 22, 48 및 7종이었다.

이들 각 group 대두 근류균의 균체단백질의 전기영동 pattern상의 차이를 1차 및 2차원 전기영동으로 분석한 결과 slow 및 fast-grower간에는 현저한 차이를 관찰할 수 있었으나 동일 생장속도를 가진 것 중 denitrifer와 nitrate respirer간에는 큰 차이가 없었다.

Introduction

The diversity of the indigenous soybean *Rhizobia* the symbiotic partner of soybean (*Glycine max*), population distributed in korean soil are poorly understood and the understanding of the *Rhizobium* ecology has agricultural importance.

In the past, the typical slow-growing *Rhizobia* nodulationg soybean have been referred to as *Rhizobium japonicum*. But recently these *Rhizobia* have been classified in a new genus, *Bradyrhizobium*, on the basis of their slow growth rate and other characteristics to distinguish them from the fast-growing, acid-pro-

ducing soybean *Rhizobia* reported by Keyser et al. [9], and recently named *Rhizobium fredii*

The most *Bradyrhizobium japanicum* (slow-grower) and *Rhizobium fredii* (fast-grower) have been reported to have dissimilatory nitrate reductase in free living cells and bactero id.[1,2,5,12,18,19] Denitrification by *Rhizobium* spp. was first noticed by the disappearance of nitrate and the subsequent production of N_2 gas in Durham tube in 1983[15] and Zablotowicz et al.[21] classified *Rhizobium* into 3 groups based on the nitrate reduction characteristics, that is, denitrifier (those that reduce nitrate to nitrogen gas), nitrate respirer (those that reduce nitrate to nitrite but do not reduce nitrite) and nitrate reductase deficient (those that lack the ability to grow anaerobically with nitrate).

Our objectives of this study were (i) to estimate the indigenous population ratio of *Rhizobium fredii* to *Bradyrhizobium japonicum* distributed in different korean soils; (ii) to estimate the ratio of denitrifier, and nitrate respirer to nitrate reductase deficient in the fast and slow growing *Rhizobia;* (iii) to investigate difference in protein patterns among the each subgroups of *Bradyrhizobium japonicum* and *Rhizobium fredii.*

Materials and Methods

Isolation of indigenous soybean *Rhizobia*

The indigenous soybean *Rhizobia* were isolated from the nodules of soybean cultivar, Danyup, cultivated in four different soils without inoculation. The fast-grower was picked up after 48 hour inoculation in AMA media at 30°C and slow-grower was picked up after 6 days of incubation. The three soil samples of indigenous *Rhizobium* source were taken from continuously soybean cultivated field and one taken from newly reclaimed soil.

Media

AMA is a yeast extract-mannitol broth medium[20] for the isolation and maintance of *Rhizobia.* RBN is a plant nutrient solution without nitrogen[22]. PBS buffer contains 6.8g KH_2PO_4, 8.7g K_2HPO_4 8.7g NaCl per liter of water, with pH 6.8.

Effectiveness assay

The effectiveness, nodulation and acetylene reduction activity of the isolated *Rhizobia* were assayed with the procedure of Wacek and Brill[20]. The surface-sterilized soybean seeds were germinated and one seedling was planted to a vial containing vermiculite with RBN solution. A loopful of each isolates from the slant culture was suspended into a 1 ml of sterile RBN solution and poured over the seedlings in the vial. A sterile plastic bag was placed over the seedling in the vial, and the vial was placed either in a green house or growth chamber. After 3 weeks of incubation, the plastic bag was removed and the plant was cut at the base of stem. A serum stopper was placed on the bottle and 0.4m*l* of 1 atm acetylene was injected into the bottle. The bottle was incubated at 25°C for 2 hours, after which 0.5m*l* of gas samples were injected into the gas chromatograph. Ethylene was detected by gas chromatograph equipped with a flame inoization detector. A Poropak N (Water Associates, Milford, Mass.) column was used to separate ethylene from acetylene. Specific activity was defined as nmole ethylene produced per hour per plant. The number of nodules and nodule weights were counted from the plant after measuring acetylene reduction activity.

