Synergetic Cultures of *Glycine max* Root Cells and Rhizobia Separated by Membrane Filters

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**ABSTRACT**

When suspension cultures of actively growing soybean (*Glycine max* L.) root cells were separated by two or three membrane filters from suspension cultures of the bacteria, a synergetic (cooperative) activation of nitrogenase was observed in the *Rhizobium japonicum* used in the bacterial side. Either plant cells or plant cell-conditioned medium was needed for this activation to take place. Both acetylene reduction and hydrogen evolution by the activated *R. japonicum* persisted for several days after removal from the apparatus when (a) a suitable carbon source was provided, (b) oxygen supply was limited, and (c) growth of bacteria was suppressed by lowering of ammonia and nitrate concentrations. Activation could also take place when the bacteria were placed in media to which plant cell-conditioned medium was added. The advantages of this method for studies on symbiosis are discussed.

**MATERIALS AND METHODS**

**Conditioned Medium.** Plant cells were placed in one chamber (P) of the apparatus "DCII" shown in Figure 1. The bacteria (*Rhizobium japonicum*) were placed in the second chamber (B) of the apparatus. These two chambers were separated by a third small connecting chamber(s) with filters interposed on each side of the connecting chamber. These filters include different combinations of cellulose nitrate-acetate (0.22 μm and 0.45 μm) which are H and G types of Millipore; Teflon (5 μm) type L Mitex and polycarbonate (GE) Membra-Fil (0.2 μm) fabricated by Johns-Manville Cellite division.

The confirmed medium was the medium taken from the chamber (P) containing the plant cells. The conditioned medium was taken between 3 to 5 days after the start of each experiment. This medium contained products secreted by plant cells in response to (a) rapidly induced growth by daily replenishment of medium B-5 (2) with pH adjusted to 6.2 and containing lowered amount of nitrogen (see below), (b) products secreted by rhizobia and diffusing across the filters from the bacterial side. In use, this medium was diluted 1:1 with conventional rhizobial medium of Valera and Alexander (16) but did not contain nitrate or ammonia (VA-N).

When nitrogenase was activated in rhizobia, the nitrate concentration of the medium from the bacterial side was measured to be less than 2 mm and decreased from its original concentration of 6 to 8 mm. The original carbon source in VA-N was mannitol and that in B-5, sucrose and mannitol. The conditioned medium was, however, found to contain a variety of reducing sugars. These reducing sugars were in turn metabolized by the rhizobia.2

The experiments of Figure 2 and Table I were conducted in the DCII apparatus with soybean root cells also present. The source of the plant cells was from subcultures of Acme root cells maintained, by repeated transfers, for 4 months in suspension culture using complete B-5 medium (4).

The source of the *R. japonicum* was as follows. *R. japonicum* R138 was originally obtained from Dr. S. Smith, Agricultural Laboratory Inc., Columbus, Ohio and maintained in our laboratory before use in the DCII apparatus. A sample of a rhizobial transfilter culture, used successfully for nitrogenase activation, was given to Dr. Deane F. Weber for serological typing. Another portion of this sample was used to inoculate Harosoy variety of soybean by Dr. M. Lamorg of our laboratory. These were then grown under greenhouse conditions together with uninoculated controls.

After 8 weeks, the uninoculated control plants were small and devoid of nodules. The inoculated plants gave large nodules which were collected and used for obtaining an isolate.

1 Contribution No. 553.

2 The sugars detected by gas chromatography are arabinose, an unidentified peak, xylose, mannose, galactose, fructose, and glucose. Mannitol and inositol may also be present as added but unconverted sugars in the medium withdrawn early in the experimental period. (Private communication from Dr. Bhuvaneshwari, Kettering Laboratory.)
Nitrates determinations were carried out after conversion of nitrates to nitrites. The latter were analyzed by the sulfanilamide and N(1-naphthyl)ethylenediamine procedure (15).

**RESULTS**

Experiments with Root Cells from Acme Soybeans and *R. japonicum* AK5-R138. Observations on the plant cells will be indicated first. Acme variety soybean root cells were used in the plant side (P) of the DCII. The period of rapid root cell division was indicated by microscopic examination to be between 2 to 5 days in the apparatus. The appearance of these cells under phase optics is shown in Figure 3. During the initiation period when the medium on side P was renewed, the plant cells which showed rapid division were the small round cells (Fig. 3). Cell division could be followed by standard techniques of Feulgen staining.

