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Expression of Rhizobial Nitrogenase: Influence of Plant Cell-Conditioned Medium†

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Conditioned medium was obtained from suspension cultures of soybean (*Glycine max* L. Merrit) cells after incubating them for 4 to 8 days with rhizobia which were separated from the soybean cells by two dialysis bags, one within another. This conditioned medium from the plant cell side (PCM) of the two membranes was used to elicit and influence nitrogenase activity (acetylene reduction) in rhizobia. When conditions for obtaining PCM from the soybean cell suspension cultures were varied, it could be shown that freshly grown rhizobia were able to induce active compounds in the PCM. These compounds caused acetylene reduction activity in test rhizobia under conditions where control rhizobia, containing various substrates, showed little or no acetylene reduction activity. Rhizobia that were already capable of acetylene reduction could not induce such compounds in the PCM when this was included with test rhizobia. The PCM from soybean cultures was also found to aid the expression of nitrogenase activity in suspension cultures of rhizobia normally associated with either peas, lupins, broad beans, or clovers. This is the first communication indicating nitrogenase activity in free-living cultures for various species of rhizobia.

When rhizobia within legume root nodules reduce dinitrogen to ammonia (or reduce acetylene to ethylene), the nodulation is termed effective. When rhizobia (which are particularly compatible with the legume in question) are in a nodule surrounded by a plant membrane and have differentiated into a form termed bacteroids (2), effective nodulation is common. Each type of legume is expected to be nodulated by its specific rhizobia, e.g., peas by various strains of *Rhizobium leguminosarum,* soybeans by *R. japonicum,* and red clover by defined strains of *R. trifolii.* Once the rhizobia invade legumes and become surrounded by a perirhizobial plant membrane, and the resulting nodules show synthesis of leghemoglobin (2), one may ask whether additional exchange of materials is yet necessary between the endosymbionts and the host legume. This would be material over and above the exchange of gases (CO_2, O_2, H_2, N_2), sugars, keto acids, amino acids, NH_4^+, and amides (1-5, 11). The interrelationships and general microbiology of different rhizobial species, the infection process, and the development of legume nodules, along with the functional biology of dinitrogen fixation, have been reviewed by Bergersen (2), Dart (6), and La Rue (12) in Section III of the *Treatise* edited by Hardy and Silver. Not much is known about the additional exchange of materials between the endosymbiont and host.

Separated in vitro suspensions of free-living rhizobia and soybean cells have been used to answer the question regarding this putative exchange of additional materials between plant cells and rhizobia in our present report. Nitrogen fixation by free-living rhizobia was demonstrated in 1975, and this literature has been reviewed by Brill (3), Burris (4), Child and Kurz (5), Evans and Barber (7), and La Rue (12) in the perspective of the general field of nitrogen fixation. The majority of experiments (5) were conducted using the bacterium $32H_1$, a rhizobial species of the cowpea group. Since $32H_1$ can be easily derepressed for nitrogenase in free-living culture, its use in associative cultures with plant cells is not a sensitive index for estimation of the materials being exchanged between the two cultures on agar surface (5).

The data presented in the present report indicate that additional exchange of materials across dialysis membranes may take place between the endosymbionts and host legumes. The majority of our data were obtained with organisms which have not been reported to exhibit nitrogenase activity in free-living cultures. These organisms include *R. japonicum* 3I-1b-138, as well as many fast-growing rhizobia.

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Apparently, Harmsen and Kolff (9) were the first (1947) to use cellophane membranes as aids to grow rhizobia, and Schultz and Gerhardt (16) have reviewed many such dialysis cultures of microorganisms. Quite recently, the use of dialysis bags to study dinitrogen fixation was also found useful by Andersen and Shanmugan (1). These workers used dialysis to remove ammonia formed by nitrogenase in Nif-derepressed mutants of *Klebsiella pneumoniae*. We resorted to a rather simple dialysis culture of rhizobia and plant cells for the majority of our experiments, and only those dialysis culture experiments have been presented. It should be emphasized that some confirmatory experiments were also carried out by the use of the double-cell transmembrane apparatus (13, 15), which has been previously used in our laboratory.

