The acetylene reduction assay for measuring nitrogen fixation in waterlogged soil

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Nitrogen fixation in waterlogged, soil-straw, and sand–clay–straw mixtures was measured with the C2H2 reduction assay, the 15N-tracer technique, and the Kjeldahl method. The reduction of 6 to 15 moles C2H2 corresponded to the fixation of 1 mole N2. The theoretical ratio is 3 moles C2H2 to 1 mole N2. A ratio of 3 moles C2H2 reduced for each mole of N2 fixed was obtained when samples of sand–clay–straw were incubated under conditions that minimized effects that were due to gas diffusion through the aqueous phase. Calculations indicated that N2 at a partial pressure of 0.2 atm above the samples was not sufficient to saturate the nitrogenase enzyme of the organisms in lower layers of soil-straw samples. Thus the concentration of N2 dissolved in the aqueous phase limited nitrogen fixation. C2H2 is more soluble in water than N2; the C2H2 reduction was not as limited by the C2H2 concentration in the aqueous phase. N2 was experimentally shown to be limited at depth in a sand–clay–glucose system in that fixation decreased from 128 to 36 pg N/g of sand–clay incubated so that the total sample depth ranged from 0.2 to 3 cm.

The C2H2 reduction assay provides a method for measuring the potential nitrogenase activity in the waterlogged soil amended with straw; however, this assay must be calibrated for specific conditions. The data also indicate that where N2 diffusion rates may limit N fixation, a normal atmosphere (80%) of N2 should be used in the experiment.

Introduction

The similarity of enzymes involved in nitrogen fixation in different organisms, and the ability of the enzyme to reduce a wide variety of substrates (8), has made possible the acetylene (C2H2) assay for nitrogenase activity. The assay is based on the reduction of C2H2 to ethylene (C2H4), which is detected with hydrogen flame ionization after gas chromatographic separation. The C2H2 reduction method has been used for in situ studies of nitrogen fixation by root systems and soil cores (9, 17). A direct comparison of C2H2 reduction and nitrogen fixation was not always reported, and a theoretical conversion factor of 1 mole N2 fixed for each 3 moles C2H2 reduced was generally used for calculating the amount of nitrogen fixed. Although work with pure cultures and cell-free extracts has demonstrated a ratio of 3.0 to 4.5 moles C2H2 formed for each mole N2 fixed, it is not known if this applied to more complex systems such as soil and nodules. The data for root nodules reported by Stewart et al. (17) indicate that often considerably more than 3 moles C2H2 were produced for each mole N2 fixed.

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Earlier work in this laboratory, with asymbiotic nitrogen fixation, demonstrated that high rates of fixation (240 to 460 µg N/g soil in 28 days) occur in soils amended with 5 to 20% straw and incubated under waterlogged conditions (14). The work reported in this paper was conducted to test the applicability of the C2H2 reduction assay for measuring nitrogen fixation under conditions where high fixation rates can occur. Kjeldahl and 15N-tracer techniques were used to confirm fixation. This made it possible to determine the factors affecting nitrogen fixation and the quantitative interpretation of the C2H2 reduction assay.

Materials and Methods

Treatment and Incubation of Samples

Asymbiotic nitrogen fixation was investigated in soil and sand–clay mixtures amended with wheat straw, ground to pass a 0.6-mm sieve. The straw contained 42% C, 0.56% total-N, 26.4 ppm NH4+-N, and 11.2 ppm NO3--N. The straw was mixed with the soil or sand–clay in a ratio of 1:5 (w/w). The soil was a Dark Brown Chernozemic soil (Weyburn Loam) containing 2.5% C, 0.21% total-N, 3.2 ppm NH4+-N, and 7.8 ppm NO3--N.

The sand–clay mixture was prepared from bentonite and pure white silica sand (60 mesh). The clay was suspended in 1 N CaCl2 for 1 week and then washed three times with distilled water, and finally suspended in distilled water (125 g clay in 300 ml H2O). This suspension was mixed with 375 g sand and then dried at 100C. An inoculum of 5% fresh soil was added to the sand–clay mixture.
Two gram samples of the soil-straw or sand-clay straw mixtures were placed in 25 ml erlenmeyer flasks and waterlogged by slowly adding 4.5 ml of liquid. Distilled water was added to the soil-straw mixture, and a salt solution of the following composition was used for the sand-clay-straw mixture: 3.6 g K$_2$HPO$_4$, 1.4 g KH$_2$PO$_4$, 0.4 g MgSO$_4$$ \cdot $7H$_2$O, 0.5 g CaCO$_3$, 10 mg Na$_2$MoO$_4$$ \cdot $2H$_2$O, and 10 mg FeSO$_4$$ \cdot $7H$_2$O per liter. With 2 g of sample in 25-ml flasks, the samples were in a layer about 5 mm thick with a 1-mm layer of water on the surface. Incubation was at 27°C for the periods of time indicated in the text.

