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Factors Affecting the Reduction of Acetylene by *Rhizobium*-Soybean Cell Associations *in Vitro*[1]

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ABSTRACT

The acetylene reduction assay was used to measure presumed N_2-reducing activity in *Rhizobium*-soybean cell associations *in vitro*. No acetylene reduction was observed in liquid suspensions of these organisms, but cells plated onto an agar medium from a liquid suspension of *Rhizobium* and soybean cells exhibited acetylene-dependent production of ethylene after 7 to 14 days. Aggregates of soybean cells 0.5 to 2.0 mm in diameter were required for this activity. Decreasing oxygen from 0.20 atm to 0.10, 0.04, or 0.00 atm completely inhibited acetylene reduction. The presence of 2,4-dichlorophenoxyacetic acid or kinetin increased endogenous ethylene production and inhibited acetylene-dependent ethylene production. Acetylene reduction was observed with three out of four strains of *R. japonicum* tested, and three rhizobial strains, which produce root nodules on cowpeas but not soybeans, formed an association capable of acetylene-dependent ethylene production.

Attempts to understand the genetic control of proteins required for the reduction of N_2 by leguminous root nodules have been thwarted by the lack of a suitable, defined experimental system. A recent report presented morphological evidence for an association between bacteria of the genus *Rhizobium* and soybean cells *in vitro* (6). Using a previously developed acetylene-ethylene assay for N_2 fixation (5) Holsten *et al.* (6) concluded that their *Rhizobium*-infected soybean cell cultures were capable of reducing N_2 on the basis of ethylene produced in the presence of acetylene. The total acetylene to ethylene reducing activity (presumed analogous to N_2-reducing activity) of the *in vitro* system was calculated to be approximately 1% of that activity found in soybean nodules. These low levels of activity were shown to be dependent upon *Rhizobium*, but unfortunately there was no discussion of acetylene dependence (6, 7). Another report has claimed marginal $^{15}N_2$ reduction in this system (4). The question of whether ethylene produced in this system arose from acetylene reduction or from other metabolic pathways is especially important because endogenous ethylene production has been reported in cultured soybean tissue (8). The present study was undertaken in an effort to verify the original observations of Holsten *et al.* (6) and to investigate endogenous ethylene production in soybean cells infected with *Rhizobium in vitro*. An understanding of the factors affecting endogenous ethylene production in this system would permit future studies to utilize the acetylene reduction assay with confidence rather than the more laborious $^{15}N_2$ assay techniques for measuring N_2 reduction in this system.

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MATERIALS AND METHODS

Tissue cultures were obtained from soybean roots (*Glycine max* cv. Acme and Sioux) in the manner described by Holsten *et al.* (6). The tissue was maintained on a medium modified from Fosket and Torrey (2), which contained the following substances in mg/l: KH_2PO_4, 300; KNO_3, 1000; NH_4NO_3, 1000; $Ca(NO_3)_2 \cdot 4H_2O$, 500; KCl, 65; $MgSO_4 \cdot 7H_2O$, 35; $MnSO_4 \cdot H_2O$, 4.4; H_3BO_3, 1.6; $ZnSO_4 \cdot 7H_2O$, 1.5; KI, 0.75; $CoCl_2 \cdot 6H_2O$, 0.025; glycine, 2.0; nicotinic acid, 0.5; thiamine·HCl, 0.1; pyridoxine·HCl, 0.1; NaFeEDTA, 32; inositol, 100; 2,4-D, 2.0; kinetin, 0.1; and sucrose, 30,000. This medium, which was designated SCP, was adjusted to pH 6.0 before adding 10 g agar/l and autoclaving. Rapidly growing clumps of tissue were used to develop a liquid suspension of cells growing in SCP without agar. Liquid suspension cultures were maintained in the dark at 25 C on a rotary shaker oscillating at 60 rpm. Stock cultures were transferred every 21 days by inoculating 50 ml of fresh medium contained in a 250-ml Erlenmeyer flask.