MATERIALS AND METHODS

Sources of rhizobia and soybeans. Rhizobia were obtained from two primary sources, Deane Weber, U.S. Department of Agriculture, Agricultural Research Service, and J. Burton, Nitragin Co., Milwaukee, Wis.

Soybean varieties Harosoy and Kanrich were obtained from the Dewine Seed Company, Yellow Springs, Ohio. Acme soybeans were provided by F. E. Porter of Northrup-King Co., Minneapolis, Minn.

Chemicals. The major ingredients for making rhizobial and plant media were obtained from Baker Co., Fisher Co., or Mallinckrodt Co.

Gases were purchased from Matheson Gas Products as prepared mixtures of argon and acetylene. Oxygen was obtained from Ohio Chemical Co. Standards for gas chromatography were obtained for H_2, O_2, C_2H_2, and C_2H_4 from Scott Research Laboratories and Applied Research Laboratories, State College, Pa.

Dialysis membranes. Spectrapor dialysis membranes used for dialysis bags were obtained from Spectrum Industries, Los Angeles, Calif. These have cellulose base and may eventually be attacked by microbial enzymes. In our checks made to show microbial containment by plating out plant cell media and by microscope examination (see below), rhizobia were undetected within our effective experimental periods. The pore size of these membranes is not drastically changed by sterilization in water, according to a company letter.

Stock culture methods. Rhizobia were maintained as slant cultures and transferred to suspension cultures before incubation with plant cells. The medium for rhizobia suspensions was a modification of that used by Valera and Alexander (17), where gluconate (46 mM) was the major carbon source and nitrogen was supplied either as glutamate (1 mM) or NH_4Cl (1 mM). The pH was adjusted to 6.5. The bacterial suspension cultures were grown on gyratory shakers (New Brunswick model G-241) at 110 rpm, 26°C.

Plant callus cultures were initiated from the hypocotyl hook region of germinating soybean seeds and were propagated on B-5 agar medium of the Prairie Regional Laboratory (PRL-B5) (8). Calluses were divided and transferred every 10 days to fresh B-5 agar-medium.

Suspension cultures of soybean cells were initiated after placing callus cells in PRL-B5 liquid medium (8). The soybean cell suspensions were grown in 100-ml batches contained in 500-ml Erlenmeyer flasks and shaken at 110 rpm, 26°C, on gyratory shakers (13).

Preparation of crude PCM. To prepare crude plant cell-conditioned medium, (PCM), the regular PRL-B5 medium (8) in the suspension culture flasks was replaced with a modified B5 medium lacking the nitrate and containing 1 mM or 0.5 mM NH_4Cl. The resulting suspension cultures were brought to 100 ml in 500-ml Erlenmeyer flasks with the modified medium. The pH was maintained between 5.8 to 6.0 during subsequent culture with rhizobia.

Rhizobia were diluted, counted, and then resuspended to a density of 3×10^9 per ml in fresh modified medium of Valera and Alexander without nitrate. The dialysis tubing was cut and clipped at one end to accommodate 3 to 5 ml of rhizobial suspension. The bags were then sterilized. The rhizobia were then added to no. 3 Spectrapor dialysis bags via a sterile funnel. The tubing was clipped shut with sterile hemostats using special Spectrapor clips. The width of this tubing was 11.5 mm, and the listed molecular-weight cut-off was at 3,500. This bag containing the rhizobia was washed with distilled sterile water (750 to 1,000 ml), then inserted using sterile forceps and hemostats into a larger dialysis bag of 25.5 mm width and molecular-weight cut-off of 6,500. The second outer bag was clipped, then extensively washed in sterile water before being introduced into the Erlenmeyer flasks containing plant cells suspended with B5 (minus nitrate) medium.

Details for harvest and handling of the crude PCM are given in Fig. 1. The PCM was easier to filter for sterile filtration when the final centrifugation was carried out for 2 h at $100,000 \times g$.

