Acetylene Reduction (Nitrogen Fixation) and Metabolic Activities of Soybean Having Various Leaf and Nodule Water Potentials[1]

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ABSTRACT

An apparatus was designed that permitted acetylene reduction (N_2 fixation) by root nodules to be measured *in situ* simultaneously with net photosynthesis, dark respiration, and transpiration of the shoot in soybean plants (*Glycine max* [L.] Merr. var. Beeson). Tests showed that acetylene reduction was linear with time for at least 5 hours, except for the first 30 to 60 minutes. Endogenous ethylene production did not affect the measurements. Successive determinations of acetylene reduction could be made without apparent aftereffects on the plant.

This apparatus was used to investigate the effects of soil flooding and desiccation on acetylene reduction under conditions where soil, nodule, and leaf water potentials could be measured. No acetylene reduction was detectable in flooded soil or in soil desiccated to a water potential of -19.5 bars. Between these extremes, acetylene reduction displayed a sharp optimum. Removing the soil eliminated the inhibitory effects of flooding, suggesting that rates of gas exchange were restricted between the nodules and the atmosphere at soil water potentials above -2 bars.

As the soil desiccated further, acetylene reduction decreased, and the decrease was correlated with decreases in photosynthesis and transpiration. Although dark respiration was inhibited, it was not affected to the extent that acetylene reduction, photosynthesis, or transpiration were. Consequently, it was concluded that photosynthesis, transpiration, or some direct effect on the nodules other than that caused by respiration were most likely to account for the inhibition of acetylene reduction at soil water potentials below -2 bars.

This study describes a system that permits *in situ* measurements of acetylene reduction (N_2 fixation) simultaneously with measurements of photosynthesis, dark respiration, and transpiration in whole plants. The method is used to determine the effects of low water potentials on these processes.

Low water availability has been reported to affect N_2 fixation

(11, 13, 22, 29, 30). Generally, plant moisture status was not determined, but in those cases where soil water potential was measured, one study showed a decrease (22) whereas the other showed an enhancement (13) in acetylene reduction (N_2 fixation) at low soil water potentials. Sprent (29) observed a decrease in acetylene reduction and nodule respiration when detached nodules lost 20% of their fresh weight during drying in air. In intact soybean, acetylene reduction decreased at soil water contents below "field capacity" (30), and Sprent suggested that direct effects of low water potentials on the nodules may account for the decrease in activity for acetylene reduction, probably because of an inhibition of nodule respiration (11, 29, 30).

Nitrogen fixation depends on a number of factors which must be supplied either by the host plant or by the soil. Photosynthates are required in order for nodule respiration to supply the reductant and ATP necessary for the reduction of N_2, and for the carbon chains that combine with NH_3 before it is exported by the nodules (3, 12). Since the amino acid products of N_2 fixation move to the shoot in the transpiration stream (26-28), transpiration may be required, particularly since the accumulation of NH_3 in the nodules might cause a repression of the synthesis of nitrogenase, as it does in free-living organisms (10, 25). In addition to water from the soil, N_2 must pass through the soil to the nodules, and O_2 (3, 12) and CO_2 (24) are also required for sustained fixation.

The complexity of this relationship between the soil, host, and nodule bacteroids causes a problem with interpreting the effects of low water potentials on N_2 fixation, largely because all the major aspects of the relationship have not been measured at the same time. We have developed a system that makes these measurements on undisturbed plants growing in soil that has been desiccated by withholding water. It permits an interpretation of the effects of desiccation and shows that, of the factors influencing nodule activity, nodule gas exchange is the major limitation to rates of acetylene reduction at high soil water potentials, but photosynthesis and/or transpiration are likely to limit acetylene reduction at low water potentials.