A second medium, which lacked mineral nitrogen, was designated SCN. It contained the following substances in mg/l: KH_2PO_4, 300; $CaCl_2 \cdot 2H_2O$, 100; KCl, 65; $MgSO_4 \cdot 7H_2O$, 35; $MnSO_4 \cdot H_2O$, 4.5; H_3BO_3, 1.5; $ZnSO_4 \cdot 7H_2O$, 1.5; $Na_2MoO_4 \cdot 2H_2O$, 0.25; $CuSO_4 \cdot 5H_2O$, 0.04; $CoCl_2 \cdot 6H_2O$, 0.025; glycine, 2.0; thiamine·HCl, 0.1; nicotinic acid, 0.5; pyridoxine·HCl, 0.1; NaFeEDTA, 32; inositol, 100; and sucrose, 30,000. The medium was adjusted to pH 6.0 before autoclaving.

Rhizobium cultures were maintained on a completely defined medium (10). Rhizobia supplied by Dr. J. C. Burton of The Nitragin Co., Milwaukee, Wisconsin included *R. japonicum* 61A76 and 61A96, *R. leguminosarum* 128C53, *R. phaseoli* 127K17, *R. trifolii* 162P17, *R. meliloti* 102F51, *R. lupini* 96B9, and rhizobial strains 29C2, 32H1, and 32Z3 which effectively nodulate cowpea (*Vigna sinensis*). *R. japonicum* R54a and 505 were obtained from Dr. H. J. Evans (Oregon State University, Corvallis, Ore.). A strain of *Agrobacterium tumefaciens*, NRRL B-37, which was isolated by the United States Department of Agriculture, was obtained from Dr. A. G. Matthysse (Indiana Medical School, Indianapolis, Ind.) and was grown on nutrient agar or nutrient broth (Difco, Co., Detroit, Mich.).

Soybean root cell-*Rhizobium* interactions which resulted in acetylene-dependent production of ethylene normally were established in the following manner. Stock cultures of cells maintained in the SCP medium for 14 days were inoculated with

0.1 ml of a rapidly growing culture of *Rhizobium* containing approximately 1.0 to 9.0 × 10^7 cells/ml. Seven days later the cells were filtered on a double layer of sterile cheesecloth and rinsed with 100 ml of SCN medium. The cells were then plated onto 5 ml of SCN containing 1% agar in 15-ml glass vials (Arthur H. Thomas Co., Philadelphia, Pa.). One 50-ml culture in a 250-ml Erlenmeyer flask produced 5 to 6 g fresh weight of soybean tissue which was divided equally into six 15-ml culture vials covered with aluminum foil caps. After 14 days on the SCN medium, serum stoppers were inserted to test for endogenous ethylene production. Twenty-four hours later the amount of ethylene produced by each replicate was determined by gas chromatographic analysis using techniques similar to those reported by Hardy *et al.* (5). Serum stoppers were removed for 10 min while the samples were flushed with sterile air to remove all traces of ethylene. Stoppers were replaced and acetylene was injected to a concentration of 0.12 atm over the tissue. Twenty-four hours later ethylene was measured again with a gas chromatograph. Dry weight of the tissue samples was determined after 48 hr at 60 C.

Acetylene-dependent ethylene production was determined in the following manner. Ethylene produced by each replicate during the first 24 hr assay (day 14–15 after plating on SCN) was subtracted from that detected in the same replicate after the second 24 hr assay was performed in the presence of acetylene (day 15–16).

The effect of oxygen on acetylene-dependent ethylene production was studied by flushing serum-stoppered vials with N_2 at the beginning of the normal assay period. A pressure of 1.0 atm was maintained in each vial by withdrawing N_2 and adding O_2 and C_2H_2 until all replicates contained 0.12 atm C_2H_2 and 0.00, 0.04, 0.10, or 0.20 atm O_2. Twenty-four hours later the ethylene concentration was determined in each replicate.

RESULTS

Initial attempts to repeat the Holsten *et al.* (6) observations using Acme soybean tissue and *R. japonicum* 61A76 grown on SCP were not uniformly successful. No acetylene-dependent ethylene production was detected in liquid cell suspensions infected with *Rhizobium*. Occasional replicates plated on SCP containing 1% agar revealed apparent acetylene-reducing activity, but statistical treatments of the data never demonstrated a significant difference between the mean of all cultures inoculated with rhizobia and the uninoculated controls.