Cellulose hollow-fiber devices (Dow Chemical Co.) have also been used in our laboratory to produce PCM, but sampling problems for rhizobia prevented further work and these experiments have not been continued.

Estimation of nitrogenase activity. Nitrogenase activity was measured on two types of rhizobial cultures: (i) rhizobia from dialysis bags that had been placed within flasks containing plant cells and (ii) test rhizobia, which were taken directly from suspension culture and not exposed previously to plant material and which were used to test the PCM after processing as in Fig. 1. The latter rhizobial cultures were standardized for testing against PCM after counting and centrifugation to 1×10^9 to 3×10^9 cells per ml before use.

These cultures for condition (ii) above were termed secondarily activated (SA) for nitrogenase phenotype, as opposed to primarily activated rhizobia (PA) sampled directly from dialysis bags. The distinctions PA and SA are important because with PA, the materials elaborated by plant cells diffused to rhizobia in optimal amounts. This was assumed because the PA rhizobia promptly reduced C_2H_2 at high rates when sampled. In the case of SA, the amount of added PCM had to be adjusted and the optimal amounts estimated within

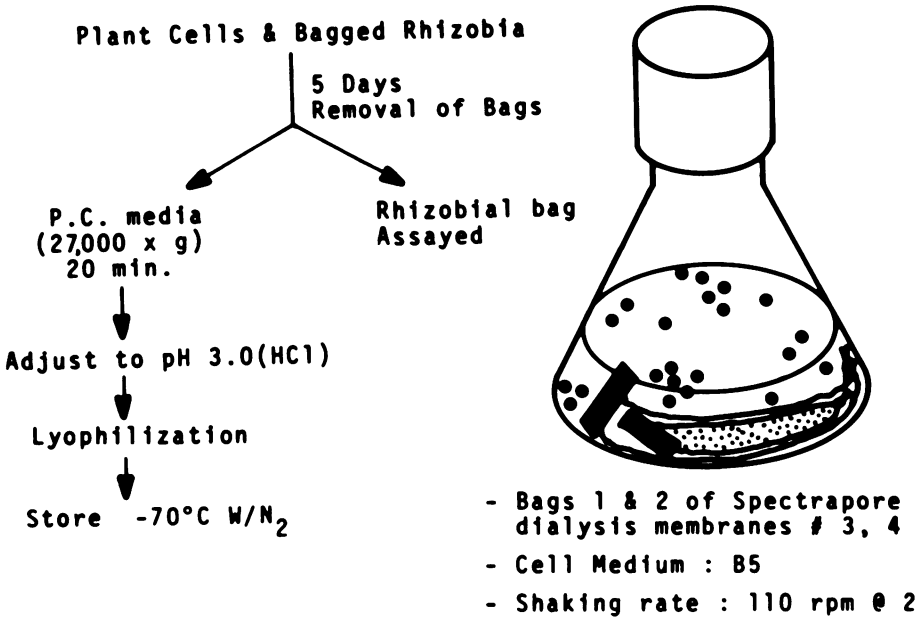

FIG. 1. *The double-bag method for production of PCM. Additional details are given in the text. The rhizobia were originally placed only in the inside of two dialysis bags. After harvest of PCM, the medium was filtered through sterilization filters and then lyophilized. The PCM was also refiltered after reconstitution.*

a narrow concentration range (Fig. 2).

The reduction of acetylene (C_2H_2) to ethylene (C_2H_4) was used to estimate nitrogenase activity. These gases were determined by gas chromatography using Porapak columns at 60°C in a model 5710A Hewlett-Packard gas chromatograph with detector at 145°C. The gas samples (0.25 ml) were drawn from 20-ml or 30-ml assay vials closed by heavy rubber serum stoppers. Each assay vial contained 1.2 ml of suspension containing the rhizobia and 10 mM succinate (pH 6.5) with or without PCM, and contained also a gas phase of 5% C_2H_2 in Ar with O_2 adjusted to 2% in most experiments. The assay conditions were as given previously (13, 14). The C_2H_2-reduction kinetics were similar to those obtained previously (5, 10, 12–15, 19).

