A NEW TECHNIQUE FOR THE INOCULATION OF *BRADYRHIZOBIUM JAPONICUM* IN THE PRESENCE OF HIGH POPULATIONS OF INDIGENOUS RHIZOBIA IN SOILS - UTILIZATION OF ANTIBIOTICS AND ANTIBIOTIC - RESISTANT STRAINS -

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ABSTRACT - A greenhouse study was carried out in a cerrado soil with an established *Bradyrhizobium japonicum* population in order to study the effect of seed treatment with antibiotics and inoculation, on soybean. Seed coating with perlite and two antibiotics (Kasugamycin and spectinomycin) increased the occurence in nodules of an inoculated strain resistant to both antibiotics.

Introduction

It is generally recognized that in soils containing well-established populations of *B. japonicum*, if more effective strains are inoculated, the replacement of indigenous soil rhizobia by the inoculated strains is very difficult due to the low competition with indigenous soil rhizobia.

The competitive nodulation ability of rhizobia is associated with the survival in soil, multiplication in the rhizosphere, tolerance to high soil temperature, nitrate sensitivity, etc. However, attachment to the root hair surface is considered to be a very important preliminary step for nodule initiation or *Rhizobium* infection to host plant. If one *Bradyrhizobium* strain becomes attached to the root surface, nodulation of subsequently attached *Bradyrhizobium* strains is inhibited by the regulation system of the host plant, with a difference in time of only 4 to 6 hours. Therefore a new technique for inoculation was developed by using antibiotics and an antibiotic-resistant strain. The aim of this study is to promete the attachment to the root hair surface of the inoculated strain while indigenoussoil rhizobia are suppressed by antibiotics.

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Materials and Methods

Experiment 1

Pot preparation: Plastic cups 200 ml in capacity containing about 150 g of soil were used for growing the soybean cultivar Doko.

Antibiotic solution: Antibiotic solution containing both 6000 ppm of kasugamycin and 6000 ppm of spectinomycin was prepared. A 6000 ppm solution and 10 fold dilution solution (600 ppm) were used for coating and immersion, respectively.

Application of antibiotics by immersion method: Seeds were immersed in a 600 ppm antibiotic solution for 0, 1, 2, 5, 10 and 20 minutes.

The amount of antibiotics incorporated into the seeds was calculated from the amount of absorbed antibiotic solution. The amount at 0, 1, 2, 5, 10 and 20 minutes after immersion was 0, 5.4, 15.0, 28.3, 30.4 and 61.0 μg per seed, respectively.

Application of antibiotics by coating method: Two ml of a 6000 ppm antibiotic solution was mixed with 5 g perlite powder. The amount of perlite powder with antibiotics coated on the seed surface at the rate of 0.055, 0.165, 0.416 was 5.824 g per 22 seeds. The amounts of antibiotics coated were 4.3, 12.9, 32.4 and 454 μg per seed in the order of application rate of perlite powder.

Planting: Seeds treated with antibiotics were sown on February 16, 1989 in soil-packed plastic cups.

Experiment 2

Three soil samples differing in the number of rhizobium populations were used in this study. The soils contained zero (few cells/g), low (5.8×10^2 cells/g) and high (3.1×10^3 cells/g) populations.

Inoculum: A kasugamycin plus spectinomycin-resistant mutant strain A1017 kas^r·spe^r (obtained from Dr. H. Maruyama, Tokyo University, Tokyo, JAPAN) of *Bradyrhibium japonicum* was grown in yeast mannitol agar containing kasugamycin and spectinomycin, 400 ppm each. For the inoculation, the bacteria on the YMA plate were suspended with 2 - 3 ml of sterilized saline. The suspension containing the bacteria was diluted 10^5 and 10^7 times, and the population of bacteria was determined by counting the colonies on a YMA plate (Table 1). The mixture of 0.12 g kasugamycin and

0.12 g spectinomycin was dissolved in 20 ml of deionized water, and the antibiotic solution (6000 ppm) was added. The inoculum was prepared by mixing 5 g of perlite, 1 ml of a 6000 ppm antibiotic solution and 1 ml (2 x 10^8 cells/g) of a suspension of the antibiotic-resistant strain A1017 $kas^r spe^r$.

TABLE 1 - Amount of antibiotics applied and number of inoculated rhizobia.

		Antibiotic		
Treatment	Perlite mg*/seed	Kasugamycin µg/seed	Spectinomycin µg/seed	Antibiotic resistant *B. japonicum* A1017 cells/seed
I	67	0	0	0
II	17	28.6	28.6	4.8×10^5
III	67	114.3	114.3	1.9×10^6
IV	150	257.4	257.4	4.3×10^6

This treatment was applied in the three soil samples

* Based on dry weight

Seeding and inoculation: About 2.7 kg of soil was added to a plastic container, 3.2 liter in capacity, for growing the soybean plants. The inoculum was coated on the seed at the rate of 0, 0.5, 2.0 and 4.5 g per 30 seeds. Three "Doko" soybean seeds per pot were planted immediately after coating of the inoculum, and covered with 1 cm of soil on Feb. 21, 1989. The plants were grown in a greenhouse for 50 days.

Results

The lesions consisted of needle-shaped leaves or leaves with chlorosis shown in Figure 1 were induced by the application of antibiotic substances. The severity of the changes differed with the method of application as shown in Tables 2 and 3.

