C₂H₂ Reduction by a "Healthy" Mixed Culture of Soybean Cells and *Rhizobium*

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A mixed culture of soybean cells and some strains of *Rhizobium* developed nitrogenase activity which was determined by reduction of acetylene. The acetylene reduction activity of the mixed culture was revealed in the period when the respiratory activity of soybean cells was maintained at their original level. Effective strains of *Rhizobium* for soybean produced higher acetylene reduction activity than ineffective strains.

Certain strains of *Rhizobium*, root nodule bacteria, have been demonstrated to develop nitrogenase activity when grown in association with cultured leguminous or non-leguminous plant cells.^{1~6)} The *in vitro* study with cultured plant cells may be helpful for understanding symbiotic plant-*Rhizobium* cell interactions *in vivo*.

In the above studies the plant cells were incubated with rhizobia for 1 to 3 weeks before assays for nitrogenase activity. The long period of mixed culture, however, results in the rupture or death of cultured plant cells and in the rapid growth of rhizobia with the production of a large quantity of slime.^{7,8)} There seems to be no "symbiotic" interaction between plant cells and rhizobia in the long period of mixed culture.

The present report shows that associations between cultured soybean cells and *Rhizobium* possess nitrogenase activity in the period when soybean cells maintain a high level of the respiratory activity.

MATERIALS AND METHODS

Organisms. Cultured soybean cells were obtained from the root tissue of *Glycine max* cv. Kingen No. 1 in the manner described by Gamborg *et al.*⁹⁾ The cells were cultured at 30°C in 100 ml of Gamborg's B5 liquid medium as described previously.⁷⁾ The cells which had been cultured for over 4 years were used in this study.

Rhizobium japonicum 311b-6, -24, -29, -31, -38,

-122, -143 were obtained through the courtesy of Dr. D. F. Weber, USDA, Beltsville, Md. Other strains of *Rhizobium* were obtained from the National Institute of Agricultural Sciences, Japan. Stock cultures of *Rhizobium* were maintained on agar slants of a yeast extract-mannitol (YEM) medium.¹⁰⁾ Rhizobia growing in the YEM liquid medium at 30°C were used in the following experiments. Effectivity of each *Rhizobium* on soybean is defined as the production of root nodules.

Mixed culture between soybean cells and rhizobia. Soybean cells were inoculated at the 6th day with 1.0 ml of exponentially growing rhizobia containing 1×10^8 cells. There were about 2×10^2 soybean cells per rhizobium. Three hours later 10 ml of the mixed culture was pipetted onto a threefold layer of sterile cheesecloth which covered one end of a glass tube (1.5 × 5.0 cm). The cells retained in the glass tube were rinsed with 10 ml of a second medium (B5-2). Composition of B5-2 was the same as that of B5, except for the lack of 2,4-Dichlorophenoxyacetic acid (2,4-D) and the substitution of NH₄Cl for (NH₄)₂SO₄. Concentration of NH₄Cl and KNO₃ in B5-2 were 0.1 mM and 2 mM, respectively. The glass tube with the cells was then plated onto 10 ml B5-2 agar in a 40 ml glass vial which was covered with an aluminum foil cap.

Assay for nitrogenase activity. Nitrogenase activity was determined by the reduction of acetylene to ethylene. The cells which had been cultured on B5-2 agar at 30°C were transferred into a 20 ml glass vial sealed with a rubber serum stopper. The gas phase was replaced with a mixture of 40% oxygen: 60% argon, and then 0.2 ml of acetylene were injected into the vial. The amount of ethylene which was produced by the mixed culture increased linearly with time for about 7 hr after the lag of about 2 hr. So ethylene measurements were usually taken at 3 and 5 hr after the injection of acetylene into the vial. The amount of ethylene was deter-

mined by gas chromatographic analysis. The cells in another vial were assayed for endogenous ethylene production.

Assay for O_2 consumption. O_2 uptake of the cells cultured on B5-2 agar was measured in a Warburg respirometer at 30°C. Each Warburg flask contained about 40 mg (dry weight) cells suspended in 3.0 ml of 50 mM potassium phosphate buffer, pH 6.5. The gas phase was air.