Dissimilatory nitrate reduction characteristics

The dissimilatory nitrate reduction in free living rhizobia was assayed by the combined procedure of Neal et al.[10], Zablotowicz et al.[21],

and Gollop and Avissar[6] with slight modification. The 5 m*l* of precultured *Rhizobia* in AMA was inoculated in serum stoppered blanched erlenmeyer flask containing 40m*l* of AMA with 2 g of KNO_3 per liter. The air in the flask was replaced by helium gas, and then incubated at 30°C for 72 hours. The nitric oxide produced during the growth of *Rhizobia* was analyzed by gas chromatograph equipped with thermal conductivity detector. The Poropak N column described in acetylene assay was used for N_2O separation. After N_2O measurement, 5 m*l* of cell culture was centrifuged at 10,000×g for 5 minutes, and the supernatant was used for the analysis of nitrate and nitrite[3,11]

SDS-polyacrylamide gel electrophoresis

SDS-PAGE of the each group of *Rhizobia* were followed by the procedure of Noel and Brill[13] with slight modification. The bacteria was grown in 2.5 m*l* of AMA to early stationary phase (approximately 2×10^9 cells/m*l*) at 30°C with shaking. Each culture was then centrifuged at 8,000×g for 10 minutes. The pellets were washed once in 10mM Tris-HCl, pH 7.6 and the cells were finally suspended in 0.1m*l* of this buffer and 0.1m*l* of SDS sample buffer containing 10% (vol/vol) glycerol, 5% (vol/vol) β-mercaptoethanol, 3% (wt./vol) SDS, and 62mM Tris-HCl, pH 6.8. The cell suspension was boiled for 2.5 minutes and quickly cooled on ice and vortexed to shear DNA. The sample were loaded without centrifugation. The acrylamide concentration of stacking and running gel were 3 and 12%, respectively. The average current for stacking and running gel were 10 and 15 mA per plate. After completion of electrophoresis, the gel were stained with 0.1% commassie blue in 25% trichloroacetic acid for 1 or 2 hours. They were destained by diffusion, first in 7% acetic acid and then in 25% ethanol-7% acetic acid until the backgroud was suitably clear.

Two-dimensional polyacrylamide gel electrophoresis

The procedure was same as described previously[4]. Bacterial strains were grown in 5m*l* of AMA to early stationary phase at 30°C with shaking. The cell was pelleted by centrifugation at 8,000×g for 10 minutes and washed once in 10mM Tris-HCl, pH 7.4, 5mM $MgCl_2$, 0.1mg of pancreatic RNase per m*l*, and 0.5 mg of DNase per m*l*. The suspension was sonicated at 4°C for 30 sec with microtip probe at 40W output. After 5 minutes of incubation at 4 to 10°C, the samples were frozen. Two hundred milligrams of urea and 0.1m*l* of lysis buffer[14] were added, and the suspension was freeze-thawed for four cycles in dry ice thanol bath. The samples were centrifuged at 3,000×g for 5 minutes, and 0.02 to 0.1m*l* of the supernatant was loaded on the isofocusing, first dimension gel. The first and second dimension gel electrophoretic procedure was followed the technique described by Roberts et al.[16,17] The acrylamide concentration in the second dimension was 12%.

Results and Discussion

Indigenous ratio of *Bradyrhizobium japanium* to *Rhizobium fredii* distributed in Korean soil

Eighty seven strains of *Rhizobia* were isolated from the nodules of soybean cultivar, Danyup, cultivated in four different soils. The population of *Bradyrhizobium* (slowgrower) was dominated over *Rhizobium fredii* (fast-grower) as indicated in Table 1. The average ratio of slow to fast-grower was 63 to 37% and the ratio in the continuously soybean-cultivated soil was higher than that in newly reclaimed soil, which suggest that slow-grower is more effective than fast-grower in symbiotic nitrogen fixation with soybean. All the fast-growers of 32 strains isolated in

Table 1. Ratio of fast to slow-growing indigenous soybean *Rhizobia* in different soils