Media Effects. Acetylene-dependent ethylene production by *Rhizobium*-infected tissue is affected by the composition of the agar medium on which the cells are grown after the 7-day infection period (Table I). Acme soybean tissue was inoculated with *R. japonicum* 61A76 in liquid SCP medium, and then 7 days later the filtered tissue was plated onto SCP medium lacking auxin and cytokinin or onto SCN medium. Only those cells on the SCN medium exhibited acetylene-dependent ethylene production significantly greater than the uninoculated control replicates ($p \leq 0.01$).

Table I. *Effect of Growth Medium on Ethylene Production and Acetylene Reduction by Acme Soybean Cells ± R. japonicum 61A76*

Each value represents a mean ± SE determined from six replicates. Endogenous ethylene production was determined prior to injecting acetylene. Acetylene-dependent ethylene data were corrected for endogenous ethylene production.

Medium	Ethylene Production			
	Control		Rhizobium-infected	
	Endogenous	C_2H_2-dependent	Endogenous	C_2H_2-dependent
	nmoles/g dry weight·24 hr			
SCP without 2,4-D and kinetin	0.0 ± 0.0	3.4 ± 0.9	0.0 ± 0.0	2.7 ± 0.7
SCN	0.9 ± 0.4	4.2 ± 1.0	0.0 ± 0.0	110 ± 25.0

Table II. *Effect of Host Tissue on Ethylene Production and Acetylene Reduction by Soybean Cells ± R. japonicum 61A76*

Cell suspensions were obtained from Acme and Sioux cultivars. The data are from five to six replicates.

Sample	Ethylene Production	
	Endogenous	C_2H_2-dependent
	nmoles/g dry weight·24 hr	
Acme control	0.0 ± 0.0	0.0 ± 0.0
Acme + 61A76	18.0 ± 7.0	218 ± 49.2
Sioux control	0.0 ± 0.0	1.8 ± 0.8
Sioux + 61A76	26.5 ± 7.5	20.3 ± 2.5

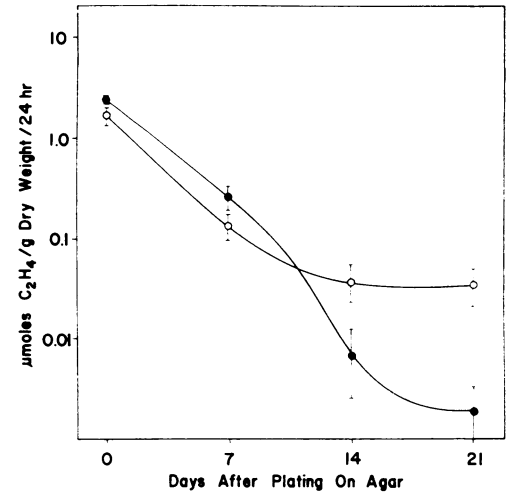

FIG. 1. Endogenous ethylene production by Acme soybean cells ± *R. japonicum* 61A76. Plant cells were infected for 7 days in liquid SCP medium before rinsing and plating on SCN medium containing 1% agar. Each point represents the mean ± SE from four replicates. Uninoculated control (●); tissue infected with *R. japonicum* 61A76 (○).

Host Tissue Effects. Acme and Sioux soybean tissues exhibit acetylene-dependent ethylene production when infected with *R. japonicum* 61A76 and plated onto SCN (Table II). This presumed acetylene reduction is significantly greater than the analogous control value ($p \leq 0.001$) for both cultivars. Because Acme tissue exhibited a much greater capacity than Sioux to reduce acetylene in the presence of *R. japonicum* 61A76, it was selected for further experiments.

Time of Assay. Data from a study of endogenous ethylene production at various times after plating the cells on SCN are shown in Figure 1. Very high levels of ethylene production observed immediately after changing the tissue from SCP to SCN declined by a factor of 100 during 14 days of incubation on SCN. The tissue infected with bacteria exhibited a brown color reportedly related to a lignification process (12), while the uninoculated controls maintained a pale yellow hue.