MATERIALS AND METHODS

Plant Tissue, Growth Conditions. Soybean (*Glycine max* [L.] Merr. var. Beeson) seeds were germinated for 3 days in vermiculite, and single seedlings were transplanted, after inoculation with a commercial preparation of *Rhizobium japonicum*, to a mixture of soil, peat, and perlite (2:1:1) in clay pots having a 15 cm top diameter. The soil had previously been heated to 90 C with steam for 1 hr. The seedlings were grown in a controlled environment chamber (day/night temperature, 29/24 C; day/night relative humidity, 30/90%; day/night irradiance, 0.40 cal cm^{-2} min^{-1}, cool white fluorescent; photoperiod, 16 hr). During the first 10 days after transplanting, the seedlings were watered with Hoagland's solution (18) containing nitrate every 3 days and with

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water all the other days. The small amounts of N supplied by the solution provided a source of N which enhanced nodulation and N_2 fixation (16, 17). After 10 days, plants were watered once daily using N-free nutrient solution (18) or water on alternate days. During the growing period, all lateral branches were excised. Plants were used for experiments when they were 4 to 5 weeks old and had shoots 40 to 45 cm tall.

CO_2 **Exchange.** Rates of net photosynthesis and dark respiration were determined twice daily (1000–1200 hr and 1500–1700 hr, CST) for whole shoots of intact plants with an IR gas analyzer and assimilation chamber (7). The construction of the semiclosed compensating gas exchange system was similar to that described earlier (2) and provided the following conditions: leaf temperature at mid-shoot was 25 ± 0.25 C; chamber air temperature was ±1 C of leaf temperature; relative humidity was 58.5 ± 0.5%; wind speed was 1.5 m·sec^{-1}; CO_2 concentration was 300 ± 3 $\mu l \cdot l^{-1}$. Light from seven 300-w incandescent spot lamps was filtered through IR-absorbing glass and 10 cm of water and provided an irradiance of 1.6 cal cm^{-2} min^{-1} at leaf height, which saturated photosynthesis. The rate of net photosynthesis was determined at approximately 30-min intervals by injecting a small amount of additional CO_2, then inactivating the CO_2 control system and measuring the time required for the shoot to decrease the CO_2 concentration in the assimilation chamber from 320 $\mu l \cdot l^{-1}$ to 280 $\mu l \cdot l^{-1}$. The rate of dark respiration was measured similarly but as the time required for an increase in CO_2 concentration from 280 $\mu l \cdot l^{-1}$ to 320 $\mu l \cdot l^{-1}$ in the dark.

Water Vapor Exchange. The rate of transpiration was determined in the same shoots in the assimilation chamber in a fashion similar to that for CO_2 exchange. About every 30 min, immediately before a measurement of net photosynthesis, the humidity was decreased slightly, the control system was then inactivated, and the time was measured for a rise in relative humidity from 57.5 to 58.5%. The system had previously been calibrated by determining the time for the weight loss of wet sponges. The time required for the rise in relative humidity gave plant transpiration in terms of the mass of water lost by the shoot.

Acetylene Reduction. Acetylene reduction was used to assay N_2-fixing activity by the soil-plant system. A chamber was designed that would enclose the undisturbed pot-soil-root system and that would permit acetylene reduction to be determined simultaneously with the measurement of CO_2 exchange and transpiration by the shoot (Fig. 1). A Plexiglas lid was sealed to a 7.2-liter polyethylene chamber with vacuum grease and clamps. The stem was inserted through a split rubber stopper in the lid and sealed in place with an inert sealing compound. Soil temperature was measured with a thermometer which also extended through the lid into the soil. Temperature was 25 ± 0.5 C for all experiments. A serum cap in the side of the chamber permitted the injection of acetylene and withdrawal of gas samples. For a determination of acetylene reduction, acetylene equivalent to 10% of the net air volume of the chamber was injected (14, 21). The net air volume was the air volume outside the pot-soil-root system plus the air-filled pore volume of the soil. The air mixture was stirred by a small fan running at a slow speed. The excess gas volume produced by the injection of acetylene was collected in two gas-receiving plastic bags on either side of the chamber. The two bags were squeezed several times to mix all the gas before they were clamped shut. Gas samples (0.5 ml) were withdrawn every 30 min and chromatographed immediately. Acetylene and ethylene were separated with a gas chromatograph having a hydrogen ionization detector at an oven temperature of 65 C. A glass column 0.8 m long and 3 mm i.d. packed with Porapak R was used for the separation. The carrier gas was N_2 flowing at 30 ml/min.

