

A Simple Technique for the Establishment of Nitrogenase in Soybean Callus Culture^{1,2}

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ABSTRACT

A nitrogen-fixing association can be readily established *in vitro* between *Rhizobium* and cultured soybean root cells. Plant cells are grown as a thin callus on the surface of solid defined medium containing low levels of inorganic nitrogen and inoculated with bacteria during the active phase of growth. Acetylene reduction activities up to 275 nmoles ethylene/hr·g dry weight of cells have been recorded.

The system is compact, uses inexpensive glassware, and facilitates multiple replication of samples. The use of a single medium throughout the experiments avoids unnecessary disturbance to the cells. The development of nitrogenase activity is relatively rapid and its activity can be recorded within a 3-week period.

Relatively little is known of the factors controlling infection or the development of nitrogenase in the nitrogen-fixing symbiosis between leguminous plants and bacteria. The sensitive acetylene reduction assay technique for the detection of nitrogenase activity (4) and the demonstration that symbiosis can be established between *Rhizobium* and plant cell tissue cultures *in vitro* (5) allow a novel approach to study these problems.

The system described by Holsten *et al.* (5) utilized relatively large volumes of soybean cells grown in a klinostat. The infection process took place in a liquid nutrient medium containing coconut milk and 2,4-D. After 3 to 7 days, the cells were transferred to a similar medium lacking 2,4-D, to allow the establishment of nitrogenase activity.

We were unable to detect nitrogenase using the Holsten *et al.* technique (5), nor could we form a symbiotic association in suspension culture in conventional vessels. Therefore, we tried establishing a symbiosis in soybean callus culture grown on solid nutrient. Nitrogenase activity could not be detected when we used the Lindsmaier and Skoog (6) and Eriksson (1) media or the Murashige and Skoog (7) medium used by Holsten (5). However, positive results were occasionally obtained using Gamborg's (3) B5 and B5c media.

These have a lower inorganic nitrogen content than the other media tested, indicating that low levels of fixed nitrogen might promote the symbiotic process. Reduction of the nitrogen level in Gamborg's (3) medium resulted in the establishment of a reproducible nitrogen-fixing system. This paper describes the medium and the experimental conditions used.

MATERIALS AND METHODS

Plant cell suspension cultures of root explants of eight varieties of soybean (*Glycine max*) were established by Gamborg's method (2). The cells used as inoculum were grown in 250-ml Erlenmeyer flasks containing 50 ml of B5 medium supplemented with 2 μ g/ml 2,4-D on a rotatory shaker (150 rpm, 1.25 inch eccentricity) at 26 C in the dark and subcultured into fresh medium (1:5 dilution) after 5 to 7 days incubation. Stock cultures of seven strains of *Rhizobium japonicum* were maintained under similar growth conditions in a liquid medium (5) and transferred at weekly intervals.

For whole plant studies, seeds were germinated singly in pots containing a vermiculite-sand (3:1) mixture under greenhouse conditions. The seedlings were exposed to *Rhizobium* at the first leaf stage, and nitrogenase activity of intact roots (4) was measured 7 weeks after planting at the time of maximum activity. After the assay, nodules were harvested and weighed, and nitrogenase activity was expressed per gram of nodule tissue.

Establishment of Symbiosis. Plant cell suspension culture (2.5 ml of a 2 to 5 day old) was pipetted onto the surface of 50 ml of LNB5 agar medium (Table I) in a 4.5 oz Gerber baby food jar, (Consumers Glass Co. Ltd., 777 Kipling St. Toronto, Ont.). To facilitate complete removal of cells for weight estimations, filter membranes of Miracloth (Chicopee Mills Inc. Broadway, N.Y. 10018) were placed aseptically on the surface of the agar prior to inoculation. A hole in the Vapack lug caps (Continental Can Co., 2740 Ontario Ave., Toronto, Ont.) contained a foam plug. Routinely, the cells were allowed to grow into a thin layer callus for 7 to 14 days prior to inoculation with 0.05 ml of bacterial suspension (approximately 10⁷ cells/ml). The cultures were incubated for 10 to 14 days after the addition of bacteria before testing for nitrogenase activity. The foam-plugged cap was replaced by one fitted with a rubber serum stopper, and 2 ml of acetylene were injected into the atmosphere above the callus culture. After 1 hr incubation, 1-ml gas samples were removed from the jar and analyzed for ethylene production using standard gas chromatographic techniques (4). Uninoculated callus cultures were used as controls. Normally, each experimental condition was tested by using a minimum of five replicates. Cultures were removed from the agar and weighed after drying.

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Table I. Composition of LNB5 Medium for the Establishment of Symbiosis between Soybean Callus and Rhizobium

Additional components were: sucrose, 30 g/l; agar, 8 g/l; the pH of the medium was 5.5.