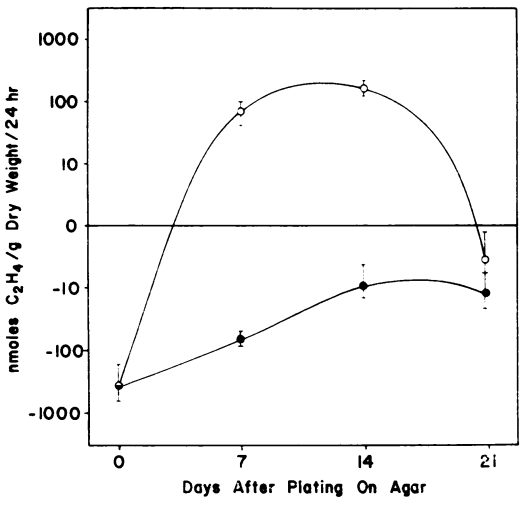

FIG. 2. Acetylene-dependent ethylene production by Acme soybean cells ± *R. japonicum* 61A76. Data were taken from the same samples represented in Figure 1. Ethylene values recorded in the presence of acetylene were corrected by subtracting the endogenous levels of ethylene production shown in Figure 1. Uninoculated control (●); tissue infected with *R. japonicum* 61A76 (○).

Figure 2 reveals the development of an acetylene-dependent production of ethylene in the tissue after plating out on SCN. Data in Figures 1 and 2 were derived from the same experiment in which four replicates were sacrificed each week to determine first endogenous ethylene production (Fig. 1) and then acetylene-dependent ethylene production (Fig. 2). Definite acetylene-dependent production of ethylene is evident at both 7 days and 14 days after plating the cells on agar. These results validate the use of a 14-day incubation period prior to testing for acetylene reduction.

Because such a long assay period was used to determine acetylene-dependent ethylene production, several trials were conducted to test whether ethylene production proceeded linearly for the entire 24-hr period. Data in Figure 3 demonstrate that acetylene-dependent ethylene production probably is linear for approximately 48 hr. Other trials occasionally showed a break in linearity in the 40- to 44-hr region.

The experiment in Figure 3 was begun 15 days after plating Acme cells ± *R. japonicum* 61A76 on SCN medium containing 1% agar. Ethylene produced in the presence of acetylene was determined on days 16, 17, and 18. Endogenous ethylene production was not detectable in any replicates of either the control flasks or the *Rhizobium*-treated cultures during the normal day 14 to 15 assay. Acetylene was introduced on day 15 (time 0, Fig. 3), and the nature of the experiment prevented further determinations of endogenous ethylene production because all replicates remained sealed until day 18. Thus the ethylene values on the ordinate represent total ethylene produced rather than the acetylene-dependent ethylene data used elsewhere in this study. Presumably, endogenous ethylene production continued at the undetectable levels observed on day 14, or at the very low levels suggested by the trace of ethylene present in the uninoculated controls at 48 hrs.

Size Effects. One very serious problem hindered experiments using the Acme tissue. Many cell suspension systems used for experimental studies consist of uniformly small aggregates of cells suspended in a liquid medium by gentle agitation. Acme tissue used in the present study contained a high proportion of small aggregates of cells, but also present were larger clumps of cells which occasionally developed to 5.0 to 10 mm in diameter. After the first positive demonstration of acetylene-dependent ethylene production in the presence of *Rhizobium*, a more uniform suspension of small cell clumps was selected from the heterogeneous suspensions used initially. Attempts to demonstrate acetylene reduction in *Rhizobium*-infected samples of this derivative line of Acme tissue were unsuccessful. Consequently, various size classes of tissue present in the heterogeneous suspension line were analyzed for their ability to associate with *Rhizobium*.