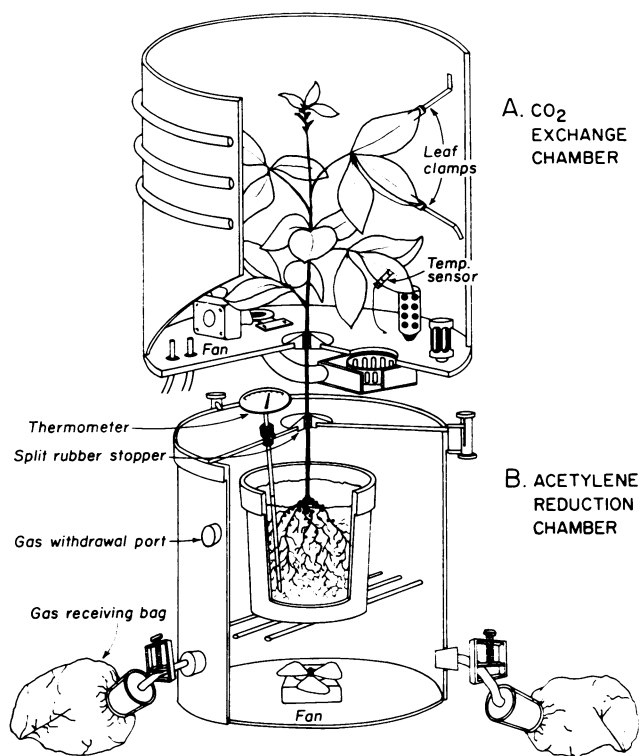**Water Potentials.** Immediately following measurements of

FIG. 1. CO_2 exchange (A) and acetylene reduction (B) chambers. All the leaves were immobilized with leaf clamps and were held perpendicular to the incoming radiation.

shoot and nodule activities, leaf water potentials were determined with thermocouple psychrometers which required excised leaf samples (4). A lower leaf was sampled by removing a disc from interveinal tissue that had been washed with distilled H_2O and permitted to dry before the measurements of gas exchange. The isopiestic technique was used (4), psychrometer chambers were coated with melted and resolidified petrolatum (5), and measurements were corrected for heat of respiration (1). After the leaf disc had been obtained, the plant was rapidly removed from the gas exchange and acetylene reduction apparatus and a soil sample was taken from the center of the pot with a 0.5-cm cork borer. The cork borer was then inserted into a humid chamber where the soil was loaded into a psychrometer chamber for an isopiestic measurement (4) of soil water potential. The plant was then returned to the constant environment chamber.

In a separate group of plants, the same procedure was followed, except that the plant was destroyed in order to obtain nodule water potentials after sampling for leaf and soil water potentials. The plant was rapidly removed from the pot to a large humid box, the soil was loosened, and the nodules were detached by using forceps. Before they were placed in a psychrometer chamber, they were blotted with tissue paper to remove soil particles adhering to the surface of the nodules.

