Endogenous Ethylene Production Is a Potential Problem in the Measurement of Nitrogenase Activity Associated with Excised Corn and Sorghum Roots

Received for publication October 27, 1987 and in revised form April 1, 1988

CHARLES SLOGER* AND PETER VAN BERKUM
Nitrogen Fixation and Soybean Genetics Laboratory, Plant Physiology Institute, United States Department of Agriculture, Building 011, HH-19, BARC-West, Beltsville, Maryland 20705

ABSTRACT

Endogenous ethylene production was evaluated as a source of ethylene during acetylene reduction assays with freshly collected roots of field-grown corn, Zea mays L. cv Funks G-4646, and sorghum, Sorghum bicolor (L.) Moench. cv CK-60A. Ethylene production was not detected when roots were incubated in air without acetylene. The presence of endogenous ethylene production was confirmed when roots were incubated anaerobically and in the presence of 40 millimolar sodium hydrosulfite. Ethylene oxidase activity was also associated with excised roots. The rate of ethylene oxidation was higher than the rates of ethylene accumulation during either acetylene reduction assays or anaerobic incubations. These results indicate that the procedure of incubating roots of grasses in air to monitor endogenous ethylene production is not a valid control in acetylene reduction studies with grasses. The presence of endogenous ethylene production during acetylene reduction assays was demonstrated by using either CO to inhibit nitrogenase activity or chloramphenicol to inhibit nitrogenase synthesis in freshly excised roots.

Most of the evidence for nitrogen fixation by bacteria associated with roots of grasses has been obtained by the measurement of nitrogenase activity using the indirect acetylene reduction method in which nitrogenses catalyzes the reduction of acetylene to ethylene. The use of a prolonged microaerophilic preincubation period or a long-term acetylene reduction assay has lead to a problem of falsely identifying the presence of nitrogenase activity in grasses or overestimation of rates of N2 fixation (11). Measurements of immediate nitrogenase activity by freshly collected grass roots are desirable because they may more closely reflect the presence of and the rate of activity associated with the roots before sampling. Low rates of acetylene reduction by corn and wheat roots have been reported using measurements made within 1 to 2 h of their excision from plants (4, 5, 12). We are concerned that ethylene attributed to ethylene reduction by nitrogenase may actually be endogenous ethylene produced by root-associated bacteria or the excised roots themselves. Endogenous ethylene production has been shown to be an important problem in measuring acetylene reduction with soil cores having low nitrogenase activity (3, 17). This has not been considered as a problem with excised roots because endogenous ethylene production has not been detected with samples incubated in air without acetylene (4, 5, 7, 12). Ethylene production in soils is not detected during aerobic incubations because the rate of ethylene oxidation is greater than ethylene production (1). It is unknown whether ethylene oxidase activity is associated with excised roots.

The objective of this investigation was to examine whether endogenous ethylene production is a source of ethylene during acetylene reduction assays with freshly excised roots of field-grown corn and sorghum. This was accomplished by assessing the impact of inhibitors of nitrogenase activity and synthesis on the accumulation of ethylene during acetylene reduction assays. Endogenous ethylene production associated with excised roots was detected when roots were incubated under anaerobic conditions in the presence of sodium hydrosulfite, a reducing agent. A preliminary report of the work has appeared (8).

MATERIALS AND METHODS

Plants. Corn, Zea mays L. cv Funks G-4646, and sorghum, Sorghum bicolor (L.) Moench. cv CK-60A, were grown in field plots without the addition of fertilizer N at the Beltsville Agricultural Research Center. Roots were collected from soybean, Glycine max (L.) Merr. cv Clark and Clark rj, (non-nodulating); peanut, Arachis hypogaea L. cv Florunner and a non-nodulating genotype; foxtail grass, Setaria sp.; lamb's quarters, Chenopodium album L.; and tobacco, Nicotiana tabacum L., which were grown in nearby plots.

Excised Roots. Root systems from corn and sorghum were collected from plants at grain filling, placed in plastic bags, and quickly transported to the laboratory. Roots were excised and placed into distilled H2O to minimize contact with air, but they were not washed. Excised roots were cut into pieces about 1 cm long. Most of the soil on the roots was dispersed by this treatment. Only some closely adhering soil remained on the root pieces. Roots from other plants were collected from actively growing plants and were treated as above. After the experiments, the roots were dried at 70°C.