Soils	Total number of isolates	Fast-grower	Slow-grower
Continuously soybean cultivated soil			
Red-yellowish soil	29	10	19
Calcareous soil I	32	13	19
Calcareous soil II	19	6	13
One year soybean cultivated soil			
Newly reclaimed soil	7	3	4
Total	87	32(37%)	55(63%)

Table 2. Effectiveness of indigenous soybean *Rhizobia* from different soils

Soils	Nodulation[1]		Acetylene reduction[1]		Nitrate reduction	
	Fresh nodule mass(mg/plant)	Amt. of bacteroid	nmole C_2H_4/ plant/hr	nmole C_2H_4/ g nodule/hr	Aerobic assay (μmole NO_2/mg protein/hr)	Anaerobic assay
Continuously soybean cultivated soils						
Red-yellowish soil	718±34	441±85	185±36	327±74	9.7	7.2
Calcareous soil I	705±194	372±18	152±15	151±19	9.5	4.0
Calcareous soil II	599±74	314±59	111±29	200±75	6.0	4.6
One year soybean cultivated soil						
Newly reclaimed soil	295±25	293±29	61±20	238±46	3.7	3.7

[1]Host plant was soybean, Dunyup

this experiment produced acid as typical physiological characteristics of fast-growing *Rhizobia*.

Effectiveness of indigenous *Rhizobia*

The nodulation, acetylene reduction and nitrate reduction activity of the indigenous *Rhizobia* from 4 different soils were assayed (Table 2), The data were means of 30 replicates of plant, Danyup, cultivated in 4 different soils. As indicated in Table 2, nodulation and acetylene reduction activity of the plants cultivated in the soils taken from the continuously soybeancultivated field were higher those of plan ts cultivated in the soil taken from the newly reclaimed field. This result suggests that the indigenous population of *Rhizobia* play great role in nodulation and nitrogen fixation. Considering the indigenous population of *Rhizobia* in soybean cultivated soil is several times more than those in newly reclaimed soil (data was not shown in this paper), inoculation benifit in newly reclaimed soil could be great.

Dissimilatory nitrate reduction characteristics

The 55 strains of *Bradyrhizobium japanicum* and 32 strains of *Rhizobium fredii* grown in AMA media with supplement of nitrate, 276.9μg per m*l*- were assayed for denitrification by analyzing the remaining nitrate and nitrite concentration in media, and the amount of N_2O gas evolved after 72 hour incubation at 30°C. Acetylene was added to inhibit nitrous oxide reductase to quantitate nitrous oxide gas as the final product. Among the 32 fast-grower, 10 strains was identified as denitrifier and 22 strains was nitrate respirer, but in the slow-grower, the denitrifier was 48 strains out

Table 3. Dissimilatory nitrate reduction characterisics of indigenous soybean *Rhizobia*

	Fast-grower			Slow-grower		
	Group I (denitrifier)	Group II (nitrate respirer)	Group III (nitrate reductase deficient)	Group I (denitrifier)	Group II (nitrate respirer)	Group III (nitrate reductase deficient)
pH in AMA culture	6.1±0.4	6.0±0.2	—	7.1±0.1	7.1±0.2	—
[1]Remaining amt. in media ($\mu g/ml$)						
NO_3	2.7± 0.9	8.1±1.5	—	9.5± 2.4	6.2±1.0	—
NO_2	79.2±23.5	150.6±9.5		74.5± 2.4	143.9±2.3	—
N_2O evolved (μM)	309.4±90.5	59.7±7.1	—	229.9±23.4	83.0±11.5	—
Number of strains from 87 isolates.	10(11.5%)	22(25.3%)		48(55.2%)	7(8.0%)	

of 55 strains and the remaining 7 strains were nitrate respirer. The nitrate reductase deficient strains were not observed in the 87 isolates. In general, free-living cultures of *Bradyrhizobium* are capable of anaerobic growth in the presence of nitrate, but were observed in final product formation[2]. The nitrate reductase probably operates under anaerobic conditions in