RESULTS

Acetylene reduction activity was observed in the association of *R. japonicum* J-5033 and soybean cell culture (Fig. 1). The highest value of the activity was obtained when the mixed cells were cultured on B5-2 agar at 30°C. The reduction activity increased rapidly with the lag of 20 hr after the mixing of these organisms. A maximum value of the activity was recorded at about 50 hr of incubation. The mixed cells cultured on B5-2 agar for over 100 hr did not reveal any acetylene reduction activity. The soybean cells without rhizobia showed no acetylene reduction activity. There was a slight endogeneous ethylene production; 0.1~1.3 nmols C_2H_4 /g dry weight/hr.

Effects of 2,4-D and inorganic nitrogens on the development of the acetylene reduction system in the mixed culture were investigated (Table I). The presence of 2,4-D in B5-2 inhibited the development of acetylene reduction system in the mixed culture. The lower

FIG. 1. Acetylene Reduction Activity of the Mixed Culture between *R. japonicum* J-5033 and Soybean Cells.

Soybean cells with *R. japonicum* J-5033 were cultured on B5-2 agar at 25°C (■—■), 30°C (●—●), and 35°C (▲—▲). Soybean cells without rhizobia cultured on B5-2 agar at 30°C were also assayed for their C_2H_2 reduction activity (○—○). Each point represents the mean $\pm$ SE from four replicates.

concentration of NO_3^- and NH_4^+ in B5-2 than those in B5 was required for reproducible production of acetylene reduction system in the mixed culture. Increasing in the concentration of NH_4Cl to 10 mM resulted in a remarkable endogeneous ethylene production by the mixed culture. Acetylene inhibited the production of endogeneous ethylene.

TABLE I. EFFECTS OF NO_3^- , NH_4^+ , AND 2,4-D ON THE DEVELOPMENT OF C_2H_2 REDUCTION ACTIVITY.

Mixed culture of soybean cells and *R. japonicum* J-5033 was grown at 30°C on B5-2 containing KNO_3 , NH_4Cl , and 2,4-D in each concentration. Maximum values of the C_2H_2 reduction activities of each culture are tabulated. Each value is the mean $\pm$ SE from four replicates.

KNO_3 (mM)	NH_4Cl (mM)	2,4-D (ppm)	C_2H_2 reduction activity (nmol C_2H_4 /g dry weight/hr)	
0	0	0	0.83 $\pm$ 3.13	(40 hr)*
0.2	0	0	0.58 $\pm$ 3.11	(40)
2	0	0	5.27 $\pm$ 3.08	(45)
20	0	0	1.08 $\pm$ 0.83	(45)
2	0.1	0	4.56 $\pm$ 0.73	(50)
2	1	0	2.71 $\pm$ 0.94	(60)
2	10	0	0.03 $\pm$ 0.27	(5)
2	0.1	0.2	2.56 $\pm$ 0.85	(50)
2	0.1	2	0.21 $\pm$ 0.34	(50)

* (), incubation time before assaying the activity.

Figure 2 shows that respiratory activity of the mixed culture rapidly decreased after about 40 hr of incubation. Soybean cells changed in their color from bright yellow to reddish brown within 60 hr of incubation. *Rhizobia* grew for over 100 hr, although the

FIG. 2. Effect of *R. japonicum* J-5033 on O₂ Uptake of Soybean Cells.

Soybean cells were cultured with (●—●) and without (○—○) *R. japonicum* J-5033 on B5-2 agar at 30°C. Each point is the average of four separate experiments.

FIG. 3. Growth of *R. japonicum* J-5033 and Soybean Cells in the Mixed Culture.

The number of rhizobia (▲—▲) was estimated by dilution plate method. The dry weight of the mixed culture (●—●) was considered that of soybean cells. To determine the dry weight, cells were washed with water on a filter paper, and dried at 90°C for 20 hr. ○—○, dry weight of soybean cells cultured without rhizobia on B5-2 at 30°C. Each point is mean of duplicate experiments.

mixed culture ceased increasing in its dry weight (Fig. 3). These results suggest that biological activities of soybean cells are inhibited in a short period of the mixed culture with *Rhizobium*.