The plant cell mass remained constant despite dilution with daily renewal of medium. Changes in pH were minimal in the P chamber when the pH of the plant medium was kept at 5.5. During this initial preparative period, diffusion of materials across the two or three membrane filters was indicated as follows. The plant cells produced a variety of reducing sugars from sucrose and these were detected first from chamber P, next from the medium in the central chamber and then from the medium on the B side.

After medium renewal in P was stopped, the majority of older plant cells showed further clumping and organized into a variety of shapes.

The changes in the bacterial isolate AK5-R138 from the same experiment are indicated in Figure 2. In this system N₂ gas was bubbled through the medium on side B until the O₂ content dropped to 5% in the liquid as well as the gas phase. The former was monitored by a Yellow Springs O₂ electrode and the latter by gas chromatography analysis with a molecular sieve (13). The decrease in bacterial absorbance at 660 nm during the initial phase of the experiment was a characteristic of this system when the VA-N medium was used and took place after treatment with nitrogen. The pH of the medium influenced the final growth density of rhizobia which prefer neutral pH for growth. The *A*₆₆₀ decrease in this case was minimized by changing the original pH of the VA-N medium to 6.2. During the later experimental period when bacterial density increased, there was some slime production as well as production of an unidentified gas mixture in B. (In initial experiments the pH drop as well as the *A*₆₆₀ decrease were rather dramatic and was accompanied often by

isolate was grown in suspension culture and labeled AK5-R138.

Acetylene reduction and H₂ evolution were measured as before (13). The liquid depth for these assays was about 5 mm and the vessels were not shaken. The acetylene concentration in the measuring gas was 1.6%; the O₂ 2%, and the remainder argon. Variation from this concentration of the gas mixture has been indicated.

Protein determinations on bacterial pellets collected by centrifugation were made after treating suitable aliquots with 0.5% deoxycholate and 0.25 M NaOH (final concentrations). These treated aliquots were placed at 90°C for 10 min and used for the protein assay according to Lowry et al. (8), with similarly treated standards of BSA.

**Fig. 1.** Assembly of DCII double chamber apparatus. A simple diagram is shown in a. *Rhizobium japonicum* were kept in one chamber (B) and cultured soybean root cells or conditioned medium in the second large chamber (P). Gas was passed thru the cell suspensions via Millipore filters in Swinny adapters (marked with stars). Sample withdrawal and additions via filters were made with the aid of syringes fitted atop long needles. Small magnetic fleas (m) were used in the two large chambers as well as the interconnecting chamber to stir the contents but not damage cells. The diagram and the photograph illustrate the basic assembly only, filters, syringes and gas lines have not been illustrated. In the mode shown in b, two small interconnecting chambers were used with three filters (f) interposed. The various chambers were held tightly together in a frame by an external pressure screw (not illustrated).

**Fig. 2.** Changes in density and pH of *R. japonicum* isolate AK5-R138 grown in the transfilter apparatus. During the period indicated by the line, N₂ gas was passed in the medium as indicated in the text. At arrow on day 5, the vitamin supplement of the medium VA-N was doubled and the culture was left alone under gentle stirring. The *R. japonicum* R138 isolate (AK5) used in the experiment reduced acetylene to ethylene as indicated in the text and Table 1.
Table 1. Effect of Added Substrates on Acetylene Reduction Activity in R. japonicum Isolate No. AK5(DCII/ACM)

<table>
<thead>
<tr>
<th>Substrates</th>
<th>% oxygen</th>
<th>3</th>
<th>5</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gluconate, 5 mM</td>
<td>Ar +</td>
<td>1.6</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>Hydroxybutyrate, 6.2 mM</td>
<td>366</td>
<td>133</td>
<td>177</td>
<td></td>
</tr>
<tr>
<td>Pyruvate, 7.5 mM</td>
<td>486</td>
<td>146</td>
<td>174</td>
<td></td>
</tr>
<tr>
<td>α-Ketoglutarate, 4 mM</td>
<td>627</td>
<td>290</td>
<td>271</td>
<td></td>
</tr>
<tr>
<td>Endogenous</td>
<td>1161</td>
<td>404</td>
<td>213</td>
<td></td>
</tr>
<tr>
<td></td>
<td>745</td>
<td>330</td>
<td>466</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. Appearance of Acme cells during the initial period of growth in the transfilter apparatus. The phase-contrast pictures are magnified 420 X (day 2). The smaller round cells were shown to exhibit rapid cell division when examined after Feulgen staining.

experimental failure if the starting $A_{660}$ was lower than 0.5.)