Nitrogen fixation was also investigated in a sand-clay-glucose system. This system was prepared by adding 2 ml of the above salt solution containing 350 ppm organic nitrogen (yeast extract), 3% glucose, and 1.7 $\times$ 10$^8$ clostridia cells/ml to 2 g sterile sand-clay. The inoculum, 3.0 ml of a 48 h culture of Clostridium pasteurianum, was added to 47 ml of salt solution. The components of the system were mixed and then incubated anaerobically for 90 h.

**Measurement of Nitrogen Fixation**

In the Kjeldahl method, nitrogen fixation was determined as the difference in total nitrogen between incubated samples and non-incubated controls (3).

For the $^{15}$N-technique, the samples were incubated in a stainless steel chamber (modified Torbal BTL anaerobic jar). The chamber was evacuated and filled with He, and then evacuated to 0.6 atm pressure. The pressure was brought to 0.8 atm with O$_2$, and the chamber was then connected to a molecular sieve pump for transfer of the $^{15}$N-labeled N$_2$ from the storage container.

Gas samples were withdrawn periodically from the chamber and analyzed for $^{15}$N$_2$ and O$_2$ with the mass spectrometer. Oxygen was added to maintain a partial pressure of 0.15 to 0.20 atm. Evolved CO$_2$ was absorbed in 20% KOH placed in the chamber at the beginning of each incubation.

For the C$_2$H$_2$ reduction assays, the samples were incubated for the desired time. Then the flasks were stopped with serum caps and a stream of He was passed through them, using a hose equipped with a hypodermic needle. A second needle act as a vent. After 3 min flushing with He, the needles were removed, the flasks and needle were removed, the flasks and needle were then connected to a molecular sieve pump for transfer of the C$_2$H$_2$ gas to the detector. The injection port and detector were temperature controlled. Oxygen was added to maintain a partial pressure of 0.15 to 0.20 atm. Evolved CO$_2$ was absorbed in 20% KOH placed in the chamber at the beginning of each incubation.

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The measurement of C$_2$H$_4$ was accomplished with a Varian Aerograph model 2100 gas chromatograph equipped with a H$_2$-flame ionization detector. The C$_2$H$_2$ and C$_2$H$_4$ were separated with a 6 mm $\times$ 2 m column of Porapak R at 55°C with a carrier gas (N$_2$) flow rate of 60 ml/min. The injection port and detector operating temperatures were 80°C and 65°C, respectively.

Five replicates were used for the Kjeldahl measurements, and the $^{15}$N measurements and C$_2$H$_2$ reduction assays were conducted in triplicate.

**Microbial Numbers**

The number of clostridia cells in the sand-clay mixture was determined by the fluorescein isothiocyanate direct count technique (1).

**Results**

**Soil-Straw and Sand-Clay-Straw Systems**

The establishment of a defined system is important in an understanding of the biological reactions occurring in soil. One of the more complex components of the system is the soil organic matter. Table 1 indicates that soil organic matter does not substantially affect nitrogen-fixing activity in soil-straw mixtures. Organic matter was removed from the soil by oxidation with H$_2$O$_2$ before addition of the straw. About 75% of the nitrogen-fixation level observed in the soil-straw system was obtained in treatments C and E where the samples were inoculated with 5% fresh soil. Fixation did not occur without the inoculum (B and D).

**TABLE 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N content before incubation, mg N/g</th>
<th>N fixation, $\mu$g N/(g X 21 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Soil + 20% straw</td>
<td>3.38</td>
<td>240 ± 36*</td>
</tr>
<tr>
<td>B. Soil (organic matter removed) + 20% straw</td>
<td>1.42</td>
<td>-50 ± 45</td>
</tr>
<tr>
<td>C. Soil (organic matter removed) + 20% straw + soil inoculum</td>
<td>1.47</td>
<td>190 ± 18</td>
</tr>
<tr>
<td>D. Sand-clay + 20% straw + soil inoculum</td>
<td>0.85</td>
<td>10 ± 27</td>
</tr>
<tr>
<td>E. Sand-clay + 20% straw + soil inoculum</td>
<td>0.86</td>
<td>180 ± 27</td>
</tr>
</tbody>
</table>

*Kjeldahl method (± standard error).
indicates that the soil and not the straw contained the organisms responsible for nitrogen fixation in these systems.

