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Preparation of Antibiotic-Resistant *Bradyrhizobium japonicum* and Its Inoculation Effects on Soybean [*Glycin max*(L.) *Merr*]

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抗生物質 標識 根瘤菌의 造製와 그 接種効果

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SUMMARY

This study was carried out to evaluate the fate of inoculant *Bradyrhizobium japonicum* and the inoculation effect on soybean in complex soil environment. To moniter *Rhizobium* strains from the root, streptomycine and streptomycine and nalidixic acid resistant marker strains were prepared by spontaneous mutagenesis. The characteristics and properties of antibiotic marked strains were not altered by the mutagenesis. The comparison of properties of wild type and antibiotic resistant *Bradyrhizobium* strains are summarized as follow :

1) The strains of USDA110K-STR^r, USDA11ON-STR^r and R318-STR^r showed weak tolerance to pH 9.0. The utilization of carbon sources by fast growing group was different from that of slow growing group. The marked strains of R214-STR^{r}NAL^r, USDA11OK-STR^r and USDA11ON-STR^r was doubtful in utilization of sorbitol and R138-STR^{r}NAL^r was doubtful in utilization of xylose as a carbon source.

2) By examining the agglutination reaction of serogroups, the strains used were identified as different ones. There were no differences between wild type and marked strains in agglutination titer values.

3) The plamid size of fast group was slightly greater than that of slow group. However, there was no differences in plasmid size between the wild type and antibiotic resistant strains. This result indicates that the antibiotic resistance was not encoded in plasmid.

4) The recovery of the inoculated strains was up to 12.5% in soybean cultivated soil and was up to 25% in soybean uncultivated soil.

5) When the wild type or marked strains were inoculated, there was no significant effect on soybean plant, whereas the inoculation effect was pronounced in soybean uncultivated soil. The inoculation effect seemed to be more pronounced in wild type strains than antibiotic resistant strains, however, the difference was not significant.

INTRODUCTION

Rhizobium japonicum, in symbiotic association with leguminous plants fix nitrogen. The largest amount of nitrogen was reported to be provided by symbiotic nitrogen fixation and the rest nitrogen is applied by adding N-fertilizer[25]. The addition of chemical fertilizer increased energy demand and production cost. This caused reductuion of nodulation and effectiveness of symbiotic nitro-

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gen fixing system[26].

It was reported that the inocualtion of rhizobia to soil, in which soybean was not cultivated, resulted in significant increases in sosybean yield and protein content of soybean. Conversely, there was no significant differences by inoculating the rhizobia to soybean cultivated soils[24]. Weaver *et al.*[24] demonstrated that if the inoculated rhizobia are to form 50% or more nodules, an inoculum rate of at least 1000 times the soil rhizobia population must be used. Eventhough a great number of efficient rhizobia were introduced to the root environment, it was often native rhizobia which dominated soybean roots because of their strong competitiveness for the nodulation site[12, 23, 24, 17].

The population dynamics of the introduced rhizobia in soils and roots are in need to be monitered[12, 23, 25] in order to make use of rhizobia as a beneficial inoculant for regume plant. An attempt was made to isolate antibiotic-resistent maker strains without losing its parental properties[4, 7, 9, 12, 21, 23]. We evaluated inoculation effects of the introduced strains in soybean cultivated and uncultivated soils.

MATERIALS AND METHODS

Strains and media : The strains of *Bradyrhizobium japonicum*, R214, USDA110K, USDA110N and R138 are slow growing group, and F1-24 is fast growing group. These strains were obtained from Agricultural Science Institute. The strains were routinely grown in Yeast-extract Mannitol Agar (YMA). For the selection of marked strains, YMA medium supplemented with 1000 ppm streptomycine (STR) for single antibiotic-resistant strains and 700 ppm STR together with 100 ppm nalidixic acid (NAL) for double antibiotic-resistant strains.

Preparation for antibiotic-resistant marker strains : The late-log phase rhizobium culture was plated on YMA+1000 ppm STR medium and was incubated at 28°C for 8 days. Colonies appeared in the presence of 1000 ppm STR were restreaked on YMA+1000 ppm STR. These colonies were used as an inoculant of single marked strains[7, 9]. The single marked strains were further used for the selection of double antibiotic marked strains on YMA+700 ppm STR+100 ppm NAL medium. Colonies grown on YMA+700 ppm STR+100 ppm NAL medium were used as an inoculant of double marked strains[9].

