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Sci. Rep. Coll. Agric. Osaka Pref. Univ. 52 : 7 ~11 , Original

Effect of the Application of Rice Straw and $(NH_4)_2SO_4$ to a Paddy Field on the Competitive Nodulation Ability of Indigenous *Bradyrhizobium* Strains

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Abstract

Competitive nodulation abilities of indigenous *Bradyrhizobium* strains in a paddy rice field at Osaka Prefectural Agricultural and Forestry Research Center were estimated by measuring both their nodule occupancy after inoculation of the soil suspensions to soybean and *N*-phenyl-1-naphthylamine (NPN) uptake activity of the isolates from the soils. Three plots (50 m^2 each) in the field had been applied annually for 18 years with $(NH_4)_2SO_4$ and rice straw at the following rate. Plot I : 0 and 0 kg, plot II: 100 and 0 kg, plot III: 100 and 7000 kg ha^{-1} y^{-1}, respectively. The inoculation test showed that bradyrhizobial population from the plot II had the highest competitive nodulation ability against *B. japonicum* strain 138 NR, and that from the plot I was the lowest. There was no significant difference in diversity of bradyrhizobial population among three plots in respect to intrinsic antibiotic resistance of the isolates of *Bradyrhizobium* from these plots. However, the plot II contained the highest proportion of the isolates which have large NPN uptake activity, indicating high competitive nodulation ability of the isolates. These findings were consistent with the previous results which indicated an increase in the competitive nodulation ability of a strain of *B. japonicum* under oligotrophic conditions.

Introduction

Inoculation of leguminous plants with rhizobial strains of N_2-fixing ability is an essential process to improve the crop productivity. However, the indigenous rhizobia must compete with the inoculant strains for the nodule formation on the host plants in most inoculation cases (Johnson *et al.*, 1965). Occasionally, an inoculum rate of more than 1000-fold over the soil population of the indigenous strain is needed in order to obtain 50% of the nodules from the introduced strain (Weaver and Frederick, 1974). The mechanisms by which the indigenous strains of rhizobia acquire a highly competitive nodulation ability against inoculant strains have not been elucidated.

Nutritional condition for bacterial growth would be one of the soil factors affecting the competitiveness of rhizobia. Rhizobial cells occur in three distinct environments, soil, root nodules, and nutrient media. Most soils except the rhizosphere soil are limited in biologically available organic matter. Rhizobia in the soil must survive starvation in the oligotrophic conditions until they encounter the host roots. Previous study shows that oligotrophic growth in purified water results in the increase of competitive nodulation ability of *Bradyrhizobium* strains which form nodules on soybean (Ozawa and Doi, 1996).

For the sake of improvement in the soil fertility of paddy fields, rice straws are sometimes plowed back into the soil after harvest season, as well as the application of nitrogen fertilizer. This performance may cause some changes in the availability of organic matter to indigenous bradirhizobia in the soil. In this study we examined the effect of application of rice straw and ammonium sulfate on the competitive nodulation ability of indigenous *Bradyrhizobium* in a paddy rice field.

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Materials and Methods

Soils

Surface soils were randomly sampled from the 3 plots of experimental rice field at Osaka Prefectural Agricultural and Forestry Research Center, Habikino, in April 1996. These soils were grey lowland soils (Gleysols). Each plot was a square of 50 m^2, being adjacent to each other in order of the plot number. These 3 plots have been supplemented annually at post harvest season since 1977 with $(NH_4)_2SO_4$ and rice straw at the following rate. Plot I : 0 and 0 kg, plot II: 100 and 0 kg, plot III: 100 and 7000 kg ha^{-1} y^{-1}, respectively. Sixty kg P_2O_5 ha^{-1} y^{-1} and 100 kg K_2O ha^{-1} y^{-1} were supplied to all the plots. Paddy rice (*Oryza sativa* L. cv. Harebare) had been cultivated every year on these plots. Some chemical properties of the collected soils were measured in the usual ways (Japn. Soc. Soil Sci. Plant Nutr., 1986), and are listed in Table 1. Soybean has not been cultivated on the field. The number of *Bradyrhizobium* cells in the soils was determined by the plant infection count method (Somasegaran and Hoben, 1994). The total viable count of aerobic bacteria in the soils was obtained by using albumin agar plates (Wellington *et al.*, 1997).