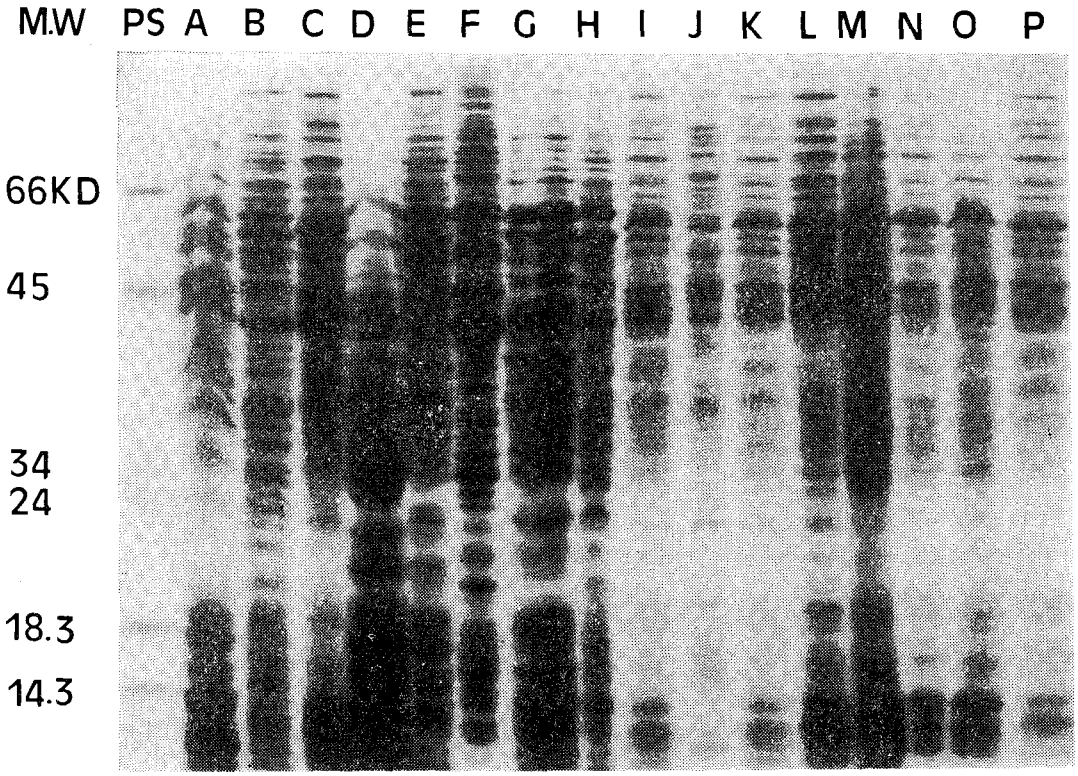

Fig. 1. Comparison of SDS-polyacrylamide gel electrophoretic pattern of the indigenous soybean *Rhizobia*.
PS: molecular weight marker proteins
A to D: denitrifying fast-grower
E to H: nitrate respiring fast-grower
I to L: denitrifying slow-grower
M to P: nitrate respiring slow-grower

the presence of nitrate to promote growth. According to the Brekum and Keyser[2], 270 strains were capable of anaerobic growth in the presence of nitrate including 11 fast-growing soybean *Rhizobia* from China out of 321 straind tested. The 51 strains of *Bradyrhizobia* belong to same serogroup were not capable of anaerobic growth in the presence of nitrate which lack of nitrate reductase.

One-and two-dimensional polyacrylamide gel electrophoretic pattern of proteins

The 87 isolates of indigenous *Rhizobia* were divided into 4 sub-groups according to growth rate and dissimilatory denitrification characteristics, that is, denitifying fast-grower(F-Ⅰ), nitrate respireing fast-grower (F-Ⅱ), denitrifying slow-grower (S-Ⅰ), and nitrate respiring slow-grower (S-Ⅱ). The four sub-groups of *Rhizobia* were analyzed by one dimensional SDS-PAGE (Fig 1) and two dimensional PAGE (Fig. 2). Close inspection of Fig. 1 revealed that the strains within the same growth rate could be distinguished from one another by SDS-gel. But distinct difference couldn't be observed between the denitrifier and nitrate respirer in the group with same growth rate. With the purpose of distinguishing the difference between the denitrifier and nitrate respirer two dimensional PAGE was conducted (Fig. 2), but any remarkable difference couldn't be observed although the difference between slow and fast-grower was more discernible than those in one-dimensional SDS-PAGE.