The C₂H₂ to C₂H₄ reduction by the soil-straw system showed an initial lag after introduction of the C₂H₂ into the system, and then became constant for the 10 to 48 h period (Fig. 1). The rate of C₂H₂ to C₂H₄ reduction in the soil-straw and sand-clay-straw systems was therefore determined by measuring the increase of C₂H₄ concentrations during the constant rate period.

The rates of C₂H₂ to C₂H₄ reduction by soil-straw and sand-clay-straw systems during a 3-week incubation period suggest two maxima of nitrogenase activity (Fig. 2). There was considerable variation in C₂H₂ to C₂H₄ reduction rates among samples; however, all samples showed the trend that is indicated by the mean values. The nitrogen-fixation rates calculated from the amount of ¹⁵N₂ incorporated during 48 to 72 h (Fig. 3) did not show two peaks of activity. However, the increased exposure time for ¹⁵N makes direct comparison with the C₂H₂ reduction assay difficult.

A comparison of the data from the C₂H₂ reduction assay with the Kjeldahl and ¹⁵N-tracer techniques is shown in Table 2. The total C₂H₂ reduced in experiments 1, 2, and 3 was obtained by integration of rate curves such as those shown in Fig. 2. Although considerable variation in the ratio of moles C₂H₂ reduced to moles

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**Fig. 2.** C₂H₂ to C₂H₄ reduction rates in the soil-straw and sand-clay-straw systems. L.S.D. (P = 0.05) = 33.5 (soil-straw) and 29.7 (sand-clay-straw).

**Fig. 3.** ¹⁵N₂-incorporation rates in the soil-straw and sand-clay-straw systems. L.S.D. (P = 0.05) = 9.8 (soil-straw), sand-clay-straw no significant differences.
N$_2$ fixed (C$_2$H$_2$ :N$_2$ reduction ratio) was observed, the C$_2$H$_2$ to C$_2$H$_4$ reduction was similar where the same incubation periods were used (experiments 2 and 3). In two cases (experiments 1 and 2), high C$_2$H$_2$ :N$_2$ ratios were observed. In experiment 1, the system may have lost nitrogen when subjected to wetting and drying. The soil-straw mixture was subjected to two 12-day periods when the water content was between 45 and 65% water-holding capacity. During these periods, mineralization and nitrification processes could have accumulated nitrate, which would have been immediately denitrified when the soil was waterlogged again (4).

The measurements of nitrogen fixation and C$_2$H$_2$ reduction were conducted on separate sets of samples in experiments 3, 4, and 5. However, in experiments 1 and 2, one set of samples was used for both the C$_2$H$_2$ assay and the Kjeldahl determinations for nitrogen fixation. It is possible that repeated 24-h exposures to C$_2$H$_2$ inhibited total nitrogen fixation without decreasing nitrogenase activity to the same extent. This could have occurred in experiment 2, where five assays were done during a 21-day incubation period, and would increase the C$_2$H$_2$ :N$_2$ reduction ratio. The results from experiment 4 indicate that the C$_2$H$_2$ :N$_2$ reduction ratios determined for a short period (48 to 72 h) were similar to those observed for a 21-day period (cf. experiment 3).

In experiment 5, the system was altered so that effects due to gas diffusion through the aqueous phase were minimized. The sand–clay-straw samples were preincubated aerobically for 9 days and then, under anaerobic conditions, were suspended in nitrogen-free liquid medium containing 1% glucose.* Ten-milliliter portions of this suspension (1 g sand–clay-straw) were placed in 25-ml flasks which were immediately stoppered and evacuated. Either 0.1 atm C$_2$H$_2$ or


