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Studies on Streptomycin Resistant Mutant Strains of *Rhizobium trifolii*

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*Rhizobium trifolii*의 스트렙토마이신 내성 돌연변이주의 특성

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ABSTRACT: Some streptomycin resistant strains of *Rhizobium trifolii* having nodulation ability were selected, and their nitrogenase activities, symbiotic effects on plant growth, and nodule *electronmicroscope* were compared with those of the wild type.

After NTG treatment, as a mutagen, at the concentration exhibiting 99.7% lethal rate, 5 strains of streptomycin resistant mutant having nodulating ability were selected.

Among these nodulating mutant strains, 3 strains produced more nodules and 2 strains showed less nodules than wild type. But their nitrogenase activities were decreased significantly, and nodule formation time was also delay compared with those of the wild type, and there was no remarkable difference in effects on plant growth. Microstructure of nodules by *electronmicroscopy* had many distinctive differences between red clover nodules inoculated with wild type and mutants.

KEY WORDS□*Rhizobium trifolii*, Mutation, Nodule formation, N_2-fixation.

In the last few years considerable interest has been devoted to studies of the genetics of root nodule bacteria, *Rhizobium* spp., which are the nitrogen fixing symbionts of legumes(Beringer *et al.*, 1980: Beringer, 1981). Scientists in this field hope to eventually genetically engineer new *Rhizobium* strains with improved properties in symbiosis.

Genetic studies depend upon the isolation of strains which carry mutations. A more serious problem with *Rhizobium* species is in the screening of survivors of mutagen treatments for specific classes of mutants. The isolation of auxotrophic and drug-resistant mutant is no more difficult than for other bacteria. The problem here is that each colony must be tested independently on a suitable legume host plant for the establishment of normal nitrogen-fixing nodules.

Despite this limitation a number of mutants of *Rhizobium* have been isolated after mutagen N-methyl-N -nitro-N-nitrosoguanidine has been used for*Rhizobia* (Beringer *et al.*, 1974). Recently transposon mutagenesis has been used for *R. leguminosarum, R. trifolii, R. phaseoli* and *R. meliloti.* (Beringer *et al.*, 1978). Maier and Brill (1976) and Beringer *et al.*, (1977) have shown that mutant of *Rhizobium* that are unable to carry out a normal symbiosis can be isolated after 'conventional' mutagenesis. Some of these mutants have been increased nitrogen fixation ability(Maier and Brill, 1978), and nodule numbers(Pariiskya, 1973). Ultrastructure of root nodule formed by mutant strains and wild strains of *Rhizobium* also have been studied. (Beringer *et al.* 1977; Downie *et al.*,

1983; Rolfe *et al.*, 1980; Verma *et al.*, 1983)

The three main methods of gene transfer in bacteria(conjugation, transformation and transduction) have been reported for many *Rhizobium* spp.(Balassa, 1963; Kowalski, 1970; Dunican and Tierney, 1974; Dunican *et al.*, 1976; Johnson and Beringer, 1977) by using mutant strains. Gene transfer mediated by plasmids has been reported extensively for *Rhizobium* species. Especially, R plasmids have probably been transferred to and between strains of all the species of *Rhizobium* (Kuykendall, 1979). It has recently been shown that nitrogenase genes and nodule genes in *Rhizobium* spp. are located on a large indigenous megaplasmid (Djordjevic *et al.*, 1982; Masterson *et al.*, 1982; Nuti *et al.*, 1977). Therefore, nif and nod gene have been transferred to other nif^- or nod^- *Rhizobia* by using these plasmids (Djordjevic *et al.*, 1983; Johnston *et al.*, 1978).

We expected that broad host range hybrid vector plasmid, RP4::Mu *cts*, could transfer nod gene of *Rhizobia* to nod^- mutants than any other vector plasmid like RP4. In this study, as a preliminary experiment, we have selected the genetically marked mutants of *R. trifolii* and experimented the characteristics of the mutant strains.

MATERIALS AND METHODS

1. Bacterial Strains

The bacterial strains used in this study are listed in table 1. *Rhizobium trifolii* T12-1 strain was obtained from Professor Uosumi, Tokyo University and *Rhizobium trifolii* T12-1K1, *R. trifolii* T12-1K2, *R. trifolii* T12-1K3, *R. trifolii* T12-1K4 and *R. trifolii* T12-1K5 were selected in our laboratory. They were the mutants of *R. trifolii* T12-1 and resistant to Streptomycin and sensitive to Tetracycline and Kanamycin.