Some of the other strains of *Rhizobium* showed the acetylene reduction activity when grown with soybean cells (Table II). Each strain of *Rhizobium* had the maximum value of acetylene reduction activity after about 50 hr of incubation with soybean cells at 30°C. Effective strains for soybean developed higher activities than ineffective strains, with the exception of some strains.

TABLE II. C₂H₂ REDUCTION ACTIVITIES OF ASSOCIATIONS OF SOYBEAN CELLS AND VARIOUS STRAINS OF *Rhizobium*

Mixed culture had been maintained on B5-2 for 50 hr at 30°C before the C₂H₂ reduction activity was assayed. Each value is the mean ± SE from four replicates.

Strain	Effectivity on soybean	C ₂ H ₂ reduction activity (nmol C ₂ H ₄ /g dry weight/hr)
<i>R. japonicum</i> J-501	+	4.98 ± 1.39
J-5033	+	4.73 ± 0.64
J-5067	-*	1.35 ± 0.86
311b-6	+	1.97 ± 0.85
-24	+	5.11 ± 0.32
-29	+	0.06 ± 0.55
-31	+	3.54 ± 0.97
-38	+	2.10 ± 0.22
-122	+	5.36 ± 1.18
-143	+	3.22 ± 2.98
<i>R. leguminosarum</i> J-303	-	1.81 ± 0.77
<i>R. trifolii</i> J-C-2004	-	0.63 ± 0.21
<i>R. meliloti</i> J-103	-	0.79 ± 1.62
J-151	-	1.91 ± 1.29
<i>R. spp.</i> J-506	-	1.99 ± 0.89
J-680	-	0.48 ± 0.39
J-1010	-	0.81 ± 0.49

* +, able to nodulate soybean *in vivo*; -, unable to nodulate soybean *in vivo*.

DISCUSSION

We have previously shown that cultured soybean cells were inhibited in their growth and ruptured by *Rhizobium japonicum* within a

few hours after the inoculation.^{7,8)} Under the condition used in this study soybean cells were also inhibited in the growth and the respiratory activity by *R. japonicum* J-5033, although the rhizobia grew rapidly in the presence of soybean cells. These results suggest that "healthy symbiotic interaction" between rhizobia and cultured soybean cells comes to an end in an early period of the mixed culture, followed by a parasitic growth of rhizobia on soybean cells.

Some of the strains of *Rhizobium* were reported to fix nitrogen even in a pure culture, when grown in a liquid medium under a low concentration of oxygen^{11,12)} or in a thick layer on an air-exposed agar surface.^{13~16)} In the long-period mixed culture, plant cells being on destruction may provide a factor (s) for rhizobia to develop the N₂-fixing system. And slime produced by rhizobia may protect nitrogenase from oxygen.¹³⁾ The long-period mixed culture can be used to elucidate the mechanism of the induction of the synthesis of nitrogenase enzymes coded in *Rhizobium*. However, it is not a favorable system in order to elucidate a factor (s) which controls the nodulation process and the symbiotic N₂-fixing activity *in vivo*.

This paper shows that acetylene reduction activity was detected in the association between soybean cells and some strains of *Rhizobium* before soybean cells decreased in their respiratory activity. Acetylene reduction assay is a simple and highly sensitive one for determining nitrogenase activity.¹⁷⁾ But until the presence of nitrogenase will be verified by ¹⁵N₂ reduction assay, we can not conclude that the acetylene reduction activity observed in this study was due to nitrogenase.

Soybean cells used in this study had been cultured for over 4 years and were completely undifferentiated, judging from their inability

to generate plant organs. Therefore, they may be different from *in vivo* tissues in various characteristics. Nevertheless, effective strains of *Rhizobium* for soybean generally produced higher acetylene reduction activity than ineffective strains. This fact suggests that certain mechanisms concerned in the host-rhizobium specificity were maintained in soybean cells after the dedifferentiation. *In vitro* study with the short-period mixed culture will afford us useful information to elucidate the specific interaction between soybean and rhizobium.

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