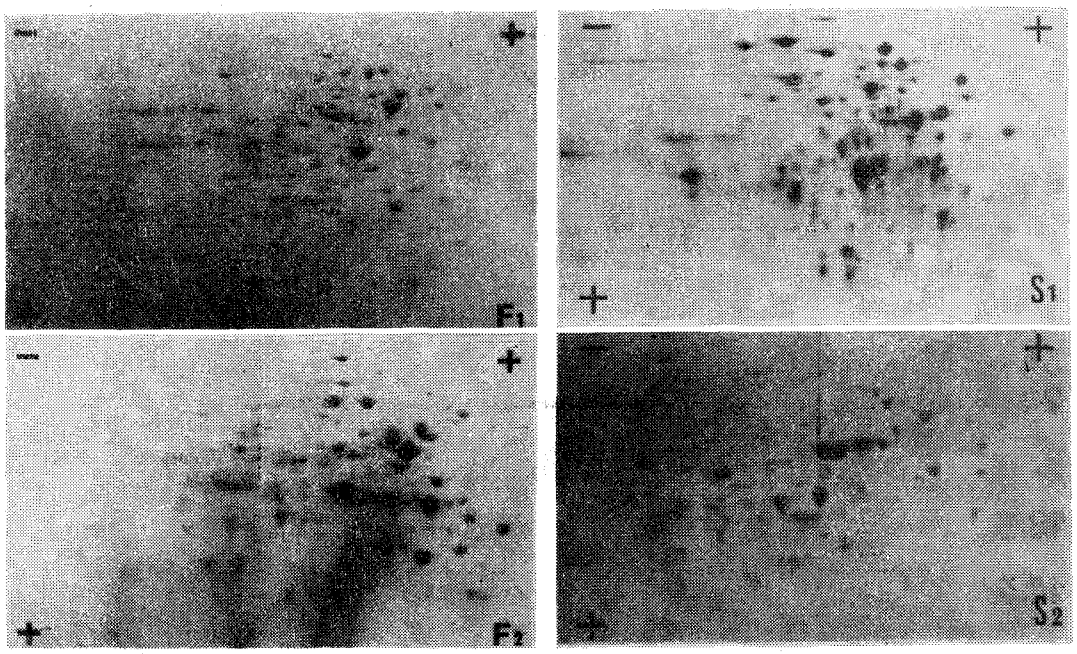

Fig. 2. Two-dimensional polyacrylamide gel electrophoretic pattern of the indigenous soybean *Rhizobia*.
X-axis is isoelectric focusing (pH 8~4) and Y-axis is SDS-dimension (MW 150,000~14,000)
F_1: denitrifying fast-grower F_2: nitrate respiring fast-grower
S_1: denitrifying slow-grower S_2: nitrate respiring slow-grower

Abstract

Eightyseven strains of indigenous *Rhizobia* were isolated from the nodule of soybean cultivar, Danyup, cultivated in four different soils sampled from continuously soybean cultivated and newly reclaimed fields. The strains were grouped into *Bradyrhizobium japanicum* (slow-grower: 55 strains) and *Rhizobium fredii* (fast-grower: 32 strains). The both groups could be divided into two sub-groups according to the denitrification characteristics, that is, denitrifying fast-grower (F-Ⅰ), nitrate respiring fast-grower (F-Ⅱ), denitrifying slow-grower (S-Ⅰ), and nitrate respiring slow-grower (S-Ⅱ). Among the 87 isolates, F-Ⅰ, F-Ⅱ, S-Ⅰ and S-Ⅱ sub-groups were 10, 22, 48 and 7 strains, respectively. The one-and two-dimensional polyacrylamide gel electrophoretic pattern of the four sub-groups were compared and discernible difference was observed between fast and slow-grower, but the difference was not discernible between sub-groups within the same growth rate group.

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