Table 1. *List of bacterial strains and their characteristics*

Strains	Markers[1)]
Rhizobium	
trifolii T12-1	Wild type, Nod^+, Nif^+
trifolii T12-1K1	$Nod,^+$ $Nif,^+$ $Sm,^+$ $Tc,^-$ Km^-
trifolii T12-1K2	$Nod,^+$ $Nif,^+$ $Sm,^+$ $Tc,^-$ Km^-
trifolii T12-1K3	$Nod,^+$ $Nif,^+$ $Sm,^+$ $Tc,^-$ Km^-
trifolii T12-1K4	$Nod,^+$ $Nif,^-$ $Sm,^+$ $Tc,^-$ Km^-
trifolii T12-1K5	$Nod,^+$ $Nif,^-$ $Sm,^+$ $Tc,^-$ Km^-

1) Sm, Streptomycin
Tc, Tetracycline
Km, Kanamycin
Nod, Nodule formation ability
Nif, Nitrogen fixation ability

2. Media and Culture

YB Broth (Yeast Extract Broth) (Vincent, 1970) for culturing *Rhizobium* was used. For plating of mutant strains of *Rhizobium*, L Broth (Luria Broth) was used and Citrate buffer (0.1M Na Citrate, pH5.5) and Phosphate buffer (0.1M Phosphate, pH7.0) were used for washing and diluting of mutating procedure. The nodulation assay is done as described by Wacek and Brill (1976).

3. Mutagenesis

The modification method of Miller(1972) was used. *Rhizobium trifolii* T12-1 suspension was treated with 50μg/m*l* N-methyl-N -nitro-N-nitrosoguanidine(NTG) at 37°C. At different time, a culture was withdrawn, spun down and washed once in phosphate buffer. 0.5m*l* diluted *R. trifolii* T12-1 suspension was spreaded on LB plate. After 3 days incubation at 30°C, colonies formed were counted.

After mutation was induced by NTG treatment at the concentration of 99.6% lethal rate, mutants were replicated on the LB media containing streptomycin (300μg/ m*l*), tetracycline (100μg/m*l*) and kanamycin (25μg/m*l*) respectively. The culture was grown for 48h at 30°C. Mutant strains which were susceptible to kanamycin and tetracycline and resistant to streptomycin, were selected. Selected individual colonies were picked and inoculated into plant for effectiveness assay.

4. Effectiveness assay

Seeds were surface sterilized by swirling them in a 20% solution of commercial bleach for 10min. The seeds were then rinsed with sterile water and soaked for an additional 10 min with 0.01N HCl. After the HCl wash, the seeds were rinsed six times with sterile distilled water and dispersed in

a sterile petri plate with moistened filter paper on the bottom. To germinate the seeds, the plates were placed in the dark at 30°C for 2 days. Each seedling was placed in a 20m*l* serum bottle which had been filled with sterile vermiculite and 16m*l* of plant nutrient solution. Before the seedling were added to the bottles they were inoculated by placing them for 1 hour in a heavy suspension of *Rhizobium* in the plant-nutrient medium. Tehn 1m*l* of this same inoculum was dispensed into each of the sterilized bottles containing the vermiculite. One inoculated seedling was placed in each bottle and a sterile plastic bag was then placed over the bottle and tied. After about 4 weeks incubation, the plastic bags were removed and the plant was cut at the base of the stem. Plants that developed abnormally were discarded. A serum stopper was placed on the bottle and 0.4m*l* of 1 atm acetylene was injected into the bottle. The bottles were incubated at 25°C for 1 to 3 hours after which 0.5m*l* gas samples were injected into the gas chromatograph (Varian Model 3700) fitted with a porapak-R column at 80°C column temperature, with N_2 as carrier gas at a flow rate of 30cc/min. (Wacek and Brill, 1976).