Components		Components	
	mg/l		mg/l
KNO ₃	1000	Na ₂ MoO ₄ ·2H ₂ O	0.25
(NH ₄) ₂ SO ₄	134	CuSO ₄	0.025
MgSO ₄ ·7H ₂ O	250	CoCl ₂ ·6H ₂ O	0.025
NaH ₂ PO ₄ ·H ₂ O	150	KI	0.75
CaCl ₂ ·2H ₂ O	150	Myoinositol	100
Fe(330) ¹	28	Thiamine HCl	10
MnSO ₄	10	Nicotinic acid	1
H ₃ BO ₃	3	Pyridoxine HCl	1
ZnSO ₄ ·7H ₂ O	2		

¹ Sequestrene 330 Fe obtained from Geigy Agricultural Chemicals, Saw Mill River Road, Ardsley, N. Y.

RESULTS AND DISCUSSION

The callus darkened within a day of microbial infection, due to the formation of lignin-like material (9). The infected calli continued to increase in weight after uninoculated controls had stopped growing (Fig. 1). These effects were also observed in associations of soybean and rhizobial strains which did not produce nitrogenase. Therefore, the increased growth is not due to nitrogen fixation but may be a response to cytokinins produced by the bacteria (8).

Nitrogenase, as determined by the acetylene reduction assay, was established with *Rhizobium japonicum* strain 61A76 and callus cultures of three varieties of soybean and with Mandarin soybean callus and five bacterial strains. Ethylene production from acetylene was linear during the assay period. Uninfected calli did not reduce acetylene nor did infected calli produce significant amounts of ethylene in the absence of acetylene. Acetylene reduction was decreased if the atmosphere was N₂:C₂H₂ 98:2 or A:O₂:C₂H₂ 78:20:2, compared to air:C₂H₂ 98:2. Acetylene was damaging to the system, for after an assay, nitrogenase was lowered or could not be detected in the sample thereafter.

Acetylene reduction was rarely observed earlier than 5 to 7 days after the addition of bacteria to the callus, and the nitrogenase activity was not improved by extending the period of postinfection incubation. Autolysis of cell cultures frequently occurred after 28 days, and results became erratic with longer periods of incubation. The time at which bacteria is added to the callus does not appear to be critical for the establishment of symbiosis, although slightly higher specific activities were recorded for cells exposed to bacteria at the end of their growth period.

Bacterial strain 61A76 was tested with callus from eight varieties of soybean, and symbiosis was established with varieties Acme, Norman, and Mandarin (Table II). Callus cultures of most other varieties did not grow well on the nutrient used. Variations occurred between replicate cultures in each experimental system, and variations from one experimental series to another were often quite large. This was particularly evident in symbiosis established with Acme and Norman cells. These gave higher nitrogenase activities than did Mandarin under the same conditions, but the frequency of successful infection was much less. Mandarin cells were used for further experiments because, although the nitrogenase activity was low, it was consistent and reproducible.

Assays of acetylene reduction activity of whole soybean

plant varieties grown on nitrogen-free media under greenhouse conditions and infected with bacterial strain 61A76 showed that the activities observed in the corresponding *in vitro* system were less than 1% of the activity of nodular tissue at the time of their maximum activity (Table III).

Not all varieties with high activities in nodules produced *in vitro* associations. However, it is interesting that the relative activities Acme>Norman>Mandarin are the same in the plant and *in vitro* system. This indicates that some of the genetic

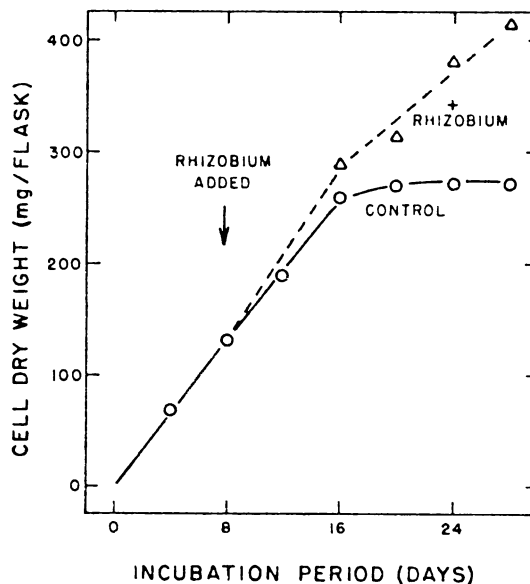


FIG. 1. Growth rate of Mandarin soybean callus on LNB5 medium.

Table II. Typical Values of Nitrogenase Activity Obtained in Experiments with Different Varieties of Soybean Callus Infected with *Rhizobium japonicum* 61A76

Figures in brackets denote values obtained from uninoculated controls. In each experiment, callus cultures were inoculated with *Rhizobium* after 10 days growth on LNB5 medium, and acetylene reduction activities were measured 14 days after bacterial addition.