Acetylene Reduction Assay. About 1 g of fresh roots was placed into 10 ml serum vials, which were then capped with serum stoppers. Assays were started by injecting 1 ml of acetylene into the vials. Gas samples (0.2 ml) were taken with 1 ml plastic tuberculin syringes at intervals during time course studies or at fixed times for other experiments. Ethylene-free syringes were stored under vacuum prior to use. The method of gas analysis was as previously described (12). Details about incubation times

1 Supported by United States Department of Agriculture, Nitrogen Fixation and Soybean Genetics Laboratory, Beltsville, MD 20705, and the Agronomy Department, University of Maryland, College Park, MD 20742, under Cooperative Agreement 58-32U3-3-370, Scientific Article No. A-4304, Contribution No. 7293, of the Maryland Agricultural Experiment Station, Department of Agronomy, College Park, MD 20742.
and temperatures are given in the figure and tables. To determine variation in acetylene reduction during the day, corn and sorghum roots were collected and analyzed at 0600, 1230, 1530, and 1830 eastern standard time on a clear day when soil moisture was near field capacity. Excised roots were incubated for 4 h at 30°C, and four replicates were used.

Acetylene reduction assays at O₂ tensions other than air were prepared by placing excised roots into serum vials and then submerging the vials in distilled H₂O to displace air. The H₂O was displaced by bubbling Ar gas into the submerged inverted vials. The vials were capped with serum stoppers under H₂O. Oxygen was added to the vials to adjust final concentrations at 0, 20, 40, and 80% O₂ before adding 1 ml of acetylene. Excised roots were incubated at 30°C for 3 h, and four replicates for each treatment were used.

Endogenous Ethylene Production. Ethylene production associated with roots was determined as previously reported (12). Excised roots were placed into 10 ml vials and incubated at 30°C for 4 h.

Endogenous ethylene production was also measured under anaerobic conditions in the presence of sodium hydrosulfite. Excised roots were placed in 10 ml vials, and the air in the vials was displaced with H₂O and Ar gas as described earlier. Sodium hydrosulfite solution (40 mm) was prepared in Ar-sparged distilled H₂O. Assays were started by injecting 1 ml of the sodium hydrosulfite solution into the capped vials. Soil was washed from roots, collected, and placed (about 1 g fresh weight) in 10 ml vials. After capping vials, air was removed from vials by purging with Ar. Assays were started when 1 ml of the sodium hydrosulfite solution was added. Vials were incubated at 30°C for 4 h before taking samples for gas analysis.

Ethylene Oxidation. Ethylene oxidation associated with excised roots in air was determined by measuring the disappearance of ethylene in 10 ml vials, containing 1 g fresh weight of roots, under an atmosphere of air containing 100 μL/L ethylene at 30°C for 4 h.

Inhibition of Nitrogenase Activity and Synthesis. The measurement of endogenous ethylene production associated with excised corn and sorghum roots during acetylene reduction assays was facilitated by the addition of 10% CO to vials to inhibit acetylene reduction as described by Nohrstedt (6). Carbon monoxide was added to vials prior to acetylene. The effect of different CO tensions on acetylene reduction by nodulated peanut root pieces was determined for 15 min incubations at 25°C using four replicates. The effect of CO on acetylene reduction by N-free enrichment cultures of N₂-fixing bacteria associated with corn and sorghum roots (16) was also tested. Root pieces were placed in 10 ml, screw-cap serum tubes containing 2 ml of sterile, N-free media (10). The serum tubes were incubated 3 days at 30°C to allow proliferation of N₂-fixing bacteria before determining acetylene reduction with or without CO as described above.

Chloramphenicol, an inhibitor of protein synthesis, was used to inhibit the development of nitrogenase activity (5). Excised corn and sorghum roots were placed in 10 ml vials and were treated with either 1 ml of chloramphenicol (100 μg/ml) or 1 ml of distilled H₂O just prior to 4 h time course measurements of acetylene reduction.