Isolation of *Bradyrhizobium* strains

Bradyrhizobium strains were isolated from nodules of soybean (*Glycine max* (L.) Merr. cv. Okuhara) inoculated with soil suspensions from the three plots (Somasegaran and Hoben, 1994). Soybean seeds were germinated in the dark at 30℃ in vermiculite tubes (2 cm i.d. x 20 cm) under sterile conditions (one seedling per tube). Ten g (fresh weight) of soil from each plot was suspended in 90 mL of distilled water. Three days-old seedlings were inoculated with 1 mL of the soil suspension, and then transferred to a growth chamber (26℃; 15,000 lx; 15 h of light, 9 h of darkness). Each plant was supplied with 20 mL of Jensen's nitrogen-free nutrient solution (Gibson, 1980) at the time of sowing and watered at appropriate times during cultivation. At 24 days after sowing, nodules found on the lateral roots as well as the taproots were detached from the roots, washed with water to remove soil, and surface sterilized by immersion in ethanol for 10 s and then treated with 5% calcium hypochlorite for 3 min. The nodules were rinsed in five changes of sterile water, and then transferred to wells (one nodule per well) of a mocrotiter tray (Coster Co.). Each nodule was crushed with a sterile glass rod. The homogenate was streaked on one-tenth strength yeast extract-mannitol (1/10 YEM) agar plates (Ozawa and Tsuji, 1992). Well-isolated colonies formed on the plates were obtained after three time repetition of the culture on 1/10 YEM agar plates. Isolates were grown and maintained on YEM agar slants.

Antibiotic resistance of isolates

Each isolate was classified according to the difference in its intrinsic resistance to the following antibiotics at the indicated concentrations (μ g/mL): ampicillin, 40; chloramphenicol, 100; erythromycine, 100; kanamycine sulfate, 50; nalidixic acid, 40; neomycin sulfate, 100; rifampicin, 100; streptomycin sulfate, 100; tetracycline, 100. Each isolate grown in YEM on a shaker at 30℃ for 5 days was diluted 10^3-fold with distilled water. Two μ l of diluted suspension, containing approximately 10^3 cells, was transferred with a multiple inoculator onto a YEM agar plate supplemented with one of the antibiotics. Thirty isolates could be inoculated simultaneously to a plate. After 7 days of incubation at 30℃, the growth of isolates on the antibiotic test plates was observed. Shannon-Weaver Index of Diversity (H) was used to measure the biological diversity of the isolates (Atlas and Bartha, 1987).

Competitive nodulation ability of indigenous population

The competitive nodulation ability of indigenous bradyrhizobia as a whole was evaluated by determining nodule occupancy of the strains after inoculation of soil suspension upon soybeans, using *B. japonicum* 138NRS as a reference strain (Ozawa, 1988). *B. japonicum* 138 NRS is resistant to 100μ g nalidixic acid mL^{-1}, 100μ g rifampicin mL^{-1}, and 100μ g streptomycin sulfate mL^{-1}. Six mixtures with the proportion of indigenous rhizobia to the strain 138NRS ranging from about 1 x 10^{-3} to 0.3 were prepared for each inoculation. The inoculum concentration of the indigenous strain ranged from 2 to 5 cells mL^{-1}. Five mL of the mixture was inoculated to a soybean seedling on vermiculite in a test

tube (2 x 20 cm), one seedling per tube. Forty to 60 nodules (>2 mm) were detached from 6 plants (25 day-old) for each treatment. Nodule occupancy was determined by the antibiotic resistance of bacteria in each nodule using 1/10 YEM agar plates.

N_{50} was used in this study as a measure of the competitive nodulation ability of indigenous bradyrhizobial population. The N_{50} value represents the proportion of indigenous bradyrhizobia to their competing strain 138 NRS in an inoculum when 50% of nodules are formed by the indigenous bradyrhizobia. N_{50} was calculated similarly for estimating ED_{50} (effective dose 50%) by the Probit method (Finney, 1978).