RESULTS

Concentration effects of PCM. When rhizobia were used under condition (ii) (SA), the PCM concentration had to be adjusted before assay. One experiment to determine optimal concentration is shown in Fig. 2. Usually, the optimal concentration for one rhizobial strain or species was determined and then used again for the same strain together with other experimental rhizobia. This concentration-effect adjustment for PCM tests could not be neglected, and each batch of PCM was tested to find out the optimal concentration. Variation in pooled batches of PCM between experiments remains a major obstacle in our attempts at purification of additional active substances from plants

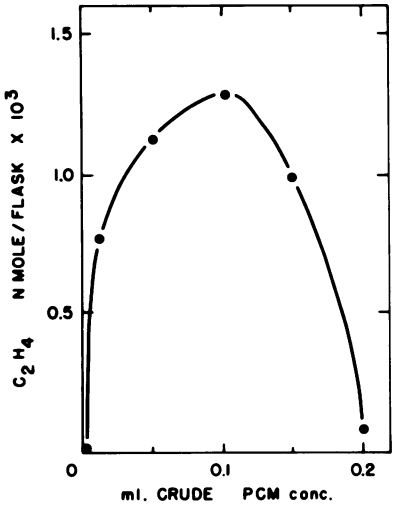

FIG. 2. *Concentration effect of PCM from Acme variety soybean cell suspensions on acetylene reduction by R. japonicum 3I-1b-138. Lyophilized stored PCM (120 mg) was dispersed in 5 ml of succinate (5 mM, pH 4). Different amounts were used as shown. The concentration of rhizobia was 2 × 10^9 cells per ml. In addition, buffered pH 6.5 succinate (10 mM) was used. The total volume of the contents of flasks was adjusted to 1.2 ml. Test rhizobia pH was 6.5. Incubation for acetylene reduction was as given in the text.*

which permit rhizobial Nif expression in liquid culture (Bednarski and Reporter, Plant Physiol. Suppl. 57:130a, 1976). The bell-shaped curve for C_2H_2 reduction with increasing amounts of PCM also makes it unlikely that PCM provides simple carbon sources for respiration that reduce O_2 concentrations, and thus explains our observations on C_2H_2 reduction (also see footnote in ref. 13).

Prior signal from rhizobia to plant cells. Several different experiments were used to find that a prior signal from rhizobia was needed for plant cells in suspension culture to make PCM. The criterion for production of PCM was whether the experimental conditions caused acetylene reduction (nitrogenase activity) at least 50-fold above the acetylene reduction for the control conditions. Rhizobia were necessary, and test media without rhizobia never reduced acetylene. Acetylene was not reduced when bacteria like *Escherichia coli* were used in the test media. These prior signal experiments were also corroborated by carrying out analogous experiments with the double-cell apparatus (13); other control conditions had already been established in a similar apparatus (15).

The control conditions where no significant C_2H_2 reduction was noted with suspensions of test rhizobia were: (i) in freshly made rhizobial or (ii) plant cell media (iii) in filter-sterilized media in which only rhizobia or (iv) only plant cells had been grown, and more important (v) in medium from plant cells which had been exposed across double dialysis bags or bacterial membrane filters to rhizobia which exhibited C_2H_2-reduction activity.

Significant C_2H_2-reduction activity (as defined above) was obtained in (vi) medium from plant cells exposed to fresh rhizobia across double dialysis bags and in medium from plant cells exposed across at least two bacterial membrane filters to rhizobia in DC III apparatus (13). (vii) C_2H_2 reduction was also obtained in medium from rhizobia which had started to show C_2H_2-reduction activity. (viii) More importantly, C_2H_2 reduction was noted in medium in which plant cells were exposed across filters or dialysis bags to filter-sterilized medium from freshly grown rhizobia. In the latter case, no rhizobia were present.