RESULTS

Acetylene Reduction Assays with Excised Roots. In preliminary experiments to detect endogenous ethylene production during acetylene reduction assays, high O₂ tensions of 40 and 80% were used to inhibit root-associated nitrogenase activity (11). Under these conditions, ethylene from endogenous ethylene production could accumulate because acetylene inhibited the oxidation of ethylene by soil bacteria (17). In the presence of acetylene, ethylene accumulated at rates of 0.27 ± 0.05, 0.20 ± 0.04, 0.15 ± 0.02, and 0.28 ± 0.11 nmol/g dry weight h⁻¹ under atmospheres containing 0, 20, 40, and 80% O₂, respectively. There was no discernable difference in ethylene accumulation among treatments, although the O₂ tensions above 20% should have inhibited nitrogenase activity (4, 11, 12). The results under high O₂ tensions indicate the presence of endogenous ethylene production.

The effect of temperature on the rate of ethylene accumulation with excised roots is shown in Table I. The rate of ethylene accumulation increased with temperature; the highest rates were recorded at 50°C. Since root-associated nitrogenase activity by excised roots of grasses is inhibited at 50°C (14), these results at high temperatures also indicate the presence of endogenous ethylene production.

Excised roots were treated with KNO₃ and NH₄Cl to inhibit nitrogenase activity in order to determine the effects on the rate of ethylene accumulation during acetylene reduction assays. Low concentrations of nitrate inhibit root-associated nitrogenase activity in cordgrass (12), and ammonia inhibits nitrogenase activity in root-associated bacteria (12, 16). In this work with excised roots, ethylene accumulation was significantly inhibited only at high concentrations of KNO₃ (100 mM) and was increased 2.5-fold with 100 mM NH₄Cl compared to controls (data not shown). These results indicate a significant proportion of endogenous ethylene formation associated with corn and sorghum roots rather than all the ethylene originating from acetylene during acetylene reduction assays.

Endogenous Ethylene Production. Ethylene production was not detectable when roots were incubated in air without acetylene. The aerobic conditions of incubation permitted ethylene oxidation by soil bacteria (1) associated with the roots. Corn roots were incubated in an atmosphere of Ar gas in an attempt to prevent ethylene oxidation to facilitate the detection of endogenous ethylene production. Ethylene production was still not detectable in Ar during 1-h incubation times. Therefore, the anaerobic conditions of the assay were improved by adding sodium hydrosulfite (40 mm) to the assay vials to lower the concentration of dissolved oxygen in the film of H₂O surrounding the roots. Under these conditions, ethylene production was measurable at rates of 0.06 nmol/g dry weight h⁻¹. A limited number of plant species were tested for root-associated ethylene production to determine whether this phenomenon was restricted to corn and sorghum (Table II). This was not the case because

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>Ethylene Corn nmol/g dry wt h⁻¹</th>
<th>Ethylene Sorghum nmol/g dry wt h⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>0.25 ± 0.01</td>
<td>0.16 ± 0.08</td>
</tr>
<tr>
<td>21</td>
<td>0.33 ± 0.07</td>
<td>0.26 ± 0.02</td>
</tr>
<tr>
<td>31</td>
<td>0.54 ± 0.41</td>
<td>0.40 ± 0.03</td>
</tr>
<tr>
<td>40</td>
<td>0.46 ± 0.14</td>
<td>0.26 ± 0.07</td>
</tr>
<tr>
<td>50</td>
<td>0.71 ± 0.19</td>
<td>0.42 ± 0.03</td>
</tr>
</tbody>
</table>
ethylene production was associated with roots of the other plants tested. Ethylene production was not detectable with rhizosphere soil from corn incubated anaerobically with 40 mM sodium hydrosulphite. The data indicate that strict anaerobic conditions during the measurements are necessary to detect ethylene production associated with roots of plants.

**Ethylene Oxidation.** Ethylene oxidation was determined with aerobic incubations of excised roots by measuring the disappearance of ethylene added to assay vials. The rates of ethylene oxidation associated with excised roots of sorghum and corn were 1.8 and 1.6 nmol/g dry weight-h, respectively. As a comparison, replicate samples of either sorghum or corn roots were incubated in air containing 10% acetylene to measure the combined ethylene production from endogenous production and nitrogenase activity. Rates for sorghum and corn were 0.6 and 1.0 nmol/g dry weight-h, respectively, indicating that the rates of ethylene oxidation associated with excised roots of sorghum or corn were higher than the rates of ethylene production from the two sources.