The changes shown in Figure 2 have also been noted in other experiments using different bacterial strains (B side) and either Harosoy or Acme root cells (P side). In the final phases of bacterial activation, movement of bacteria into the central, small interconnecting chamber has sometimes been noted when cellulose nitrate filter is used at the B side of the apparatus.

Characteristics of Activated R. japonicum. Table I summarizes results obtained with activated R. japonicum AK5-R138 after removal from DCII on the 10th day of the experiment (Fig. 2). In this experiment two polycarbonate filters were used, both of 0.2 μm pore size and activation time for bacteria was longer. The conditions under which replicate samples were tested influenced the amounts of acetylene reduced.

Since the rhizobia were taken freshly out of the DCII apparatus, endogenous substrate was still present in the bacterial suspension even after 10 days and acetylene was reduced in the absence of added substrate. When extra substrate was added after this period, α-ketoglutarate usage resulted in more acetylene reduction than pyruvate or β-hydroxybutyrate, all of which were superior to gluconate.

Conditioned Medium and R. japonicum Strain 61A76. The first experiment showing the effect of conditioned medium was carried out in the same manner as with plant cells. The R. japonicum strain 61A76 was placed in the bacterial side (B) of the DCII apparatus at an initial cell density to give a 600 nm absorbance reading at 1.16 on day 1 in a 100-ml volume. N2 gas (100 ml) was introduced in side B over a 15 min period four times daily for 3 days. At the start of the experiment, 100 ml of Millipore-filtered conditioned medium was added in the plant side (P) of the DCII apparatus. This conditioned medium was obtained in the previous experiment from the P side of the
apparatus. On each of the following 4 days, 30 ml of the medium were removed and 30 ml of "fresh" conditioned medium were added. No plant cells were used in this experiment.

**EFFECTS OF CULTURE AGE**

The bacterial suspension was removed from the DCII apparatus on day 5 and used for two kinds of acetylene reduction experiments. In the first experiment shown in Table II, one portion of the bacterial suspension culture was diluted to 100 ml in a milk-dilution bottle with VA-N medium and placed on a stirrer (about 90 rpm). The suspension was kept at constant volume by additions of VA-N medium to replace the bacterial suspensions removed for acetylene reduction assays shown in Table II. Results from days 8 and 10 are shown to indicate that whatever original carbon source was present in the medium after 8 days was unavailable to the rhizobia by 10 days. Thus, acetylene reduction could be obtained on day 8 without addition of extra substrate but this could not be repeated on day 10.

The acetylene reduction data are expressed on basis of nmoles per hr per mg dry wt. Note that the various substrates affect acetylene reduction activity differently at these two intervals. On day 8, succinate was not important as a substrate. The endogenous supplies were absent when no reducing sugars were detected in the B side on day 10 and no acetylene was reduced (Table II). On day 10, succinate was acting as a better substrate for acetylene reduction than the previously important β-hydroxybutyrate. The preferred endogenous substrate(s) from the bacterial cell-suspension have not yet been identified. 2

**EFFECTS OF EXTRA O₂ AND CO ON ACETYLENE REDUCTION**

The second experiment on the activated *Rhizobium* 61A76 was conducted on day 13 after the experiment was started (6 days after removal from DCII apparatus). Addition of substrate was therefore a requirement.

In the second experiment with *R. japonicum* 61A76 the effects of further O₂ addition and the effects of CO on acetylene reduction were examined in the presence of various substrates (Fig. 4). Oxygen inactivates unprotected nitrogenase while CO inhibits nitrogenase activity reversibly. The effects of 2% CO on the functioning respiratory pigments have not yet been assessed.

The effect of extra added O₂ is indicated in the left panel of Figure 4. Acetylene reduction assays were begun at A with 2.1% O₂ and 1.6% C₂H₂ in argon. Extra O₂ was then added at the "O" mark. The tactic of rapidly adding extra O₂ decreased the rate of acetylene reduction at once but did not entirely stop the reduction in these intact bacteria with either pyruvate or glucose. Later, when the vials were evacuated and refilled with the original gas mixture as in A, rapid acetylene reduction continued. Note that the reduction rate with succinate and glucose remained proportional throughout.