Assay for *N*-phenyl-1-naphthylamine (NPN) uptake activity

The uptake of NPN into bacterial cells was estimated by a modification of the method of Loh *et al.*, (1984). Two mL of HEPES buffer (pH7.2) with 1 mM KCN and 0.1 mL of the cell suspension were mixed in a cuvette. Temperature of the cuvette was maintained at 30℃. The reaction was started by adding 40 μ L of 500 μ M NPN to the mixture. Increase in fluorescence intensities of the mixture was measured with a Hitachi Fluorescence Spectrophotometer 650-10(S). Excitation and emission wavelengths for NPN were set at 350 nm and 420 nm, respectively, with slit widths of 4 nm. As described previously, the addition of NPN to the cell suspension resulted in a time-dependent increase in fluorescence intensity due to the NPN incorporation into the outer and inner membranes of bacteria (Ozawa *et al.*, 1995). The fluorescence intensity was expressed as a function of time in the following equation.

$$\log (F_{max} - F_t) = \log F_{max} - kt$$

where t is the reaction time, F_t is the fluorescence intensity at time t, F_{max} is the maximum fluorescence intensity, and k is a constant, representing NPN uptake activity of the cell.

Results and Discussion

We isolated 57, 69, and 67 strains of *Bradyrhizobium* from the plot I, II, and III, respectively. These isolates were classified into 47 types of different intrinsic antibiotic resistance (IAR, Table 2). Bacteria belonging to larger number of IAR are tolerant to more antibiotics. Figure 1 shows the frequency distribution of these IAR types of the isolates from these 3 plots. Although there was no significant difference in Shannon-Weaver index, bradyrhizobial populations from the 3 plots were distinguished by the characteristic profiles of IAR. Thirty-nine % of the isolates from the plot I belonged to the IAR type 7. The isolates from the plot II and III were dispersed in the other IAR types. The IAR types accounting for over 10% of the isolates from the plot II were the type 7, 13, 16, and 28, while the plot III had the type 11 and 19 instead of the type 16 and 28. The IAR type 13 was the major one in the plot III.

Table 3 presents the difference of N_{50} value of the indigenous bradyrhizobia in the 3 plots when inoculated with *B. japonicum* 138NRS strain to soybean. These N_{50} values show the competitive nodulation ability of the indigenous bradyrhizobia as a whole population. Brady-

Table 1. Chemical properties and bacterial densities of the soils from the experimental plots.

	Plot		
	I	II	III
pH (H_2O)	5.90	5.91	6.30
pH (KCl)	5.49	5.45	5.66
Total C (g kg^{-1})	12.1	12.2	16.3
Total N (g kg^{-1})	1.2	1.2	1.2
CEC (cmol (+) kg^{-1})	10.4	10.2	11.6
Exchangable cations (cmol (+) kg^{-1})			
Ca	6.9	6.9	8.7
Mg	1.7	1.8	2.1
K	0.1	0.1	0.2
Available P_2O_5 (g kg^{-1})	0.26	0.22	0.26
Available SiO_2 (g kg^{-1})	1.13	1.07	1.24
Available N (g kg^{-1})	0.064	0.057	0.158
Total aerobic bacteria (CFU g^{-1})	2.5×10^7	1.8×10^7	3.2×10^7
Bradyrhizobium cells (CFU g^{-1})	1.0×10^2	3.1×10	5.8×10

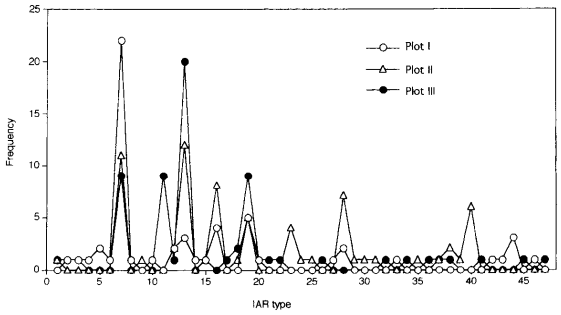

Fig. 1. Frequency distribution of intrinsic antibiotic resistance types of *Bradyrhizobium* isolates.

rhizobial population from the soil of the plot II had the highest competitive nodulation ability, and that of the plot I was the lowest.

We have estimated competitive nodulation abilities of individual isolates by measuring NPN uptake activity of the cells. NPN was used as a hydrophobic probe to estimate surface hydrophobicity of bacteria. In the previous study, we found the positive correlation between the NPN uptake activity, that is surface hydrophobicity of the strain, and the competitive nodulation ability of *Bradyrhizobium* strains (Ozawa *et al.*, 1991). Thirteen, 15, and 16 isolates from the plot I, II and III, respectively, were assayed for NPN uptake activity expressed by the parameter k, Bradyrhizobial population from the plot II contained the highest proportion of the cells which have large NPN uptake activity, and the lowest in the population from the plot I (Table 4). These results suggest that the higher competitive nodulation ability of bradyrhizobial population is due to the higher proportion of much more competitive strains in the population.