RESULTS

To test the apparatus, the reduction of acetylene was followed in soybean having a leaf water potential of −4.4 bars (Fig. 2A), and another having a leaf water potential of −18.2 bars (Fig. 2B). When 10% acetylene was present, reduction occurred and was linear for at least 5 hr. Since rates during the first 30 min were sometimes different from the steady rates thereafter (see insets, Fig. 2, A and B), the acetylene reduced between 60 and 120 min was adopted as the assay. When 0.001% ethylene was injected into the chamber in the absence of acetylene, no change in con-
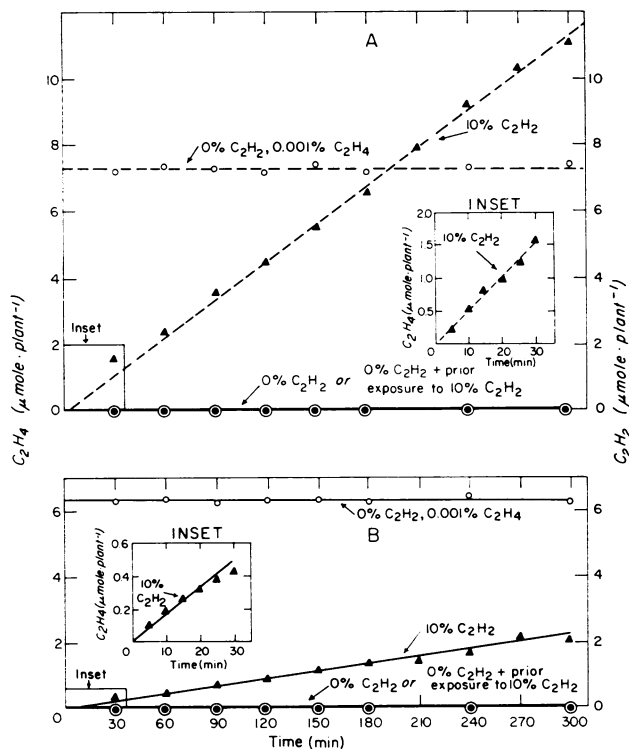
FIG. 2. Ethylene production by intact plants having the pot-soil-root system in an acetylene reduction chamber. A: Plant with leaf water potential of −4.4 bars; B: plant with leaf water potential of −18.2 bars. Insets represent ethylene production during first 30 min of 300 min time course. The prior exposure to C_2H_2 in one of the treatments was for 6 hr, and 17 hr elapsed before measurements began at 0% C_2H_2.

tentials, as water was withheld from the plants. Figure 4 shows that nodule water potentials followed soil water potentials but remained about 2 bars lower. Leaf water potentials became progressively more negative than soil water potentials until the soil became quite dry (soil water potentials = −13 bars), after which leaf water potentials began to approach soil water potentials. Figure 4 also shows leaf and soil data from experiments conducted later in this work. Their confluence with the data from the plants that were destroyed suggests that the potential relationships could be applied to other plants, provided they were maintained
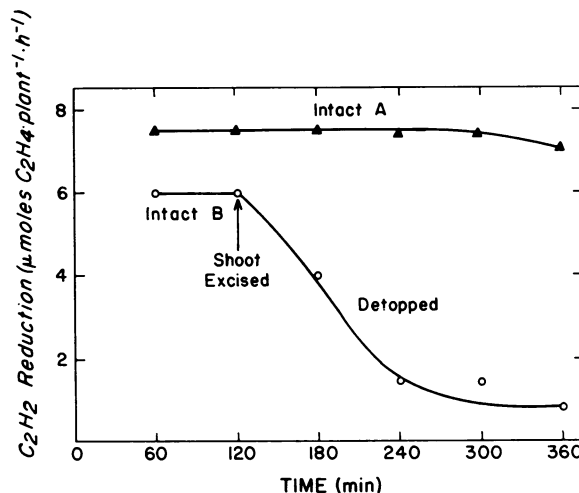

FIG. 3. Rates of acetylene reduction in well watered soybean plants. Intact plant (A) was illuminated at an irradiance of 1.6 cal cm^{-2} min^{-1} for 6 hr. Intact plant (B) was supplied with the same irradiance for 2 hr, after which its shoot was excised at the base of the stem.

centration occurred over a period of several hours, which suggested that the polyethylene containers and pot-soil-root systems neither gave off nor adsorbed appreciable amounts of ethylene (Fig. 2, A and B). There was little endogenous ethylene produced by the assay system in the absence of acetylene and ethylene, and exposure to acetylene left no residue, as measured by ethylene production, after 17 hr (Fig. 2, A and B). Desiccation of the plant had no effect on the linearity of the assay or on the endogenous production of ethylene, but it caused a decrease in the rate of acetylene reduction (Fig. 2, A and B).