Table 1 shows that PCM was produced after a prior chemical stimulus from rhizobia because these experiments were conducted either with membrane filters in DC III apparatus or across dialysis tubing for separating the media and cultures. The bacterial supernatant by itself was inactive before exposure to plant cells. The experimental details were as follows. (a) Kanrich variety soybean callus cells were dispersed and grown in suspension culture as in Materials and Methods. The plant cells were then placed in B5-PRL medium (8) without nitrate or hormones and containing only 0.5 mM NH_4Cl. The plant cells were divided into two sets, one set used for the double bag with rhizobia and the other with just bacterial medium as in (v) above. (b) The bacterial strain for use in the double bag as well as in the test was USDA 3I-1b-138. The rhizobia were reisolated from nodules, and single colonies were regrown in the medium of Valera and Alexander (17). (c) The concentration for test rhizobia (Table 1, sets A to D), freshly suspended as in Materials and Methods, was 3×10^9 cells per ml. (d) Soy_{japn} refers to PCM obtained from the plant cell medium as given in Materials and Methods. (e) The rhizobia originally in the medium were removed by centrifugation at 17,000 × *g* for 20 min on a Sorvall Centrifuge (SS-34 rotor) and then at 100,000 × *g* for 60 min in a Beckman Ultracentrifuge. The supernatant from this centrifugation was placed in the double bags after sterilizing by membrane filtration (Millipore Corp.; Table 1, sets C, D, and E). After 5 days the media from plant cell side and bags were centrifuged, filter sterilized, and used with fresh test bacteria at the same time as with sets.

TABLE 1. *Formation of active soybean PCM after exposure to medium from suspension culture of R. japonicum*[a]

Set	Rhizobia used	Acetylene reduction (nmol/mg of protein per h)
A. Plant cells and rhizobia R138 in double bags	From bag	165
B. Filtered supernatant from plant cells as in A (Soy_{japn})	Test	171
C. Bacterial supernatant from bags after exposure to plant cells	Test	125
D. Bacterial supernatant before being placed in bags	Test	3
E. As in C	None	0

[a] Details given in text. "Test" refers to test rhizobia used for assay of PCM. The C_2H_2 reduction was measured between 72 and 96 h.

Soybean cell medium conditioned by other species of rhizobia. Conditioned medium from soybean cell suspension cultures was produced when various strains of the natural symbiont *R. japonicum* were used. The plant cell suspensions were also changed successfully to produce PCM, e.g., from soybean varieties Kanrich, Harosoy, and Beeson, rather than Acme. (Other species of plant cells have not been used, aside from soybeans. Some experiments to obtain PCM with Little Marvel variety

of peas are being conducted in our laboratory.) We have also started growing tobacco cells for use with *R. japonicum* (also see ref. 5, 10, 12, and 19).

Other species of rhizobia have been used successfully in the double bags to produce PCM from Acme variety soybean cell suspensions by primary activation (Table 2). For example, we incubated Acme cell suspensions with *R. trifolii*, a symbiont which naturally associates with clover, sometimes with peas, and on occasion with beans (*Phaseolus vulgaris*). The PCM from this combination aided nitrogenase activity of *R. japonicum* and vice versa. The experiment of Table 2 shows that PCM from Acme soybean cell suspensions would aid acetylene reduction in *R. japonicum* (secondary activation) after exposure of Acme plant cells to other species of rhizobia even though these species sampled from the double bags after the plant cell exposure did not reduce C_2H_2 at a significant rate. The PCM (Soy_{japn}) is PCM produced by the usual combination from soybean cell suspensions exposed to *R. japonicum*. PCM (Soy_{japn}) was repeatedly used with other species of rhizobia leading to C_2H_2 reduction by these species (secondary activation). In addition, if a combined nitrogen source was included in the incubation medium during the test, e.g., glutamate or casein hydrolysate, an increase in rhizobial protein was obtained.

In different experiments three batches of lyophilized, stored PCM were used (Table 3). The PCM (Soy_{japn}) was prepared as in Fig. 1 and refiltered through a membrane filter before use.