**Table II. Acetylene Reduction by *R. japonicum* 61A76 after Activation with Conditioned Medium**

Two-ml portions of rhizobial suspensions were placed in vials with a total volume of 15 cc. The vials were evacuated twice for each 5 min and filled by a mixture containing 2% CO₂, 1.6% acetylene in argon. The hourly rate was calculated from the steep portion of the ethylene accumulation curve. The readings for light scattering at 660 nm were 1.18 and 1.58 for 8-day and 10-day samples. The concentrations of the substrates were as follows: succinate, 1.5 mm; L-malate, 3.1 mm; β-hydroxybutyrate, 1.5 mm; and α-ketoglutarate, 1.6 mm.

<table>
<thead>
<tr>
<th>Acetylene Reduction</th>
<th>Additions</th>
<th>None</th>
<th>Succinate</th>
<th>L-malate</th>
<th>β-Hydroxy-</th>
<th>α-ketoglutarate</th>
</tr>
</thead>
<tbody>
<tr>
<td>nmole C₂H₄ per hr per absorbance unit</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 8</td>
<td>46.7</td>
<td>36.8</td>
<td>16.9</td>
<td>90.5</td>
<td>12.0</td>
<td></td>
</tr>
<tr>
<td>Day 10</td>
<td>0</td>
<td>228</td>
<td>75</td>
<td>194</td>
<td>92</td>
<td></td>
</tr>
</tbody>
</table>
activated if they were precipitated by cold centrifugation and resuspended in air. In the case of AK5, the loss of acetylene reducing ability took place within 8 hr. Bacterial growth was not inhibited in VA, and the doubling time ($A_{660}$) in these stirred cultures, with lost ability to reduce acetylene, was 7 hr. In other experiments not shown here, 10 mm ammonium chloride was shown to inhibit acetylene reduction within 6 hr.

**DISCUSSION**

*R. japonicum* strains R138 and 61A76 were used for the demonstration of nitrogenase activation (acetylene reduction) when cultivated in conjunction with soybean root cell suspensions under transfiler conditions. These strains have been used successfully to nodulate soybean roots in the greenhouse as well as in our field experiments. We have also used them to show invasion of cultured soybean root cells. When reisolated from *in vitro* cultures, rhizobia could be shown surrounded by an additional membrane presumably of plant origin. Such enclosed rhizobia could also be shown to reduce acetylene to ethylene.

When similar conditions were used without plant root cells in culture or without "conditioned medium," the same rhizobia failed to exhibit nitrogenase activity.

Rhizobia activated via the transfiler apparatus and with the aid of plant material were shown to have the following characteristics. The activated *R. japonicum* in the system required (a) continued presence of conditioned medium to remain activated for many days; (b) when substrates in the medium (indicated by presence of reducing sugars) were depleted, addition of suitable carbon sources were needed to give acetylene reduction (Table I); (c) the system was successful when O$_2$ concentration was between 2 to 5%, the nitrate was decreased by metabolic demands to lower than 2 mm and ammonia to lower than 1.6 mm on the B side of the apparatus (Fig. 4, Tables I and II); and (d) the doubling time of rhizobia was 20 hr or longer (Fig. 2).

With regard to the substrates elaborated by plant cells and used by the *R. japonicum* in the apparatus, we have identified several reducing sugars crossing over from P side of the apparatus which are eventually exhausted in later stages of the experiments.

The rhizobia from DCII apparatus have now been shown to give the best rates of acetylene reduction with $\alpha$-ketoglutarate, pyruvate, and $\beta$-hydroxybutyrate. None of these substrates or amino acids, such as glutamine or asparagine, have been actually identified crossing over from the P side of the DCII system. It is not known whether these substances are used directly or whether their metabolic products are used.

The unique contributions of synergetic transmembrane method are that rhizobial strains which can be activated are: (a) also the strains which can demonstrate active nodulation in plants; (b) the plant cells used in the method are from the same plants in which nodulation can be demonstrated; (c) invasion of these plant cells in direct contact with rhizobia can also be demonstrated *in vitro* and used for further comparative studies; (d) the level of acetylene reduction activity (nitrogenase) in the rhizobia from transfiler synergetic cultures are similar to the levels obtained with bacteroids in the absence of facilitated diffusion (without including leghemoglobin).

Diverse combinations of eukaryote cells and nitrogen-fixing microorganisms can thus be used for *in vitro* experiments to aid in understanding why symbiosis is often specific (14) and why endosymbioses has developed for legumes but not for other types of crop plants.

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**LITERATURE CITED**