To test if acetylene reduction by the nodules was affected by the shoot of the host plant, acetylene reduction by an intact plant and a detopped plant were compared. Figure 3 shows that acetylene reduction was constant when the host plant was exposed to an irradiance of 1.6 cal cm^{-2} min^{-1}. However, 2 hr after detopping the host plant, the rate of acetylene reduction declined to about 23% of that of the intact system. Repeated experiments showed that the time lag between detopping and the first detectable effects on acetylene reduction varied between 30 to 60 min in our apparatus. Acetylene reduction was intimately dependent on the presence of the shoot, and the following experiments, which extended over a period of days, should have reflected the effects of shoot activities as well as the direct response of the nodules to the treatment conditions.

It would be desirable to know nodule water potentials as well as leaf and soil water potentials in the following experiments. However, plants would have to be destroyed to obtain nodule water potentials, preventing subsequent experimentation with those particular plants. To circumvent this problem, several plants were placed under the experimental conditions for 6 hr and were destroyed to measure soil, nodule, and leaf water po-
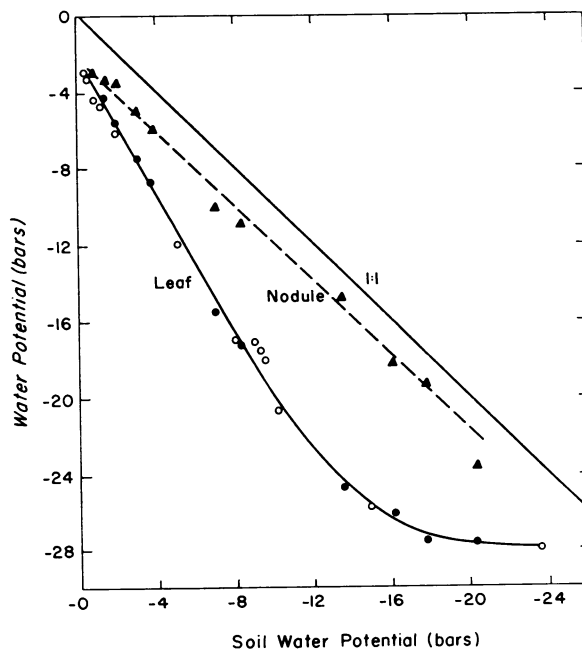

FIG. 4. Effect of soil water potential on leaf water potentials and nodule water potentials of soybean plants that had been exposed to the conditions for measurement of photosynthesis, transpiration, and acetylene reduction for at least 6 hr. Leaf water potentials obtained from those plants utilized for daily measurements are shown by (O). Leaf water potentials taken from a separate group of plants are shown as (●). These latter plants were destroyed for determining the nodule water potentials (▲).

under identical environmental conditions. Therefore, the relationships of Figure 4 were used to estimate nodule water potentials in subsequent experiments under the same conditions, where leaf and soil water potentials were known but the plants could not be destroyed.

Figure 5 shows that net photosynthesis, transpiration, and dark respiration by the shoot decreased as soil, nodule, and leaf water potentials decreased. Acetylene reduction first increased, then decreased. The decrease in acetylene reduction was correlated with the change in net photosynthesis, transpiration, and dark respiration. However, although net photosynthesis, transpiration, and acetylene reduction ultimately became negligible, dark respiration by the shoot did not and retained about half its original activity even at the lowest leaf water potentials. The initial rise in rates of acetylene reduction was reproducible, but was not related to changes in photosynthesis, respiration, or transpiration. Although the soil water potential was -1 bar under the wettest conditions (Fig. 5), it was determined primarily by the osmotic component, which was -0.8 bars (measured with the thermocouple psychrometer on water draining from the soil). The matric component could be calculated by difference and was -0.2 bars, which is close to that expected for a well watered soil that has been allowed to drain freely ("field capacity"). Consequently, a considerable amount of H_2O should have been available to the nodules, but this should also have been accompanied by a considerable amount of air-filled pore space (we measured 164 cm³, or 22% of the total pore space of the soil when air-dried, in this instance).