Biological and morphological characteristics : Biological and morphological characteristics of the strains of *R. japonicum* were examined according to the methods of "Manual of Methods for General Bacteriology"[8] and "Microbiology, a Laboratory Manual"[5]. Serogroup test was done by Sato *et al.*[20].

Plasmid pattern : The late-log cell cultures were centrifuged and the pellets were suspended in TE buffer. Lysis buffer was added to remove cell wall. Plasmid was prepared according to the methods of large plasmid extraction[1, 2, 6, 14, 15, 16, 18, 19]. To avoid the fraction of plasmid, phenol extraction procedure was omitted.

Inoculation effect on soybean plant : For this study, pot experiment was carried out. Two different soils, previously cultivated with soybean and uncultivated soil, were used. The chemical properties of the soils are shown in Table 1. Each soil was received 44 mg $(NH_4)NO_3$, 175mgKH_2PO_4, 5,000mg$Ca(OH)_2$and 6 mg $Na_2MoO_4.2H_2O$/500 g dry soil. Soybean cv. Milyang was used as a host plant for the pot experiment. Seeds were surface sterilized by soaking in 20% of commercial Rox solution for 10 minutes. Seeds were rinsed three times and soaked in 0.01N HCl for 10 minutes followed by through rinsing with sterile water. These seeds were placed in a sterile petri dish with a moistened filter at the bottom. For germination of seeds, the plates were placed in the dark at 30°C for one day. After germination, only the healthy seedlings were selected. Three seedlings were transplanted in a pot filled with soybean cultivated or uncultivated soil. One ml of cell suspension (ca. log 8 CFU/ml) was inoculated to each seedlings. Fourty five days after transplanting, a number of nodules, fresh weight of nodules, plant height, nitrogen and

Table 1. Chemical properties of soybean cultivated and uncultivated soils

Soils	pH (1 : 5 H_2O)	O.M (%)	T-N (%)	Chemical constituents						
				Available (ppm)		Exch. Cations (me/100g)				
				P_2O_5	SiO_2	K	Na	Ca	Mg	CEC
Soybean cultivated	6.1	1.6	0.08	98	72	1.2	1.8	3.2	1.3	7.4
Soybean uncultivated	6.4	2.1	0.09	82	67	2.1	0.8	3.7	2.7	9.8

Table 2. Biochemical and morphological characteristics of wild and antibiotic resistant strains of *Bradyrhizobium japonicum*

Characteristics	Strains				
	SDA				
	R214	F1-23	USDA110K	USDA110N	R138
	WSN	WSN	WSN	WSN	WSN
BIOCHEMICAL PROPERTIES					
Catalase	+++	+++	+++	+++	+++
Urease	+++	+++	+++	+++	+++
Oxidase	+++	+++	+++	+++	+++
Production					
H_2S	---	---	---	---	---
Acid	---	---	---	---	---
Alkali	+++	---	+++	+++	+++
Utilization of					
Citric acid	---	---	---	---	---
Tolerence					
2% NaCl	---	+++	---	---	---
pH 9	+++	+++	+±+	+±+	+±+
MORPHOLOGICAL PROPERTIES					
Flagella	+++	+++	+++	+++	+++
Motility	+++	+++	+++	+++	+++
Gram staining	---	---	---	---	---
Shape of cells	RRR	RRR	RRR	RRR	RRR

W : Wild type, S : Streptomycine resistance(1000 ppm)
N : Streptomycine (700 ppm) and Nalidixic acid resistance (100 ppm)
+ : Positive, − : Negative, ± : Doubtful, R : Rod

chlorophyll contents, acetylene reducing activity (ARA) and recovery of the introduced strains were examined.

Acetylene reduction activity : Nodules were collected in Erlenmeyer flasks. Serum stoppers were placed on the flasks and 10 ml of air in the flask was replaced with 10 ml acetylene gas[10]. Flasks were incubated at 28°C for 2 hrs and then 1 ml gas sample was injected into a gas chromatography with a hydrogen flame detector. As a calibration standard, 64.2 ppm ethylene was injeted[23].