Results of assaying different size classes of cells from the same suspension are shown in Table III. Acme tissue was infected with *R. japonicum* 61A76 and plated on SCN in the normal manner. During the rinsing and plating procedure, however, three size classes of tissues (<0.5 mm, 0.5–2.0 mm, and 5.0–10 mm) were selected with stainless steel screens. Only the 0.5- to 2.0-mm clumps of tissue infected with *Rhizobium* showed significantly greater ($p \leqslant 0.01$) acetylene-dependent ethylene production than the uninoculated controls. Fortunately, cell aggregates of this size formed most of the biomass in the heterogeneous line of Acme tissue. In later experiments, the largest clumps of tissue (>2.0 mm) were removed easily by hand after rinsing away the smallest aggregates of cells (<0.5 mm) through a double layer of cheesecloth.

Auxin and Cytokinin Effects. Apparent acetylene reduction

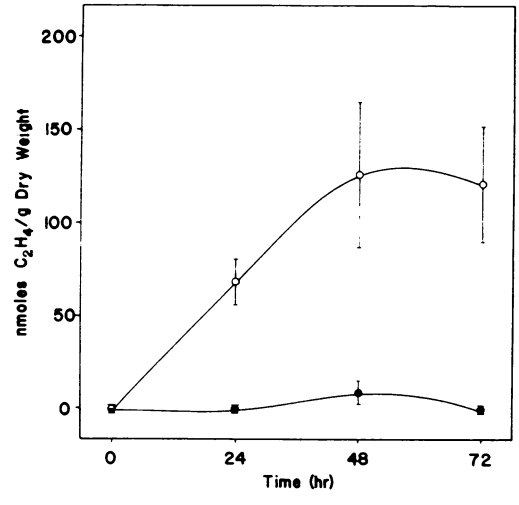

FIG. 3. Ethylene produced in the presence of acetylene over a 72-hr period by Acme soybean cells ± *R. japonicum* 61A76. Endogenous ethylene production was not detectable at time 0. The tissue had been exposed to bacteria for 21 days at time 0: 7 days in liquid SCP medium and 14 days on SCN medium containing 1% agar. Each point represents the mean ± SE from three replicates. Uninoculated control (●); tissue infected with *R. japonicum* 61A76 (○).

Table III. *Effect of Cell Aggregate Size on Ethylene Production and Acetylene Reduction by Acme Soybean Cells ± R. japonicum 61A76*

The data are from four to eight replicates.

Size	Ethylene Production			
	Control		Rhizobium-infected	
	Endogenous	C_2H_2-dependent	Endogenous	C_2H_2-dependent
mm	*nmoles/g dry weight·24 hr*			
<0.5	22.6 ± 5.6	−5.4 ± 4.1	20.6 ± 3.7	−9.1 ± 3.4
0.5–2.0	4.4 ± 1.1	12.3 ± 3.5	42.5 ± 6.4	44.3 ± 7.0
5.0–10	4.6 ± 2.9	−2.4 ± 5.2	36.9 ± 15.0	−43.4 ± 11.9

Table IV. *Effect of Auxin and Cytokinin on Ethylene Production and Acetylene Reduction by Acme Soybean Cells ± R. japonicum 61A76*

The data are from four replicates.

Medium	Ethylene Production			
	Control		Rhizobium-infected	
	Endogenous	C_2H_2-dependent	Endogenous	C_2H_2-dependent
	nmoles/g dry weight·24 hr			
SCN	0.0 ± 0.0	−5.1 ± 5.2	3.8 ± 0.1	58.0 ± 21.6
SCN + 2,4-D, 2 mg/l	149 ± 81.5	−63.0 ± 64.0	187 ± 67.5	−74.0 ± 77.5
SCN + kinetin, 0.1 mg/l	131 ± 68.0	−110 ± 60.2	142 ± 121	−68.0 ± 88.5
SCN + 2,4-D, 2 mg/l; kinetin, 0.1 mg/l	338 ± 132	−80.6 ± 79.2	250 ± 97.2	−87.1 ± 71.0