**Inhibition of Nitrogenase Activity and Synthesis.** CO was used to inhibit nitrogenase activity to determine endogenous ethylene production associated with excised roots in the presence of acetylene. The inhibitory effect of CO on nitrogenase activity was established by first using nodulated peanut plants. Acetylene reduction activities by nodulated root pieces in the presence of 0, 5, 10, and 20% CO were 45.7, 1.4, 0.5, and 0.3 mol ethylene/g dry weight-h, respectively. A concentration of 10% CO resulted in 99% inhibition of acetylene reduction. Similarly, acetylene reduction activity was inhibited by 97% when N2-fixing bacteria in N-free bacterial enrichment cultures of excised corn and sorghum roots were exposed to 10% CO (Table III). However, this CO treatment caused only a 50 and 60% inhibition of acetylene reduction by excised corn and sorghum roots, respectively (Table III). This indicates the presence of endogenous ethylene production, which is unaffected by CO, during the acetylene reduction assays. CO did not inhibit endogenous ethylene production associated with corn roots incubated at 50°C in the absence or presence of acetylene (data not shown).

Time course profiles of acetylene reduction with excised corn and sorghum roots showed nonlinear rates of ethylene production during the first hour of assay (data not shown). The initial nonlinear rates of ethylene production may indicate the synthesis of nitrogenase proteins and initiation of its activity superimposed upon a linear rate of endogenous ethylene production. Therefore, excised roots were treated with chloramphenicol, and time-course measurements of acetylene reduction were repeated. The results in Figure I indicate that ethylene production was partially inhibited by chloramphenicol after the first hour. Linear rates of ethylene production were observed in the presence of chloramphenicol. One source of ethylene depended upon protein synthesis during the acetylene reduction assays, while a second source was not sensitive to the action of chloramphenicol. The inhibitory effects of CO (Table III) and chloramphenicol (Fig. 1) on ethylene production during acetylene reduction assays were similar; ethylene production by treated samples was 50% and 59% of the controls for corn and 40% and 44% of the controls for sorghum, respectively.

**DISCUSSION**

Our results indicate that endogenous ethylene production is associated with freshly collected roots from field-grown corn and sorghum as well as from other plants. This was verified by detecting endogenous ethylene production under anaerobic conditions in the absence of sodium hydrosulphite and the absence of acetylene. Endogenous ethylene production during acetylene reduction assays was demonstrated by the continued production of ethylene after inhibiting nitrogenase activity either with CO or inhibiting nitrogenase synthesis with chloramphenicol.

Endogenous ethylene production was expected to be associated with roots, because ethylene is produced by microorganisms at anaerobic microsites in soil and in the rhizosphere of plants (9). Ethylene production in soil is inhibited by NO3- but is increased by NH4+ and high temperatures (9). In our studies, ethylene production associated with root samples during acetylene reduction assays was inhibited by 100 mM KNO3 and increased by

---

**Table II. Ethylene Production Associated with Roots of Various Plants**

Excised roots were incubated in Ar at 30°C for 4 h in 50 mM sodium hydrosulphite. Values are the means ± SE of 3 replicates.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Ethylene nmol/g dry wt-h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peanut, non-nodulating</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>Peanut, nodulating</td>
<td>0.17 ± 0.03</td>
</tr>
<tr>
<td>Soybean, non-nodulating</td>
<td>0.16 ± 0.05</td>
</tr>
<tr>
<td>Soybean, nodulating</td>
<td>0.20 ± 0.03</td>
</tr>
<tr>
<td>Foxtail grass</td>
<td>0.19 ± 0.02</td>
</tr>
<tr>
<td>Lamb's-quarters</td>
<td>0.30 ± 0.08</td>
</tr>
<tr>
<td>Tobacco</td>
<td>0.08 ± 0.02</td>
</tr>
</tbody>
</table>

**Table III. Effect of Carbon Monoxide on Ethylene Production during Acetylene Reduction Assays with Roots of Corn and Sorghum, and N-Free Enrichment Cultures of Corn and Sorghum Roots**