Identification of the introduced maker strains from nodules : Nodules were taken at random regardless of their size. Nodules were surface sterilized with 3% H_2O_2 solution for 30 min. followed by through washing with sterile water. The nodules to be examined were palced in cap tubes containing 0.2ml sterile saline solution (0.85 %) and squashed with sterile glass rod[4]. The turbid suspensions were streaked on YMA+1000 ppm STR medium or YMA+700 ppm STR+100 ppm+NAL medium. Plates were incubated at 28°C for 5days.

RESULTS AND DISCUSSIONS

Preparation of antibiotic resistant marker strains : The wild type strains used in this study were all sensitive to antibiotics. Marker strains obtained *via* spontaneous mutation with antibiotic(s) grew on a medium containing antibiotics. The single marked strains could grow on YMA+1000 ppm STR and the double marked strains grew on YMA+700 ppm STR+100 ppm NAL.

Biochemical and morphological characteristics : As shown in Table 2, all strains were positive in catalase,

urease, and oxidase, but negative in H_2S production. All strains examined could not utilize citrate as a carbon source. The strains of slow group are sensitive to 2% NaCl and those of fast group are resistant to salt. Most of the strains were tolerant to pH 9, however, strains of USDA110K-STR^r, USDA110N-STR^r and R138-STR^r were rather doubtful. All strains were rod shaped, motile, gram-negative, and had flagella.

Utilization of carbohydrates : Glenn reported that there are no metabolic systems to utilize disccharides in slow group strains while fast group utilizes wider range of carbon sources. As shown in Table 3, both slow and fast group utilized fructose, mannose, xylose, mannitol, sorbitol and dextrose. However, the slow group did not utilize cellobiose, lactose, raffinose, inositol and USDA110N-STR^r were doubtful in utilization of sorbitol. Fast group utilized cellobiose as a carbon source but slow group did not use it. These results indicate that the antiobiotic marked strains are virtually not altered by antibiotics for the utilization of carbon sources.

Table 3. Utilization of carbon sources by wild type and antibiotic-resistant strains of *Bradyrhizobium japonicum*

Carbon sources	R214		F1-23		USDA110K		USDA110N		R138	
	W	S	W	S	W	S	W	S	W	S
Frouctose	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+
Xylose	+	+	+	+	+	+	+	+	+	±
Mannitol	+	+	+	+	+	+	+	+	+	+
Sorbitol	+	±	+	+	+	±	+	±	+	+
Dextrose	+	+	+	+	+	+	+	+	+	+
Cellobiose	−	±	+	+	−	±	−	±	−	±
Sucrose	−	±	+	+	−	−	−	−	−	−
Lactosee	−	−	+	+	−	−	−	−	−	−
Raffinose	−	−	+	+	−	−	−	−	−	−
Inositol	−	−	+	+	−	−	−	−	−	−
Dextrin	−	−	+	+	−	−	−	−	−	−
Starch	−	−	−	−	−	−	−	−	−	−

W : Wild type, S : Streptomycine resistance(1000ppm),
N : Streptomycine (700 ppm) and Nalidixic acid resistance (100 ppm)
+ : Positive, − : Negative, ± : Doubtful.

Table 4. Cross agglutination reaction between wild type strains

Antibodies	Antigens (Agglutination titer* (Relative unit))				
	Strains				
	R214	F1-23	USDA110K	USDA110N	R138
R214	1024	—	128	128	128
F1-23	—	1024	—	—	—
USDA110K	64	—	1024	1024	—
USDA110N	64	—	1024	1024	—
R138	128	—	32	32	1024

* The highest dilution leading to distinctive agglutination reaction

Table 5. Cross agglutination reaction between parent and antibiotic-marked strains

Antibody	Antigens (Agglutination titer* (Relative unit))		
	Wild type	STR^r	STR^{r}NAL^r
R214	1024	1024	1024
F1-23	1024	1024	1024
USDA110K	1024	1024	1024
USDA110N	1024	1024	1024
R138	1024	512	512

* The highest dilution leading to distinctive agglutination reaction

Determination of serogroup : The antigens of USDA 110N and USDA110K reacted strongly with their own antibodies (Table 4), but they did not react or weakly reacted with other antibodies. USDA-strains may be identified as the same strain. The antigen of fast group reacted with its own antibody but not with the others. On the otherhand, antibody of R214 reacted weakly (agglutination titer : 128) with other antigens. All the antibiotic marked strains reacted strongly with their own antigens except R138-STR^r and R138-STR^{r}NAL^r (Table 5). However, there was no remarkable differences in agglutination titer value. In general, the antibodies did not react with other antigens and vice versa[3].