To test whether the amount of H_2O available to the nodules was insufficient to maintain high rates of acetylene reduction, additional H_2O was added to the soil of a well watered plant. Figure 6 shows that flooding further reduced the rate of acetylene reduction by the nodules. The inhibition was reversible if the
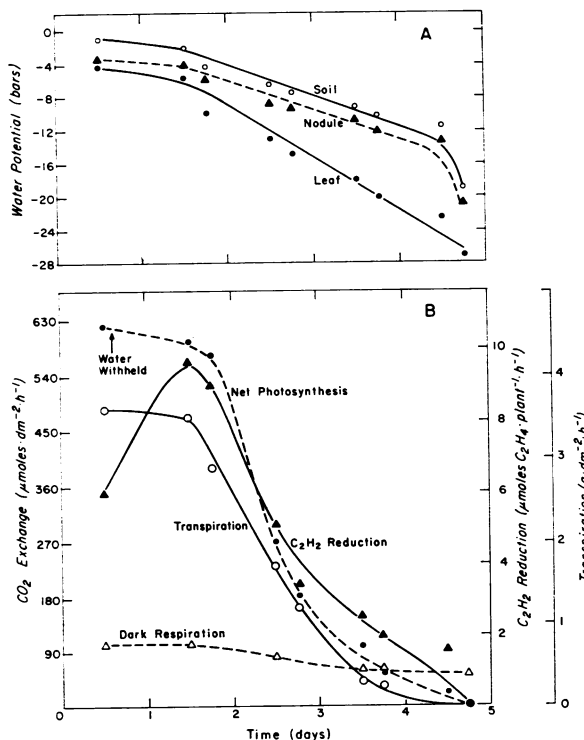

FIG. 5. Transpiration, CO_2 exchange, and acetylene reduction of a soybean plant at various times after water was withheld from the soil. A: Soil, nodule and leaf water potentials (nodule water potentials were determined from the relationship of Fig. 4); B: net photosynthesis, transpiration, acetylene reduction, and dark respiration of the shoot.
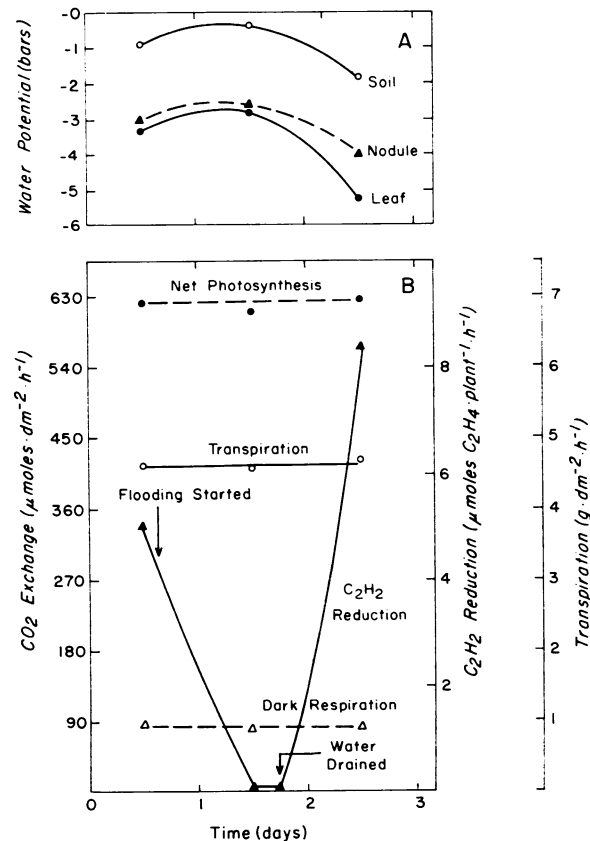
FIG. 6. Effects of flooding on CO_2 exchange, transpiration, and acetylene reduction. After the measurements of the first day, the pot-root-soil system was immersed in water for 24 hr under growth conditions. Measurements of CO_2 exchange, transpiration, and acetylene reduction were then repeated while the soil remained flooded. Following these measurements, the excess water was drained from the soil, the plant was returned to the growth conditions, and the measurements were repeated the next day. A: Soil, nodule, and leaf water potentials (nodule water potentials were determined from the relationship of Fig. 4); B: net photosynthesis, transpiration, acetylene reduction, and dark respiration of the shoot.

excess H_2O was drained from the soil. There was no effect of flooding on photosynthesis, dark respiration, or transpiration. This result suggested that additional H_2O could not overcome the initial inhibition of acetylene reduction shown in Figure 5, and indeed it worsened the problem. It appeared that gas exchange in the pore space of well watered soil may have controlled the rate of acetylene reduction by the nodules.