It has been known that chemical nitrogen fertilizer such as $(NH_4)_2SO_4$ would decrease available organic matter for soil microorganisms. The previous study suggest that *Bradyrhizobium* in oligotrophic conditions increase their competitive nodulation ability (Ozawa and Doi, 1996). The carbon starvation may explain a smaller population of aerobic bacteria and *Bradyrhizobium* (Table 1) as well as a higher competitive nodulation ability of bradyrhizobial population in the soil of plot II. On the other hand, application of rice straw to paddy fields increases the content of soil organic matter (Yoneyama *et al.*, 1977). There was no significant difference in the chemical features of the soils from the three plots except the higher values of total carbon and available nitrogen in the plot III (Table 1). The results in this study suggest that the increase in the amount of available organic matter in soil environment by applying rice straw together with $(NH_4)_2SO_4$ decreases the proportion of more competitive strains in *Bradyrhizobium* population.

Table 2. Intrinsic antibiotic resistance of *Bradyrhizobium* isolates.

IAR type	Amp*	Chl	Ery	Kan	Nal	Neo	Rif	Stm	Tet
1	−	−	−	−	−	−	−	−	−
2	−	−	−	−	−	+	−	−	−
3	−	−	−	−	+	−	−	−	−
4	+	−	−	−	−	−	−	−	−
5	−	−	−	+	+	−	−	−	−
6	−	−	+	−	−	−	−	+	−
7	−	−	+	−	−	+	−	−	−
8	−	−	+	−	+	−	−	−	−
9	+	−	−	−	+	−	−	−	−
10	−	−	−	+	+	+	−	−	−
11	−	−	+	−	−	+	+	−	−
12	−	−	+	+	−	+	−	−	−
13	−	+	+	−	−	+	−	−	−
14	−	+	+	−	+	−	−	−	−
15	+	−	−	+	+	−	−	−	−
16	+	−	+	−	−	+	−	−	−
17	−	−	+	+	−	+	+	−	−
18	−	+	+	−	−	+	−	−	+
19	−	+	+	−	−	+	+	−	−
20	−	+	+	−	+	+	−	−	−
21	+	−	−	+	+	−	−	−	+
22	+	−	−	+	+	+	−	−	−
23	+	−	+	−	−	+	+	−	−
24	+	−	+	−	+	−	−	+	−
25	+	−	+	−	+	+	−	−	−
26	+	+	−	−	−	+	+	−	−
27	+	+	−	+	+	−	−	−	−
28	+	+	+	−	−	+	−	−	−
29	+	+	+	+	−	−	−	−	−
30	−	−	+	+	+	+	−	+	−
31	−	−	+	+	+	+	+	−	−
32	−	+	+	−	−	+	+	−	+
33	−	+	+	+	+	+	−	−	−
34	+	−	+	−	+	+	−	+	−
35	+	−	+	−	+	+	+	−	−
36	+	−	+	+	−	+	+	−	−
37	+	−	+	+	+	+	−	−	−
38	+	+	+	−	−	+	−	−	+
39	+	+	+	−	−	+	−	+	−
40	+	+	+	−	−	+	+	−	−
41	−	+	+	+	+	+	+	−	−
42	+	−	+	+	+	+	−	+	−
43	+	+	+	−	−	+	+	+	−
44	+	+	+	+	+	+	−	−	−
45	−	+	+	+	+	+	+	−	+
46	+	−	+	+	+	+	+	+	−
47	+	+	+	−	+	+	+	+	−

+ or − : An isolate grew or did not grow on the YEM plate supplemented with each antibiotic.
* : Amp = ampicillin, Chl = chloramphenicol, Ery = erythromycine, Kan = kanamycine sulfate, Nal = nalidixic acid, Neo = neomycin sulfate, Rif = rifampicin, Stm = streptomycin sulfate, Tet = tetracycline

Table 3. Competitive nodulation index (N_{50}) of the soil suspensions from the experimental plots.

Plot	N_{50}	95% Confidence interval
I	0.82	0.12~5.8
II	0.0064	0.0038~0.011
III	0.071	0.030~0.17

Table 4. Frequency distribution of the isolates in relation to NPN-uptake activity (k).

Plot	k (min^{-1})			
	k <1	1≦ k <2	2≦ x <3	3≦ x
I	7	5	1	0
II	5	4	2	4
III	3	7	5	1

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(Recieved Dec. 1, 1999; Accepted Feb. 11, 2000)