Plasmid pattern : The plasmid of fast group was

about 23 kb and that of slow group was slightly greater than 23 kb (Fig. 1). There was no differences in plasmid size between the wild type and the marked strains indicating that the antibiotic resistance was not coded in the plasmid[2].

Inoculation effect of the introduced strains on soybean plant : The inoculation effect of the introduced strains on soybean plant is shown in Table 6 and 7. The inoculation effect was more pronounced in soybean uncultivated soil. In soil previously cultivated with soybean (Table 6), a number and fresh weight of nodules, plant height, nitrogen and chlorophyll contents were lower than those in soil without soybean cultivation (Table 7). The inoculation effect of antibiotic marked strains showed similar pattern as their wild type strains.

Acetylene reduction activity : ARA in soybean cultivated soil was in the range of 200~250 nmol/g

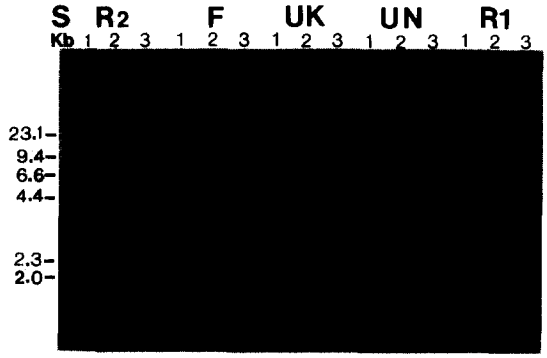

Fig 1. Agarose-gel electrophoresis of plasmid DNA from *Bradyrhizobium japonicum* and its marked strains
S : Molecular standard, R2 : Strain R214,
F : Strain F1-23, UK : Strain USDA110K,
UN : Strain USDA110N, R1 : Strain R138
1 : Wild type strains, 2 : Streptomycine resistant strains
3 : Streptomycine and nalidixic acid resistant strains

Table 6. Inoculation effect of R. japonicum on soybean plant grown in a soil previously cultivated with soybean.

	Control	Strains				
		R214	F1-23	USDA110K	USDA110N	R138
A. Soybean cultivated soil						
Nodule numbers (No. of nodules/plant)						
WLD	133± 10	125± 90	137± 90	128± 22	145± 12	132± 39
STR^r		130± 12	115± 34	125± 13	114± 48	171± 17
STR^rNAL^r		142± 45	137± 17	143± 39	153± 28	132± 68
Fresh weight of nodules (g/plant)						
WLD	1.74± 0.30	1.57± 0.46	1.66± 0.21	1.98± 0.07	2.33± 0.42	1.87± 0.19
STR^r		1.69± 0.36	1.87± 0.21	1.75± 0.33	1.91± 0.81	1.86± 0.44
STR^rNAL^r		1.56± 0.60	1.89± 0.40	1.45± 0.30	1.65± 0.50	2.01± 0.90
Plant height (cm)						
WLD	43.0± 1.0	48.0± 3.5	49.0± 1.0	49.0± 4.6	44.5± 2.3	44.5± 2.6
STR^r		41.0± 4.0	42.0± 3.0	41.0± 5.8	44.2± 0.7	50.0± 6.0
STR^rNAL^r		41.0± 3.3	45.0± 2.0	41.0± 2.0	39.0± 1.0	49.0± 3.6
Nitrogen contents (mg-N/g dry leaf)						
WLD	43.3± 3.1	45.2± 2.5	44.1± 2.2	47.6± 2.5	42.7± 3.6	50.8± 2.4
STR^r		47.1± 2.0	58.2± 1.2	43.3± 4.3	40.9± 3.7	51.2± 1.8
STR^rNAL^r		46.2± 9.1	43.1± 9.6	40.9± 3.7	42.5± 1.5	50.5± 4.4
Chlorophyll contents (mg/g fresh leaf)						
WLD	1.94± 0.3	1.62± 0.3	1.96± 0.2	1.33± 0.4	2.20± 0.1	1.97± 0.1
STR^r		1.96± 0.1	1.42± 0.2	2.30± 0.1	2.00± 0.1	2.04± 0.3
STR^rNAL^r		1.88± 0.4	1.80± 0.2	1.89± 0.2	2.20± 0.7	1.60± 0.2

Values are mean of three replicates and standard deviation.