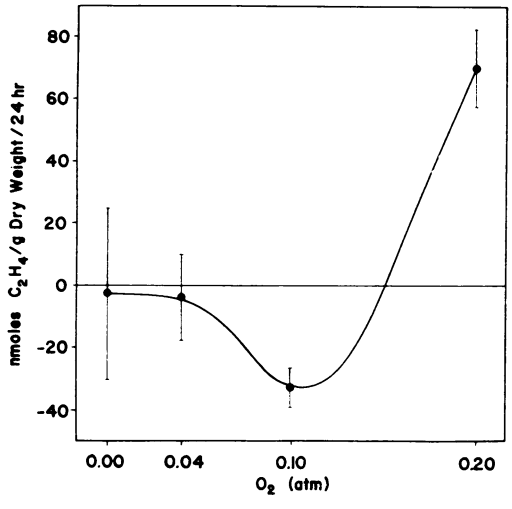

FIG. 4. Effect of oxygen concentration on acetylene-dependent ethylene production by Acme soybean tissue ± *R. japonicum* 61A76. Ethylene produced in the presence of acetylene by uninoculated control samples was subtracted from values recorded for tissue infected with *R. japonicum* 61A76. The tissue had been exposed to bacteria for 21 days as in Fig. 3. Each point represents the mean ± SE from six replicates.

was demonstrated only in the *Rhizobium*-infected tissue grown on SCN (Table IV). The presence of either 2,4-D or kinetin inhibited any measurable acetylene-dependent ethylene production and promoted endogenous ethylene production. The large standard errors for all data collected in the presence of auxin and cytokinin emphasize the importance of avoiding the variability under these conditions by plating the soybean cells on SCN after the infection.

Oxygen Effects. Data presented in Figure 4 suggest that oxygen concentrations less than 0.20 atm during the assay period do not increase acetylene-dependent production of ethylene. It seemed undesirable to determine a true value for endogenous ethylene production by subjecting the tissue to low levels of oxygen prior to injecting acetylene. For this reason, the data in Figure 4 are expressed as ethylene produced in the presence of acetylene by *Rhizobium*-infected tissue minus the ethylene produced by uninfected controls under the same conditions. A separate experiment demonstrated that in either infected or uninfected tissue essentially zero levels of endogenous ethylene production were not altered by decreasing the available oxygen.

The possibility that decreased levels of oxygen inhibited acetylene reduction after 48 hr (Fig. 3) was examined by restoring O_2 levels to 0.20 atm in each flask after 48 hr. This procedure, which was analogous to a successful experiment performed by Sprent (11) on excised soybean nodules, was unsuccessful in restoring the ability to reduce acetylene.

Bacterial Effects. Table V reveals the capacity of various strains of *Rhizobium* and *Agrobacterium tumefaciens* NRRL B-37 to induce an acetylene-dependent production of ethylene in Acme soybean cells. Two important results of this experiment should be noted. First, *R. japonicum* R54a, a strain that effectively nodulates soybean, did not establish any association with the soybean cells which resulted in detectable acetylene reduction. Second, the rhizobial strains which nodulate cowpeas but not soybeans did exhibit acetylene-dependent ethylene production in association with Acme cells. Several other rhizobial strains, most notably *R. trifolii* 162P17, exhibited levels of acetylene-dependent ethylene production which were significantly greater than the uninoculated control tissue but less than those obtained with *R. japonicum* 61A76. A question remains about results obtained with *R. phaseoli* 127K17, *R. leguminosarum* 128C53, and *R. trifolii* 162P17 because these organisms grew much more rapidly than the soybean tissue in the two weeks after plating the cells on SCN. *A. tumefaciens*

Table V. *Effect of Bacterial Strain on Ethylene Production and Acetylene Reduction by Acme Soybean Cells*

Inoculum values are expressed as viable cells × 10^{-6}/50 ml of soybean cell suspension. Data were compared with the uninoculated control by a two-tailed *t* test. Tests in which $p \leq 0.01$ are indicated. The ethylene data are from five replicates.