5. Electron Microscopy

Ultrastructure of root nodule was detected as described by Mackenzie and Jordan (1974). The nodules were fixed 2 hours at 4°C in 2.5% paraformaldehyde-glutaraldehyde (phosphate buffer, pH 6.8), postfixed for 2 hours in 2% osmium tetraoxide in the 0.1M buffer (phosphate buffer pH6.8), dehydrated in a graded series of acetone in the same buffer and embedded in Epon-Araldite mixture solution. Embedding samples were cut with Sorvall proter Blum MT-II or Ultramicrotome, stained with methylenblue-basic fuchsine. Silver sections were stained with uranyl acetate and Reynolds' lead citrate (Reynolds, 1963) and examined with a JEM 100CX-II (80Kv) Electron Microscope.

RESULTS AND DISCUSSION

1. Effects of NTG on the Survival rate of *R. trifolii* T12-1 and Primary Selection of the mutants.

R. trifolii T12-1 was sensitive to the mutagen, NTG. It exhibited 50% lethal rate at the concentration of 50mg/m*l* Citrate buffer, after 5 min, and lethal rate was increased up to 99.6%, after 20 min.

R. trifolii T12-1, induced by NTG, were replicated on nutrient agar medium containing antibiotics in order to select mutant strains. About 100 mutants were selected. They could grow on nutrient agar containing streptomycin but not on tetracycline and kanamycin.

2. Nodule formation ability

Nodule formation ability was experimented by inoculating the *Rhizobium* into host plant (Red clover). After 3-4 weeks the plants were scored for nodulation and the ability to reduce acetylene. One hundred colonies were tested and five isolates had nodule formation ability.

We wished to determine whether mutagenesis of the bacterium *R. trifolii* T12-1 would produce strains capable of changing the N_2-fixing root nodule symbiosis between this organism and red clover, having antibiotics resistance at the same time.

Mutant *R. trifolii* T12-1K1, *R. trifolii* T12-1K2 and *R. trifolil* T12-1K5 formed 3.4, 3 and 2.3 nodules, it showed more nodules than the wild type on red clover. And mutant *R. trifolii* T12-1K3 and *R. trifolii* T12-1K4 formed the almost same

Table 2. *Nodulation Characteristics and growth of Red clovers inoculated with the mutant strains of R. trifolii.*

Strains	Number of plants	Total no. of nodules	Average No. of nodules	Average plant height
Rhizobium				
trifolii T12-1K1	5	17	17/5=3.4	9.1
trifolii T12-1K2	6	18	18/6=3.0	11.0
trifolii T12-1K3	6	6	6/6=1.0	10.5
trifolii T12-1K4	2	2	2/2=1.0	NM*
trifolii T12-1K5	3	7	7/3=2.3	11.0
trifolii T12-1 (wild type)	6	9	9/6=1.5	11.9

*NM=Not measured.

nodule numbers as the wild type (Table 2). In the case of nodule formation times, nodules inoculated with mutant strains have appeared later than normal nodules.

In the plant hight, however, our result have shown that there are little differences between mutants and wild type Rhizobium trifolii T12-1 (Fig. 2). And there is only good growth comparing with the non-inoculated plant (Fig. 1). Maier and Brill, (1976) had reported that plant inoculated with the mutant strains were smaller as compared to the wild type. Our result about plant growth was some consistent with Maier and Brill's.

3. Nitrogen fixing ability of mutant strains

Selected mutant strains were tested for symbiotic acetylene reduction by a rapid screening effectiveness assay with 4 weeks old bottle-grown red clover plants (Wacek and Brill, 1976). Acetylene reduction is an index of N_2 fixation. Thus, lack of acetylene reduction activity correlated well with lack of the ability of the bacterium-legume system to fix N_2.

As shown in the table 3, mutant strains were

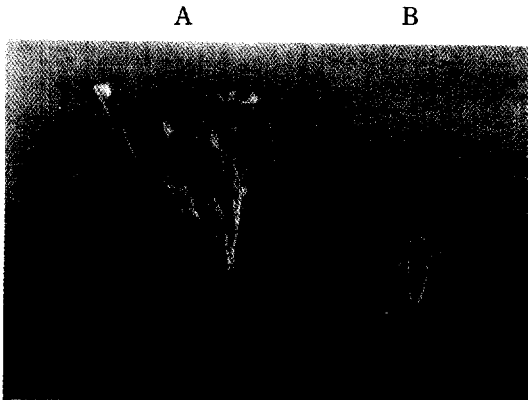

Fig. 1 *Comparison between red clover plants inoculated with the wild-type and non-inoculated.* Seeds were surface sterialized in 1N H_2SO_4 10m*l* for 20 mins and rinsed with sterile water. To germinate the seeds, the moisturizing plate were placed in the dark at 30°C for 2 days. Each seedling was placed in 20m*l* serum bottle which had been filled with sterile vermiculite and 16m*l* of plant nutrient solution 0.5%-0.65% agar medium can be substituted for vermiculite. Before seedling were added to the bottles, we inoculated 1m*l* of ***Rhizobium*** suspension. Plant were grown with an 18 hours light photoperiod (15,000 Lux), the temperature was 23-25°C and the relative humidity was 50%.