Table 7. Inoculation effect of *Bradyrhizobium japonicum* on soybean plant grown in a soil without previous cultivation with soybean

	Control	Strains				
		R214	F1-23	USDA110K	USDA110N	R138
B. Soybean uncultivated soil						
Nodule numbers (No. of nodules/plant)						
WLD	95± 29	120± 30	136± 11	109± 46	142± 12	112± 28
STR^r		109± 31	104± 28	105± 23	115± 22	101± 18
STR^rNAL^r		112± 27	115± 22	108± 20	101± 34	105± 44
Fresh weight of nodules (g/plant)						
WLD	1.36± 0.5	1.67± 0.3	1.63± 0.6	2.09± 0.6	1.84± 0.2	1.59± 0.5
STR^r		1.63± 0.2	1.49± 0.5	1.70± 0.5	1.66± 0.5	1.51± 0.3
STR^rNAL^r		1.47± 0.1	2.61± 0.2	1.36± 0.5	1.55± 0.4	2.44± 0.2
Plant height (cm)						
WLD	38.0± 4.8	39.0± 2.3	51.0± 4.0	48.0± 1.0	44.5± 4.8	45.0± 2.0
STR^r		37.0± 3.1	39.0± 2.0	40.0± 2.0	39.0± 1.7	45.2± 2.2
STR^rNAL^r		41.0± 3.5	41.0± 1.7	38.3± 1.5	38.0± 4.0	41.0± 6.0
Nitrogen contents (mg/g dry leaf)						
WLD	40.2± 4.2	41.3± 3.0	47.2± 6.6	47.7± 3.5	41.2± 0.9	44.5± 6.8
STR^r		40.6± 2.9	41.0± 9.2	40.7± 4.9	42.4± 4.9	42.2± 3.1
STR^rNAL^r		41.2± 3.0	40.2± 5.0	39.6± 4.5	36.7± 2.9	42.8± 0.9
Chlorophyll contents (mg/g fresh leaf)						
WLD	1.32± 0.4	1.61± 0.4	1.88± 0.4	1.36± 0.1	2.11± 0.3	1.46± 0.1
STR^r		1.77± 0.4	1.49± 0.1	1.74± 0.3	1.42± 0.1	1.35± 0.3
STR^rNAL^r		1.33± 0.6	1.58± 0.3	1.36± 0.3	1.50± 0.4	1.70± 0.3

Values are mean of three replicates and standard deviation.

fresh nodule, hour (Fig. 2). In soybean uncultivated soil, the ARA was in the range of 100~200 nmol/g fresh nodule, hour. However, the values are not significantly different. The highest value of 280 nmol/g was obtained in a soybean cultivated soil with the inoculation of strain R138. The lowest value was obtained in soybean uncultivated soil with strain R138. The ARA in soybean cultivated soil inoculated with marked strains was significantly higher than that of soybean uncultivated soil. Weaver *et al.*[24] reported that eventhough a greater number of strians were inoculated, they could not influence root systems because the soil contains native strains which are very competitive and active[11,12,24].

Recovery of the introduced strains from nodules : Fig. 3 shows the recovery rate of introduced strains. The recovery of single marked strains was up to 25% and that of double marked strains was up to 22%. Only 12.5% of the marked strains was recovered from the soil previously cultivated with soybean, while 25% of the introduced strains was recovered from soybean uncultivated soil. The marked strians of R138-STR^r, R214-STR^rNAL^r, and USDA110N-STR^rNAL^r were not able to form nodules in soybean cultivated soil. The highest recovery among the strains was obtained from the strain USDA110-STR^r. The recovery of the strains in soybean uncultivated soil was, in general, higher than that of the strains in soybean cultivated soil. These results are in consistent with those of Janssen and Strijdom[11], Kuykendall and Weber[12], Turco *et al*[21,22], and Watanabe and Yoshida[24], and Weaver and Friedrick[25].