**TABLE 2**
Comparison of the C$_2$H$_2$ assay with the Kjeldahl and $^{15}$N-method for measuring nitrogen fixation

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>System</th>
<th>Incubation period</th>
<th>µmoles N$_2$ or C$_2$H$_2$ reduced/g*</th>
<th>Kjeldahl</th>
<th>$^{15}$N$_2$</th>
<th>C$_2$H$_2$</th>
<th>C$_2$H$_2$:N$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Soil-straw</td>
<td>63 days</td>
<td>22 ± 3</td>
<td>—</td>
<td>170 ± 20(2.7)†</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Soil-straw (wet-dry cycle)</td>
<td>21 days</td>
<td>5 ± 1</td>
<td>—</td>
<td>160 ± 20(2.5)</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Soil-straw</td>
<td>21 days</td>
<td>13 ± 5</td>
<td>79 ± 8(3.8)</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Sand-clay-straw</td>
<td>48 h</td>
<td>1 ± 0.2</td>
<td>6.7 ± 0.1(3.4)</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Sand-clay-straw</td>
<td>72 h</td>
<td>1 ± 0.5</td>
<td>9.9 ± 0.7(3.3)</td>
<td>6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Figures in parentheses = µmoles C$_2$H$_2$ reduced/(g X day).

**TABLE 3**
Nitrogen fixation and C$_2$H$_2$ reduction by C. pasteurianum in a sand–clay–glucose system

<table>
<thead>
<tr>
<th>Gas phase (atm)</th>
<th>Clostridia, 10$^8$ cells/g</th>
<th>N$_2$ and C$_2$H$_2$ reduction, µmoles/g</th>
<th>N$_2$ (Kjeldahl)</th>
<th>C$_2$H$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>He N$_2$ C$_2$H$_2$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.6 0.4 0</td>
<td>75 ± 15*</td>
<td>1.16</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>0.9 0.1</td>
<td>14 ± 5</td>
<td>—</td>
<td>26.50</td>
<td></td>
</tr>
<tr>
<td>0.6 0.4 0.005</td>
<td>42 ± 9*</td>
<td>0.57</td>
<td>—</td>
<td>3.3</td>
</tr>
</tbody>
</table>

* Figures in parentheses = Standard error.
0.6 atm $^{15}$N-labeled $N_2$ (with the balance being He) were introduced into the flasks, which were then incubated on a rotary shaker for 24 h. During this period, 2.0 μmoles $C_2H_2$ was reduced and 0.7 μmole $N_2$ was incorporated per gram of sand–clay–straw. This is a $C_2H_2:N_2$ reduction ratio of 3:1, which is the same as the theoretical value, whereas the ratio for the undisturbed systems were 6:1 or greater. This indicates that the high ratio of 3:1, or greater than 0.2 cm the concentration of $N_2$ or $C_2H_2$ at the sites of reduction.

**Sand–Clay–Glucose System**

Table 3 shows the results of experiments with *C. pasteurianum* in a sand–clay–glucose system exposed to various gas phases. When incubated in the presence of 0.1 atm $C_2H_2$, the number of *Clostridium* cells was much lower than in the samples incubated in $N_2$ and He. In the samples incubated in 0.1 atm $C_2H_2$, the cells were pleomorphic and 2 to 3 times larger than normal. Incubation of the samples in an atmosphere of $N_2$ and He, or a low concentration of $C_2H_2$, plus $N_2$ and He, resulted in cell numbers which were similar in both treatments, and there was no evidence of pleomorphic cells (experiment 2). A $C_2H_2:N_2$ reduction ratio of about 23:1 can be calculated from the data for experiment 1.

This high ratio may reflect differences in the rates of diffusion of the gases to the nitrogen-fixing site, particularly if the concentration of $N_2$ in the aqueous phase was limiting nitrogen fixation.

Figure 4 shows the effect of the depth of sample on nitrogen fixation by *C. pasteurianum* in a sand–clay–glucose system. The samples (2 g sand–clay) were incubated for 90 h in containers of varying size so that the sample depth ranged from 0.2 to 3.0 cm. The atmosphere was $O_2$-free, and $N_2$ was at a partial pressure of 0.4 atm. The nitrogen content of samples was measured by the Kjeldahl method before and after incubation. Nitrogen fixation decreased as the sample depth was increased, indicating that at depths greater than 0.2 cm the concentration of $N_2$ at the nitrogen-fixing site was limiting. A similar situation may have existed in the soil–straw and sand–clay–straw systems.