The plants are 28-30 days old.

A. the red clover inoculated with wild-type *R. trifolii* T12-1

B. the red clover non-inoculated.

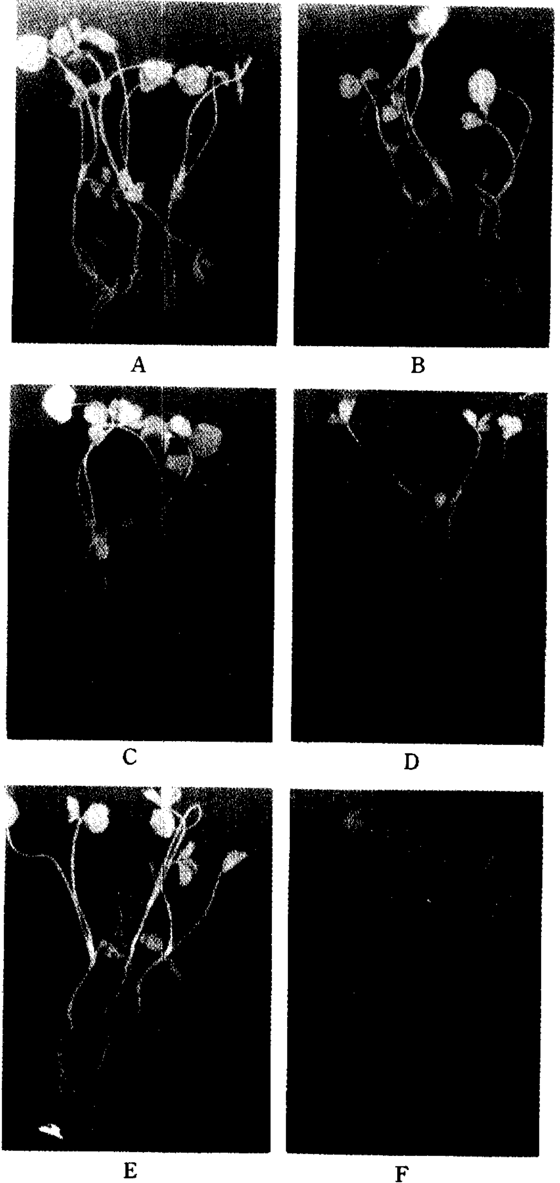

Fig. 2. *Comparison between red clover plants inoculated with the mutant strains of R. trifolii.*

a. *R. trifolii* T12-1K1 b. *R. trifolii* T12-1K2

c. *R. trifolii* T12-1K3 d. *R. trifolii* T12-1K4

e. *R. trifolii* T12-1K5

f. wild-type *R. trifolii* T12-1

The plants are 4 weeks old.

Table 3. *Comparison of nitrogenase activities of red clover inoculated with several mutant strains of R. trifolii.*

Strains	C_2H_2 reduced per plant (n mole/hour)
Rhizobium trifolii T12-1K1	2.40(*n* mole/hour)
T12-1K2	1.72
T12-1K3	0.29
T12-1K4	0
T12-1K5	0
Wild-type *Rhizobium trifolii* T12-1	14.61

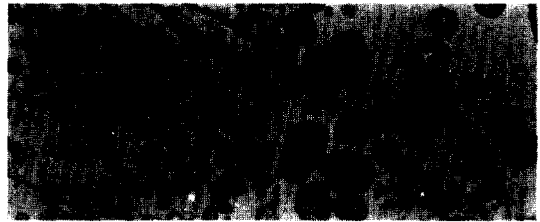

Fig. 3. *Ultrastructure of red clover nodule infected with wildtype Rhizobium trifolii* T12-1.
A: X8500, **B:** X20800.

BD	:Bacteroid	L	:lipid
PCW	:plant cell wall	V	:vacuole
LBD	:lysed bacteroid		

less active than wild type *R. trifolii* T12-1 when tested by the effectiveness assay, although they had more nodules than will type.