To test whether gas exchange in the air-filled pore space of well watered soil limited acetylene reduction, the soil was gently washed from root systems, and the rate of acetylene reduction was measured before and after removal of the soil. This treatment should have removed any restriction in gas exchange imposed on the nodules by the soil. Acetylene reduction was increased by a factor of 1.6 to 1.7× after the soil had been removed (data not shown). This compares with an increase of 1.6× when the nodules and soil had been desiccated initially *in situ* (Fig. 5). Therefore, the initial inhibition of acetylene reduction in well watered soil (Fig. 5) can be attributed to inadequate rates of gas diffusion in the available pore volume of the soil.

Although photosynthesis and transpiration are known to continue at fairly rapid rates throughout the life cycle of plants, acetylene reduction frequently changes markedly. Soon after germination, little fixation can be detected. It then reaches a maximum but declines during the reproductive phase of the life
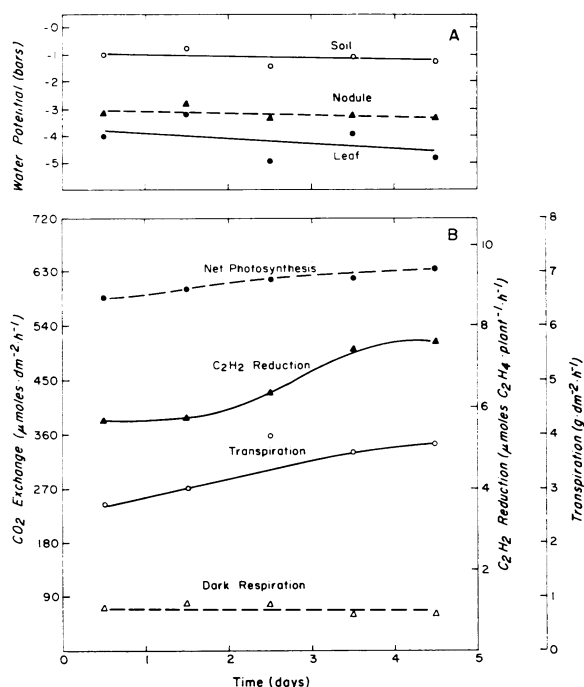
FIG. 7. Transpiration, CO₂ exchange, and acetylene reduction of a soybean plant maintained in well watered soil throughout the time shown in Fig. 5. A: Soil, nodule, and leaf water potentials (nodule water potentials were determined from the relationship of Fig. 4); B: net photosynthesis, transpiration, acetylene reduction, and dark respiration of the shoot.

cycle (14–16). To test whether the rise and decline in acetylene reduction observed here (Fig. 5) could be caused by a similar developmental phenomenon, the measurements were repeated, but H₂O was not withheld from the soil. Figure 7 shows that acetylene reduction increased somewhat during the last half of the treatment period. The increase came at a time when acetylene reduction decreased during desiccation (Fig. 5). The constant rates in the first 2 days also could not account for the initial increase in acetylene reduction during desiccation (Fig. 5).