**Theoretical Consideration**

A consideration of the laws describing transfer of gas through water-saturated soil helps to explain the high $C_2H_2:N_2$ reduction ratios observed for the soil–straw and sand–clay–straw mixtures. Several diffusion equations are available and have been used for soils and plants. All are some form of the following statement of Fick's first law (6):

$$q = -D \left( \frac{dc}{dx} \right),$$

where $q$ is the amount of gas which passes across a unit area where the concentration gradient is $dc/dx$. $D$ is the diffusion coefficient, and is a property of the medium and the gas. Relative magnitudes of the coefficients predict that diffusive transfer of $N_2$ and $C_2H_2$ will be about the same with equal gradients and areas of diffusion. The diffusion coefficients for $N_2$ and $C_2H_2$ in water at 27°C are $1.98 \times 10^{-5}$ and $1.89 \times 10^{-5} \text{cm}^2/\text{s}$, respectively (12). Because the solubility of $N_2$ in water at 27°C and 0.2 atm pressure is 0.12 μmole/ml and the solubility of $C_2H_2$ in water at 27°C and 0.1 atm pressure is 3.8 μmole/ml (18), the gradients for $N_2$ and $C_2H_2$ will differ greatly. The greater solubility of $C_2H_2$ has the effect of increasing the concentration of $C_2H_2$ in the water at the atmosphere–water interface. This increases the rate of transfer of $C_2H_2$ over that of $N_2$ for equivalent distances and areas, and at the partial pressures used in these experiments. If the concentration

![Fig. 4. The effect of sample depth on nitrogen fixation by *C. pasteurianum* in a sand–clay–glucose system.](image-url)
of N₂ at the site of nitrogen fixation is limiting as Fig. 4 suggests, then a high C₂H₂:N₂ ratio will result.

The relationship between the distance below the surface and the concentration of dissolved N₂ and C₂H₂ in the soil-straw mixture was calculated, and is illustrated in Fig. 5. For the purposes of calculation, it was assumed that no N₂ or C₂H₂ consumption occurred in the top 0.2 cm. This follows from two observations made on an identical system, and reported elsewhere (14);* direct microscopic examination of the soil-straw system by the contact slide technique showed that clostridia occurred only at depths greater than 0.2 cm from the air-water interface, and it was established that clostridia were the only nitrogen-fixing organisms present in the system. Magdoff and Bouldin (11), using a similar system found high fixation rates in the top 0.2 to 0.3 cm, but their system contained aerobic nitrogen-fixing organisms.

Fick's first law, in the following form, describes the diffusion of N₂ and C₂H₂ through the surface layer (0.0 to 0.2 cm) where no nitrogenase activity was present:

\[ \frac{dc}{dy} = -q_0 y / D_s \]

where \( c \) is the concentration of gas (µmole/ml) at a distance \( y \) cm below the surface, and \( c_0 \) is the concentration of gas (µmole/ml) at the surface. The flux of gas through the surface is \( q_0 \) µmole/(cm² x s), and \( D \) is the diffusion coefficient (cm²/s).

The fluxes \( q_0 \) of N₂ and C₂H₂ into the soil-straw system were estimated from the data given in Table 2 (experiment 4), where the area of sample exposed to the gaseous phase was 4.8 cm²/g of material. The flux values were calculated to be \( 1.3 \times 10^{-6} \) µmole N₂/(cm² x s) and \( 8.1 \times 10^{-6} \) µmole C₂H₂/(cm² x s). In considering the diffusion through the soil-straw mixture, the diffusion coefficient must be modified to account for the effective path length of diffusion and for the porosity. The diffusion coefficient for the waterlogged soil-straw, \( D_s \), is equal to (0.66) (0.715) \( D \). The effective path length factor is generally taken as 0.66 and the porosity (71.5%) was determined by the air pycnometer method (2).

In the 0.2- to 0.6-cm depth, the rate at which N₂ and C₂H₂ are consumed must be considered. For the steady state, the diffusion equation is as follows (2):

\[ \frac{d^2c}{dy^2} = -\alpha / D_s \]

where \( \alpha \) is the "activity," expressed in µmole/(cm³ x s). If \( \alpha \) is assumed to be constant with depth, then \( q_2 = -L \), where \( q_2 \) = flux of gas at the 0.2 cm depth (\( q_2 = q_0 \), since there was no activity in the 0.0 to 0.2 cm layer), and \( L \) is the distance below the 0.2 cm level at which \( \alpha \) becomes zero. By integrating the above equation twice with the boundary conditions, when \( y = L \) then \( dc/dy = 0 \) and when \( y = 0 \) then \( c = c_2 \) (\( y \) is the distance below 0.2 cm level), we obtain the following relationship:

\[ c_y = c_2 - (\alpha y^2 / 2 D_s) - (q_2 y / D_s), \]

where \( c_y \) is the concentration of gas at depth \( y \) cm below the 0.2 cm level, and \( c_2 \) is the concentration of gas at the 0.2 cm level. The activity, \( \alpha \) was taken as 2.5\( q \), since \( q \) represents the quantity of gas consumed by 0.4 cm³ (1 cm² x 0.4 cm thick).

The Michaelis constant \( (K_m) \) for the nitrogenase enzyme is estimated to be about 0.1 µmole/ml for N₂ and C₂H₂ (9). The \( K_m \) for whole cells may be 4 to 10 times lower than the \( K_m \) for the cell-free enzyme (10), thus the \( K_m \) values for N₂ and C₂H₂ are expected to be in the range 0.01 to
0.03 μmole/ml, respectively. The calculated concentration of N₂ and C₂H₂ in the soil–straw system (Fig. 5) indicate that N₂ could have been limiting but C₂H₂ was not. Hence, ¹⁵N₂-incorporation measurements determined the actual nitrogen fixation under the given conditions, whereas the C₂H₂ reduction assay measured the nitrogen-fixing potential of the organisms.

Discussion

In the past, partial pressures of 0.1 to 0.2 atm N₂ were considered adequate for nitrogen-fixation studies in soils, using the ¹⁵N-tracer technique (5, 13, 14, 16). However, measurements made with the ¹⁵N-tracer technique and the C₂H₂ reduction assay indicated that the N₂ concentration may have limited nitrogen fixation in waterlogged soil when the N₂ above the soil was at a partial pressure of 0.2 atm. It also is possible that the N₂ concentration could be a factor limiting nitrogen fixation in specific microsites within the soil with moisture contents near water-holding capacity. Greenwood and Goodman (7) calculated that water-saturated soil aggregates of 3-mm radius can become O₂ free at their centers. Thus, it is also possible that the N₂ concentration within natural soil aggregates when exposed to low partial pressures of N₂ could limit the nitrogen-fixing activity of the organisms present. The information available for nodules (17) also suggests that the N₂ concentration in the center of the nodules was not sufficient to saturate the nitrogenase enzyme when incubated in 0.2 atm N₂. Estimation of nitrogen fixation in soil and nodules may be low if 0.2 atm N₂ are used. In attempting to correlate C₂H₂ reduction with actual nitrogen fixation, 0.8 atm N₂ should be used in the ¹⁵N-tracer technique.

Hardy et al. (9) suggest an incubation period of 1 h for the C₂H₂ reduction assay of soil cores. However, for waterlogged soil samples, several hours are required to reach equilibrium after introduction of the C₂H₂ into the gaseous phase above the soil. In such cases, the concentration of C₂H₂ usually used (0.1 atm) was shown to inhibit cell division of nitrogen-fixing clostridia. This effect was overcome by using 0.005 atm C₂H₂ and 0.4 atm N₂. A similar technique has been used for measuring nitrogen fixation in continuous cultures of Azotobacter (T. A. La Rue, personal communication), and may be applicable to long-term measurements in soil and soil–plant systems. However, the effects of C₂H₂ and C₂H₄ on soil microorganisms and plants should be considered before such a method is attempted.

It has been shown that 10 ppm C₂H₄ inhibits root elongation in barley plants (15). Although no endogenous C₂H₄ production by soil–straw mixtures was detected in this study, Smith and Scott Russell (15) measured C₂H₄ concentrations of 9.3–10.6 ppm in the soil atmosphere of anaerobically incubated soil. If endogenous C₂H₄ production is significant in natural soils, a long-term C₂H₂ reduction assay, based on low concentrations of C₂H₂, may be impractical.

The C₂H₂ reduction assay, as used in this study provided a measurement of the potential nitrogenase activity of the nitrogen-fixing organisms in waterlogged soil–straw and sand–clay–straw mixtures. Because of the sensitivity and facility of the assay, it was possible to make periodic determinations during a 3-week incubation period. Thus, the effects of various factors on asymbiotic nitrogen fixation in soil can be studied over short time periods.

Acknowledgments

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