Mutant strains *R. trifolii* T12-1K4 produces nodules appearing similar to wild-type nodules.

The nodules formed by these mutant strains, however, showed decreased acetylene reduction or did not at all. It suggest that mutation in *Rhizobium* presumably is located in a structural gene for nitrogenase. The phenotype of all mutants tell us that N_2 fixation is not necessary for a nodule to be formed.

There is an observation that ineffective mutant strains of *Rhizobium* generally produce more nodules per plant than are found when the plant is inoculated with an effective wild strains (Maier and Brill, 1976). This seem to be the case with our ineffective or less effective strains as well. There must be some type of control of nodule formation by the effective symbiosis.

4. Ultra structure of root nodules

Fig. 3 shows a infected plant nodule cell with wild type *R. trifolii* T12-1. The cell contains many infected bacteroid, bacteroid distributed throughout the cytoplasm. The lysed bacteroids had not been appeared in the 4 weeks old nodule with inoculated wild type. Typical bacteroid were elongated rods ranging from 4 to 7μm in length and were individually enclosed in tight membrane envelopes. This stage is very active in nitrogen fixation (Paau, *et al.*, 1978).

Fig. 3 shows that 4 weeks wild type *Rhizobium trifolii* is relatively active in N_2 fixation.

In the case of mutant *Rhizobium trifolii* T12-1K1(Fig. 4. A, B) and *R. trifolii* T12-1K2(Fig. 5. A, B), many lysed bacteroids were shown. (Beringer *et al.*, 1977) These bacteroids had less densed cell contents and more vacuoles than in nodule of wild type *Rhizobium trifolii*. *R. trifolii* T12-1K2, which was less active in nitrogen fixation than *R. trifolii* T12-1K1, had more lysed becteroid. Generally, in the infected cells by wild *Rhizobium*, senescent bacteroids contain large membraneous vesicles (Paau *et al.*, 1980) and are greatly dis-

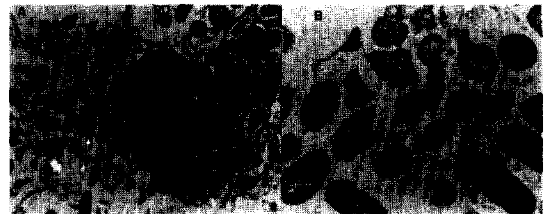

Fig. 4. *Red clover nodule infected with mutant Rhizobium trifolii T12-1K1.*
A: X8500, **B:** X20800.

Fig. 5. *Red clover nodule infected with mutant Rhizobium trifolii* T12-1K2.
A: X8500, **B:** X20800.

organized and structurally empty (Vance *et al.*, 1979). By our electron microscopic results, it was guessed that nodules inoculated with mutnat strains were less active in N_2-fixation because of more lysed bacteroids and vaculoes.

적 요

뿌리혹 형성능을 가지면서 스트렙토마이신에 내성을 보이는 *Rhizobium trifolii*돌연변이주들을 선별하였고, 야생주와 비교하여 Nitrogenase활성과 식물체의 생장에 미치는 돌연변이주들의 영향, 그리고 뿌리혹의 변화를 전자현미경으로 관찰하였다.

돌연변이 유발원으로서 *Nitrosoguanidine*를 사용하여 99.6%의 치사율을 나타내는 농도로 처리하였다. 그 결과, 뿌리혹 형성능이 있으면서 스트렙토마이신에 내성을 갖고 테트라싸이클린과 카나마이신에 민감한 다섯 균주를 선별하였다.

이들 뿌리혹을 형성하는 돌연변이주 중, 세 균주는 뿌리혹의 수가 야생형 보다 많았으나 식물체의 생장에서는 약간의 감소를 보였고 Nitrogenase 활성은 현저히 감소하는 것으로 보아 질소고정유전자가 뿌리혹 형성에 필수적인 것은 아님을 알 수 있었다.

야생주와 돌연변이주를 각각 접종한 Red Clover의 뿌리혹을 전자현미경으로 관찰하였을 때, 질소 고정능이 감소된 뿌리혹 속의 박테로이드가 야생형이 감염된 뿌리혹의 것보다 시기적으로 빠르게 노쇠되어 용균세포현상을 나타내었다.

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