Bacterial Strain	Inoculum	Endogenous C_2H_4	C_2H_2-dependent C_2H_4
		nmoles/g dry weight·24 hr	
Control	None	0.5 ± 0.4	1.9 ± 1.1
R. japonicum 61A76	1.1	1.7 ± 0.3	33.3 ± 2.5 $p \leq 0.001$
R. japonicum 61A96	8.7	7.0 ± 1.0 $p \leq 0.001$	135 ± 26.6 $p \leq 0.01$
R. japonicum R54a	3.7	0.0 ± 0.0	0.0 ± 0.0
R. japonicum 505	0.6	15.5 ± 4.6	52.4 ± 2.8 $p \leq 0.001$
R. lupini 96B9	2.9	0.0 ± 0.0	7.2 ± 2.7
R. sp. 29C2	0.2	4.0 ± 1.3	45.0 ± 6.0 $p \leq 0.001$
R. sp. 32H1	0.6	3.3 ± 1.3	503 ± 92 $p \leq 0.001$
R. sp. 32Z3	0.2	7.8 ± 1.1 $p \leq 0.001$	30.6 ± 8.9 $p \leq 0.01$
R. meliloti 102F51	40	0.0 ± 0.0	2.7 ± 1.4
R. phaseoli 127K17	21	5.4 ± 1.4 $p \leq 0.01$	9.8 ± 2.9
R. leguminosarum 128C53	4.6	3.4 ± 0.2 $p \leq 0.001$	12.8 ± 3.4
R. trifolii 162P17	8.6	4.9 ± 1.5	11.6 ± 1.8 $p \leq 0.01$
A. tumefaciens NRRL B-37	26	134 ± 22 $p \leq 0.001$	9.8 ± 10.8

NRRL B-37 also completely covered the soybean cells plated on SCN, and, in addition, it exhibited very high levels of endogenous ethylene production.

Other trials with rhizobial strain 32H1 confirmed that it established a reproducible relationship with Acme soybean cells which resulted in acetylene-dependent ethylene production. In all cases strain 32H1 was much more active in the reduction of acetylene than accompanying replicates infected with *R. japonicum* 61A76.

Stability of Host Cell Line. A comparison of the acetylene-dependent ethylene production values recorded in various experiments throughout this study revealed large variations between trials for those data collected from the Acme-*R. japonicum* 61A76 association. Differences in the amounts of endogenous ethylene production did not account for the observed variation. Figure 5 reveals a general decline in acetylene-dependent ethylene produced by the most active associations of these organisms during an 8-week period. Although this unfortunate situation developed in later studies, statistical evaluation of data still permitted the demonstration of significantly greater levels of acetylene-dependent ethylene production in *Rhizobium*-infected tissue (Table V).

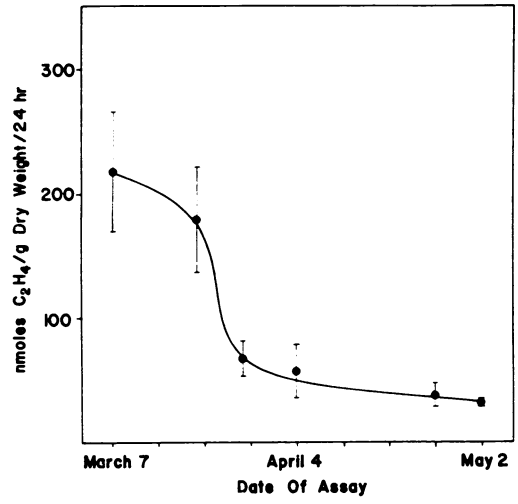

FIG. 5. Variation in acetylene-dependent ethylene production by one line of Acme soybean cells infected with *R. japonicum* 61A76. The standard infection and assay procedures were used. Each value was significantly greater than the uninoculated controls which had values insignificantly different from 0.0 nmoles ethylene/g dry weight·24 hr. Each point represents the mean ± SE from three to six replicates.

DISCUSSION

The results from this study lend support to the claim (6) that certain *Rhizobium*-soybean cell associations are capable of reducing acetylene. Although there were highly significant differences between control replicates and *Rhizobium*-infected samples in the present study, the values obtained for acetylene-dependent ethylene production fell near the lower end of the range of values reported by Holsten *et al.* (6) for reduction of acetylene to ethylene. A direct comparison is not possible without data on endogenous ethylene production in the latter experiments.