TABLE 2. *Conditioning of soybean cell suspension culture medium by different species of rhizobia*[a]

Set	Rhizobia used	Acetylene reduction (nmol/mg of protein per h)
R. trifolii + soybean	From bag	4.2
R. trifolii + soybean = Soy_{trif}	*R. japonicum* test	1,715
R. meliloti + soybean	From bag	13
R. meliloti + soybean = Soy_{mel}	*R. japonicum* test	492
R. japonicum + soybean = Soy_{japn}	From bag	1,334
Control	*R. japonicum* test	0.2

[a] *R. trifolii* 16217 and *R. meliloti* 102F7 were grown in the medium of Vincent (18). *R. japonicum* 3I-1b-138 was used for the test organism as well as for production of PCM (Soy_{japn}). The same suspension culture of Acme variety soybean cells was divided and used for the experiment. The various PCM were harvested after 7 days. The test organism was *R. japonicum* 3I-1b-138, used at a concentration of 2.6×10^9 cells per ml. The acetylene reduction was calculated between 48 and 96 h.

TABLE 3. *Acetylene reduction by various rhizobial species with three unfractionated PCM Soy_{japn} preparations*

Rhizobia	Acetylene reduction (nmol/mg of protein per h)[a]		
	Prepn 1	Prepn 2	Prepn 3
R. japonicum 3I-1b-138	0.1	1.6	0.7
R. japonicum + PCM	26.7	34.0	11.7
R. meliloti 102F7	0.1		0.1
R. meliloti + PCM	286		3.4
R. trifolii 162P17	0.2	+	+
R. trifolii + PCM	273	296	253
Bacterium $32H_1$	5.7		
Bacterium $32H_1$ + PCM	530		
R. japonicum 61A76		10.3	17.1
R. japonicum + PCM		242	66.0
R. lupini 96B9		+	0
R. lupini + PCM		182	158
R. leguminosarum 175G10			+
R. leguminosarum + PCM			157

[a] Each preparation was used to give ca. 8 mg of PCM per ml. Other conditions were as given in the text. The rate between 72 and 96 h is given and based on final protein content from duplicate vials. Duplicates agreed within 10%.

Even though the nitrogenase activity of various rhizobial species was not optimized in each case, duplicates in all cases agreed within 10%.

It could also be shown (data not given) that stored lyophilized PCMs (Soy_{trif} and Soy_{mel}) could be reconstituted and were active in the elicitation of nitrogenase from fresh cultures of *R. trifolii* and *R. meliloti*, respectively. These crude PCM preparations were also used to give acetylene reduction with other fresh suspension cultures from species such as *R. leguminosarum*.

DISCUSSION

Our results in this and other papers (14) indicate that rhizobia of different species elaborate chemicals which induce plant cells into producing materials that eventually influence the expression of Nif activity in other rhizobia. The expression of nitrogenase activity in vitro in the rhizobia listed in Tables 2 and 3 has not been previously reported.

This in vitro expression of nitrogenase activity was exhibited under our conditions of experiment by a wide range of rhizobial species in liquid culture, including fast growers like *R. trifolii*. Reports of secondary activations have not been found in the literature (5, 10, 12).

Our preliminary reports (Bednarski and Reporter, Plant Physiol. Suppl. **57**:130a, 1976) have indicated two sizes of compounds called H and L from PCM which aid nitrogenase expression

(14). The identification of these species will be facilitated by using liquid culture media as in a previous method (13, 14, 15) or in the easier method we have just described. Under our conditions simple carbohydrates (including pinitol) have not, by themselves, aided in inducing C_2H_2 reduction by most of the rhizobia listed above. The exceptions are 32H$_1$, 61A76, and 61A110 from Nitragin and USDA strain 3I-1b-83.

Tests for the expression of nitrogenase (acetylene reduction activity) in various genetic strains and crosses of rhizobia and other bacteria should be facilitated by the use of either PCM or its isolated active ingredients. Fast-growing rhizobia have been previously found difficult to derepress for nitrogenase (10). This difficulty can be circumvented by the use of PCM or appropriate substances isolated from the plant cell culture media with the aid of dialysis cultures.

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