	Immersion						Coating			
Time Minutes	0	1	2	5	10	20	--	--	--	--
Amounts	0	5	15	30	35	61	4	13	32	454

FIG. 1 - Symptom of disorder induced by apllication of antibiotics.

TABLE 2 - Effect of antibiotics on growth of soybean plants.

N° treatment	Application method	Antibiotics mg/seed		Germination	Needle-shaped leaves	Plants with chlorosis %
		Kasugamycin	Spectinomycin	%	%	
1	Control	0	0	85	0	0
2	Immersion	5.4	5.4	90	6	28
3	Immersion	15.0	15.0	85	7	67
4	Immersion	28.3	28.3	75	73	87
5	Immersion	30.4	30.4	70	100	100
6	Immersion	61.0	61.0	60	100	100
7	Coating	4.3	4.3	80	0	0
8	Coating	12.9	12.9	80	0	20
9	Coating	32.4	32.4	90	11	67
10	Coating	454.0	454.0	75	20	67

1) Germination rate was observed at 8 days after sowing.
2) Lesions were observed 11 days after sowing.

TABLE 3 - Effect of antibiotics on dry matter, stem length and nodulation of Doko soybean at 3 weeks after sowing.

N° treatment	Application method	Main stem length cm	Plant dry matter mg/plant	Nodule	
				Number n°/plant	Weight mg/plant
1	Control	9.5 ± 2.7	307	14.3 ± 5.5	11.0
2	Immersion	12.1 ± 2.2	290	11.7 ± 4.5	12.0
3	Immersion	13.6 ± 1.5	294	15.1 ± 4.6	10.4
4	Immersion	5.3 ± 4.1	116	5.9 ± 3.6	1.5
5	Immersion	2.4 ± 1.2	95	10.0 ± 3.0	2.4
6	Immersion	2.4 ± 2.4	113	10.0 ± 3.8	1.6
7	Coating	10.9 ± 1.0	268	8.9 ± 5.1	7.1
8	Coating	11.9 ± 1.2	276	14.0 ± 8.0	9.3
9	Coating	11.9 ± 1.6	201	9.3 ± 3.9	4.7
10	Coating	10.9 ± 1.7	214	10.2 ± 4.0	3.4

The rate of germination decreased in proportion to the antibiotic contents in the immersion method, while no differences were observed in the coating method. All the plants which received more than 30 μg of antibiotic substances by using the immersion method showed needle-shaped leaves and/or chlorosis, while the percentages of these lesions in plants for which the antibiotics were applied by the coating method remarkably decreased (20% and 67%) even at a concentration of 454 μg (Table 2).

As the contents of antibiotics in treatments 5 and 9 were almost equal, main stem length and dry matter were compared in treatments 5 and 9 (Table 3). Elongation of the main stem in treatment 5 was strongly suppressed unlike in treatment 9. Also the dry matter content in treatment 5 was remarkably lower than that in treatment 9. These results indicate that the application of antibiotics with perlite reduced the severity of the lesions induced by the antibiotics.

Since the severity of the lesions caused by the application of antibiotics was reduced by the coating of perlite powder, a new method of inoculation was examined using antibiotics and an antibiotic-resistant strain (Figure 2).

Zero Rhizobia in virgin soil.

Middle Rhizobia in soil.

High Rhizobia in soil.

FIG. 2 - Pot experiment of inoculation method using antibiotic resistant strain A1017 kasr.sper and antibiotic substances.

Table 4 shows the length of the main stem and dry weight of herbage 24 days after sowing and Table 5 shows the proportion of nodules containing the antibiotic-resistant strain A1017 24 days after sowing. The length of the main stem was not reduced with the antibiotics. The weight of herbage, namely, plant growth was reduced, and the degree of reduction differed with the soils used (Table 4). Strains resistant to both kasugamycin and spectinomycin were not detected in the soils used (Table 5).

TABLE 4 - Length of main stem and dry weight of herbage 24 days after sowing.

Treatment	Length of main stem (cm)			Dry weight of herbage (mg/plant)		
	A soil	B soil	C soil	A soil	B soil	C soil
I	15.9	15.1	16.6	493	340	395
II	18.2	16.6	17.9	408	303	373
III	17.6	16.8	16.5	368	127	234
IV	16.4	16.2	15.9	248	151	228

Note: For the details of the treatment see Table 1. Populations of soybean rhizobia in A, B and C soils were 0, 5.8×12^2 and 3.1×10^3 cells/g, respectively.

Though it was expected that all the nodules in soil A except in treatment I could be formed with the inoculated strain (antibiotic-resistant strain A1017), this strain was not detected in about 40% of the nodules in treatments II and III for unknown reasons. Almost all the nodules in treatment IV where a large concentration of antibiotics was applied were occupied by the inoculated strain, suggesting that the utilization of antibiotics and an antibiotic-resistant strain was more suitable than the usual inoculation method.

TABLE 5 - Proportion of Doko soybean nodules containing antibiotic-resistant strain A1017 at 24 days after sowing.

Treatment	Number of nodules examined			Number of nodules containing antibiotic-resistant strain			Percentage of nodules containing inoculum strain		
	A soil	B soil	C soil	A soil	B soil	C soil	A soil	B soil	C soil
I	1	40	30	0	0	0	0	0	0
II	12	40	30	7	4	3	58	10	10
III	20	10	20	11	1	19	55	10	95
IV	24	20	20	24	16	20	100	80	100