Variety	Acetylene Reduction Activity			
	Expt 1	Expt 2	Expt 3	Expt 4
	nmoles C ₂ H ₄ /hr-gm dry wt			
Acme	274.6 (0.4)	87.3 (0.2)	47.2 (0.3)	15.9 (0.1)
	256.8 (0.3)	84.8 (0.4)	50.1 (0.3)	14.4
	170.1 (0.1)	30.2 (0.1)	0.7	
	249.3	0.5		
	217.2	0.7		
	0.2	0.1		
Mandarin	0.3	0.4		
	3.8 (0)	2.5 (0.2)	3.6 (0.4)	5.0 (0.3)
	2.9 (0.1)	4.7 (0.2)	7.8 (0.3)	2.5 (0.1)
	0.6 (0.1)	2.2 (0.1)	4.1 (0.4)	2.1
	2.7	3.1	4.9	4.3
	2.3	2.4	2.9	6.5
Norman	2.4	4.2	0.3	0.6
	2.1	0.8		
	5.0 (0.1)	20.7 (0.1)		
	26.8 (0.2)	19.4 (0.1)		
	42.9	0.1		

Table III. *Relative Acetylene Reduction Activities of Nodules and Callus of Soybean Varieties Inoculated with Rhizobium japonicum Strain 61A76*

Variety	Specific Activity	
	Nodule	Callus
	<i>nmoles C₂H₄/hr·g dry wt</i>	
Acme	15030	48.9
Norman	9630	24.8
Morsoy	8800	1.8
Portage	6670	0.6
Altona	4270	0.5
Fiskebye	3820	1.9
Kent	3230	0.5
Mandarin	2620	3.1

Table IV. *Nitrogenase Activity of Mandarin Soybean Callus Exposed to Various Strains of Rhizobium japonicum*

Bacterial Strain	Acetylene Reduction Rates
	<i>nmoles C₂H₄/hr·g dry wt</i>
83 ¹	10.2
85 ¹	22.4
86 ¹	8.4
10324 ²	3.5
54071 ²	0.1
11927 ²	0.2
61A76 ³	3.3
None	0.1

¹ U.S.D.A. Beltsville, Md., courtesy of Dr. D. Weber.

² American Type Culture Collection.

³ Nitragin Sales Corporation, Milwaukee, Wisconsin, courtesy of Dr. J. C. Burton.

Table V. *Effect of Inorganic Nitrogen Concentration on the Establishment of Symbiosis between Mandarin Soybean Callus and Rhizobium japonicum 61A76*

Salt Conc'n		Acetylene Reduction Activity	
KNO ₃	(NH ₄) ₂ SO ₄	Inoculated	Uninoculated
<i>mg/l</i>		<i>nmoles C₂H₄/hr·g dry wt</i>	
2000	150	0.3	0.0
1000	150	3.9	0.1
500	150	5.9	0.3
0	150	0.2	0.0
1000	0	3.2	0.2
1000	50	16.6	0.3
1000	100	9.6	0.4
1000	300	2.1	0.2

contribution to fixation by the plant may still be expressed in tissue culture.

Five of seven strains of *Rhizobium* tested with Mandarin callus gave positive acetylene reduction activities (Table IV). Strains 83, 85, and 86 were particularly effective. Strains

61A76 and 10324 gave lower nitrogenase activities, whereas strains 54071 and 11927 gave no evidence of nitrogen-fixing ability, although the callus appeared to respond in the same way to bacterial invasion.

The inorganic nitrogen status of the growth medium is important for the establishment of a nitrogen-fixing symbiotic association. Reducing the level of potassium nitrate to 1000 mg/l resulted in the establishment of a nitrogen-fixing symbiosis with strong acetylene reduction activity (Table V). At this lowered level of nitrate ion, a reduction of the ammonium sulfate concentration to 50 mg/l gave significantly increased nitrogenase activity. The establishment of symbiosis is clearly governed by the supply of both medium constituents at levels lower than those required for optimum growth of plant cells alone.

Holsten *et al.* (5) reported that 2,4-D inhibited the formation of nitrogenase in their infected tissue cultures. Soybean requires 2,4-D or a related auxin for continued multiplication in tissue culture. However, the cells are able to carry over sufficient auxin to permit growth for one or two transfers in auxin-free nutrient (Gamborg, personal communication). This is fortuitous. In our system, plant cells are maintained in Gamborg's B5 medium containing 2,4-D, but infection and formation of nitrogenase takes place on an auxin-free nutrient. Our procedure will therefore be convenient for the study of plant growth regulators; their effect on infection or fixation will not be obscured by the presence of auxin.

The LNB5 medium is chemically defined, and coconut milk or similar adjuncts are not necessary to obtain a symbiotic association. The additions of several types of protein hydrolysate to the medium were without beneficial effect. The technique described by Holsten *et al.* (5) requires the use of a klinostat, and investigators lacking this equipment may have been discouraged from obtaining nitrogenase in tissue culture. The necessity to transfer cells from one nutrient to another increases the chance of contamination and disturbance to the cells. The simple, single-nutrient procedure described here should permit researchers to readily apply the techniques of plant tissue culture to the problems of symbiotic nitrogen fixation.

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