Attempts to increase the acetylene-dependent production of ethylene were not successful, but they did clarify several of the variables present in this system. The results in Table I suggest that a more detailed investigation of the media components may promote the *Rhizobium*-soybean association *in vitro*. Preliminary indications (Table I) that the acetylene-dependent production of ethylene is inhibited by mineral nitrogen have been supported in subsequent experiments. The relationship of these results to the acetylene reduction reported by Holsten *et al.* (6) on a medium which contained 60 mM nitrogen is not clear.

The negative values for acetylene-dependent ethylene production by the control replicates in Figure 2 deserve comment. It is probable that these values are a product of the method of presentation rather than a demonstration that acetylene inhibits endogenous ethylene production. Figure 1 shows that endogenous ethylene production decreased logarithmically during the 1st week after the cells were plated onto SCN. Assays for endogenous ethylene production were conducted on day 0 to day 1. It is apparent from Figure 1 that this value will be greater than endogenous ethylene production during the test for acetylene reduction on day 1 to day 2. Thus, when the value for endogenous ethylene production is subtracted from the ethylene produced in the presence of acetylene, the result is a negative number. It is possible to calculate a correction factor for this decrease in endogenous ethylene production by interpolation in Figure 1. Corrected values for the control replicates in Figure 2 would have produced a line which did not deviate significantly from 0.0 nmole C_2H_4/g dry weight·24 hr. Similar corrections for the *Rhizobium*-treated cultures suggest that the capacity to reduce acetylene (presumed nitrogenase activity) begins to develop from the day cells are plated onto SCN.

The observation that very small aggregates of soybean cells did not reduce acetylene in association with *Rhizobium* (Table III) suggested that a certain mass of tissue may be required to protect any nitrogenase present from ambient oxygen. Possibly leghemoglobin could perform this function as Wittenberg *et al.* (13) have suggested that it does in root nodules. Alternatively a less efficient form of general respiratory protection might be operating. If the latter be the case, then lowering the ambient oxygen level should produce a significant increase in acetylene reduction as has been demonstrated in *Azotobacter-Paspalum* associations (1). Lower concentrations of oxygen, however, completely inhibited any demonstrable acetylene-dependent production of ethylene (Fig. 4).

The promotion of endogenous ethylene production by soybean cells in the presence of 2,4-D (Table IV) parallels the responses reported for *Ruta graveolens* and *Acer pseudoplatanus* cells in culture (3, 9). No acetylene-dependent production of ethylene was observed with the auxin and cytokinin levels used in the present study. The increases in endogenous ethylene production with hormonal treatments were not dependent upon *Rhizobium* (Table IV). Whether development of the symbiosis is inhibited directly by the cytokinin and auxin or indirectly through increased ethylene production remains to be determined.

The results in Table V emphasize one potential advantage of the *in vitro* study of *Rhizobium*-soybean cell associations. No natural interactions between rhizobial strains 29C2, 32H1, or 32Z3 and intact soybean plants have shown that these organisms can interact to produce high levels of acetylene-dependent ethylene (presumed nitrogenase activity). In the present study, this genetic capability was revealed. Such observations eventually may be helpful in understanding the genetic control of the nitrogenase complex.

Extrapolation of the indirect acetylene reduction assay to actual N_2 fixation is a difficult matter. As one would predict, microkjeldahl nitrogen determinations on tissues in the present study showed no significant differences between control replicates and *Rhizobium*-infected cells. Calculations reveal that if the nitrogenase complex is responsible for the observed acetylene-dependent ethylene production in this system, then a considerable increase in activity would be required to detect

$^{15}N_2$ reduction in the attenuated Acme tissue represented in Figure 5.

It must be concluded that associations between soybean root cells and *Rhizobium* were established *in vitro*. Under certain conditions acetylene-dependent production of ethylene was observed. Presumably, this ethylene was produced by the reduction of acetylene as claimed by other workers (6, 7). Several experimental variables have been examined, which should assist future attempts to study the symbiotic nitrogenase complex *in vitro*.

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