Excised roots and 3-d enrichment cultures were incubated at 30°C for 4 and 1 h, respectively, in 10% acetylene without (control) or with 10% CO. Values are means ± SE of four replicates.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Ethylene nmol/g dry wt-h</th>
<th>Percent of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>CO</td>
</tr>
<tr>
<td>Excised roots</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sorghum</td>
<td>0.10 ± 0.01</td>
<td>0.04 ± 0.00</td>
</tr>
<tr>
<td>Corn</td>
<td>0.04 ± 0.00</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>Enrichments</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sorghum</td>
<td>28.8 ± 0.0</td>
<td>0.8 ± 0.6</td>
</tr>
<tr>
<td>Corn</td>
<td>88.1 ± 7.1</td>
<td>1.9 ± 0.7</td>
</tr>
</tbody>
</table>

**Fig. 1.** Time course of ethylene production during acetylene reduction assays with excised roots with (○) or without (□) chloramphenicol. Values are the means of three replicates ± SE.
addition of 100 mM NH₄Cl and incubations at 60°C. These characteristics are similar to those reported for ethylene production in soils. In contrast, roots with immediate detectable linear rates of acetylene reduction as well as nitrogen fixation by bacteria suggested to associate with roots of grasses are inhibited by NO₃⁻, NH₄⁺, and incubation at 50°C (11–13). Therefore, endogenous ethylene production is a major source of ethylene during acetylene reduction assays with corn and sorghum roots. Bacteria are probably mostly responsible, but cut roots also produce ethylene and may contribute to ethylene accumulation during acetylene reduction assays. Ethylene accumulation could not be detected during aerobic incubations of soils without acetylene because the rate of ethylene oxidation by soil microorganisms is greater than the rate of its production (1). In our study, the rates of ethylene oxidation associated with the excised roots were also higher than the rates for endogenous ethylene production measured with anaerobic conditions. Thus, ethylene does not accumulate when excised roots are incubated in air without acetylene. However, in the presence of acetylene, endogenous ethylene produced during incubation accumulates because acetylene inhibits ethylene oxidation by soil microorganisms (2, 3, 17). Therefore, there are two important points with regard to acetylene reduction studies with freshly excised roots of grasses. First, the procedure of incubating excised roots in air to monitor endogenous ethylene production is not valid. Second, the combination of low rates of acetylene reduction and the presence of endogenous ethylene production may lead to an overestimation of the nitrogenase activity, or more seriously, may indicate the presence of nitrogenase activity where none exists. This problem has been described with measurements of acetylene reduction in soils (2, 3, 17).

Both CO or chloramphenicol inhibited ethylene production during acetylene reduction assays to the same extent. Probably, CO inhibited nitrogenase activity, while chloramphenicol prevented the synthesis of the nitrogenase proteins. The rates of ethylene production observed when nitrogenase was inhibited by these treatments were similar to the rate of endogenous ethylene production measured when anaerobic conditions eliminated ethylene oxidation. The addition of CO to samples prior to the acetylene reduction assays with excised roots was an easy and practical method to determine the presence of endogenous ethylene production. Lethbridge et al. (5) used chloramphenicol to inhibit the development of nitrogenase activity associated with washed excised roots of wheat and barley. Endogenous ethylene production was reported not to occur when chloramphenicol was present. With corn and sorghum roots, we observed ethylene production in the presence of chloramphenicol. The chloramphenicol studies reported here and by Lethbridge et al. (5) indicate that acetylene reduction activity associated with roots was due to de novo nitrogenase synthesis during the assay. Thus, nitrogenase activity associated with freshly excised roots does not accurately reflect the rate of activity in undisturbed roots.

Acknowledgments—The technical assistance of Jeffery N. Powers is gratefully appreciated. Dr. Robert Howell kindly provided the peanut plants.

LITERATURE CITED
12. VAN BERKUM, C SLOGER 1979 Immediate acetylene reduction by excised grass roots not previously preincubated at low oxygen tensions. Plant Physiol 64: 184–189
16. VON BELOW JWF, J DORMEER 1975 Potential for nitrogen fixation in maize genotypes in Brazil. Proc Natl Acad Sci USA 72: 2389–2393