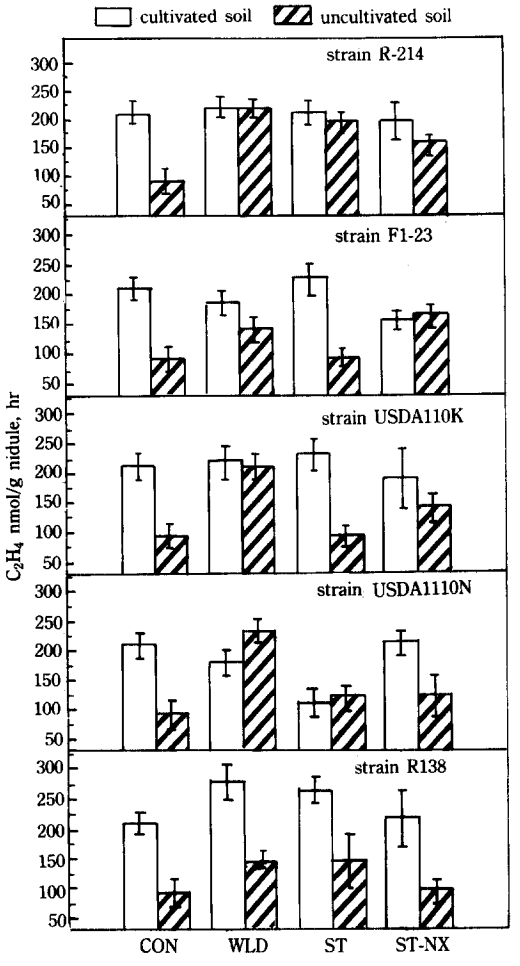

Fig 2. Inoculation effects of *Bradyrhizobium japonicum* and its marked strains on acetylene-reducing activities in different soils. CON : Control, WID : Wild type strains, ST : Streptomycine resistant strains ST-NX : Streptomycine and nalidixic acid resistant strains

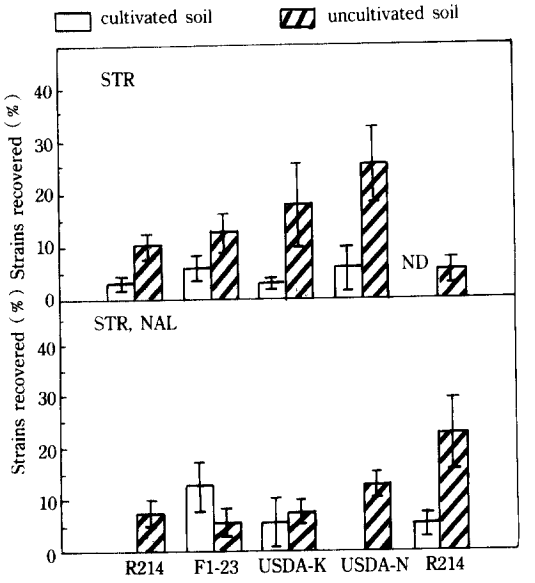

Fig 3. Recovery (%) of antibiotic-resistant *Bradyrhizobium japonicum* strains from soybean nodules in different soils.
STR : Streptomycine resistant strains
STR, NAL : Streptomycine and nalidixic acid resistant strains
USDAK : Strain USDA110K,
USDAN : Strain USDA110N
ND : Nodules are not formed

摘　　要

현재 대두근류균 접종제로서 이용되고 있는 *Bradyrhizobium japonicum*의 토양중에서 그 추이를 밝히고자 항생제 내성균주를 조제하여 그들의 특성을 모균주와 비교하고 아울러 토양에서 접종효과를 추적한 결과는 다음과 같다.

1. USDA110K-STR^r, USDA110N-STR^r, R138-STR^{r}은 pH 9에 내성이 약했으며, R214-STR^r, NAL^r, USDA110N-STR^{r}은 sorbitol에 R138-STR^{r}NAL^{r}은 xylose에 대한 자화성이 불분명했다.

2. Serogroup에 의해 각 균주들은 서로 다른 균주로 동정되었으나 원균주와 標識菌株간에는 거의 차이가 없었다.

3. Plasmid size는 생육 촉진형 균이 지연형보다 약간 컸으나, 야생균주와 그들의 標識菌間에는 각각 차이가 없었다.

4. 5년이상 대두 재배 토양에 標識菌株 接種時 0～12.5%, 대두무재배 토양에서는 5～22.5%의 접종률을 보였다.

5. 원균주와 標識菌株를 각각 접종시 5년이상 대두재배 토양에서는 접종효과가 없었으나 대두 무재배 토양에서는 상당한 접종효과가 있었으며 원균주가 標識菌株보다 더 큰 효과를 나타냈다.

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