Since the lag in the response of acetylene reduction to changes in shoot activity was on the order of 2 to 3 hr (Fig. 3), and the desiccation experiments extended over several days, nodule activities should have reflected any influence of shoot transpiration and CO₂ exchange on the process. It therefore was possible to compare directly the sensitivity of acetylene reduction and shoot activities as a function of soil, nodule, and leaf water potentials without the complicating effects of possible time differences between nodule and shoot responses to desiccation. Figure 8 shows that net photosynthesis, transpiration, and acetylene reduction were correlated with each other when the initial stages of desiccation are ignored and only those water potentials are considered that give maximum rates of acetylene reduction or desiccation-inhibited rates of acetylene reduction (leaf water potentials between −5.4 and −27.6 bars). There was relatively little effect of leaf water potentials between −5.5 and −10.5 bars on net photosynthesis, transpiration, or acetylene reduction. At leaf water potentials of −13 bars, however, net photosynthesis was 53%, transpiration 57%, and acetylene reduction 56% of the maximum rates (Fig. 8). Symptoms of leaf wilting first appeared at leaf water potentials of about −13 bars. The response of net photosynthesis and transpiration was similar to that described by Boyer (6) for soybean var. Harosoy, except that at leaf water potentials of −25 bars, photosynthesis became negligible in Beeson but remained about 20% of the maximum in Harosoy.

DISCUSSION

Of the studies of N₂ fixation that have dealt with the effects of water deficits, most have concerned N₂ fixation itself and have not included other aspects of the Rhizobium-host relationship. Sprent (29, 30) used detached soybean nodules, which precluded the additional measurements, whereas others (13, 23) have enclosed whole plants for measurements of acetylene reduction. There is a problem with enclosing whole plants for repeated measurements of acetylene reduction, because ethylene, the product of the reduction, may cause side effects such as leaf aging and abscission (20), or interactions with auxin transport and metabolism (8). Advantages of our apparatus were that the possible side effects of ethylene on the shoots were eliminated, the soil-root system was undisturbed, and simultaneous measurements could be made of the metabolic activities of the shoot. The acetylene reduction system was small (7.2 liters), and simple to operate. The plastic bags used to collect excess gas kept the system virtually at atmospheric pressure throughout the determinations. Therefore evacuation of air in the chamber was not required before acetylene was added to the system and the bags could be sealed after acetylene reduction commenced, so that the acetylene reduction chamber had a constant volume. Acetylene reduction was linear for a long period in this system and other effects such as adsorption of ethylene, endogenous ethylene production, and pretreatment effects were virtually undetectable. Furthermore, these conditions were met regardless of the degree of desiccation of the soil.

Nevertheless, in spite of these advantages and the fact that acetylene reduction measures nitrogenase activity, the assay does not completely reflect N₂ fixation. Ethylene, the product of acetylene reduction, diffuses away from the nodules rather than
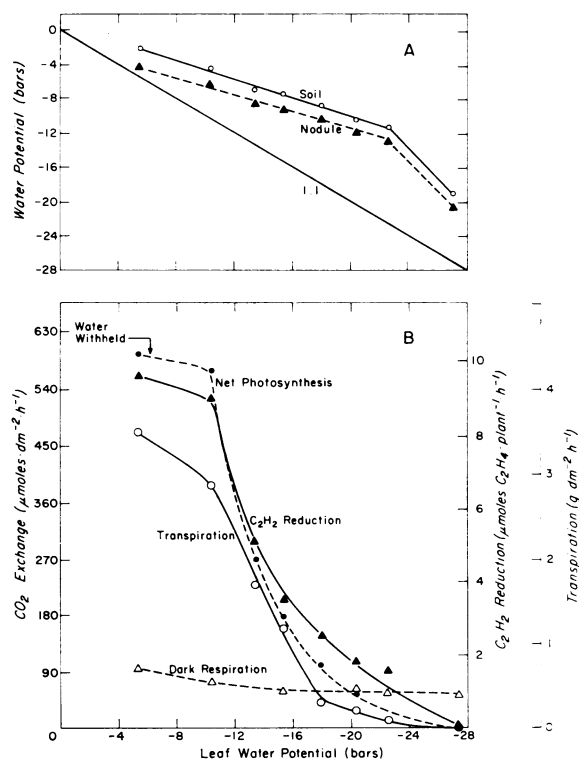

FIG. 8. Transpiration, CO₂ exchange, and acetylene reduction of a soybean plant at various leaf water potentials. Data are shown only for those leaf water potentials that permitted maximum or desiccation-inhibited rates of acetylene reduction. A: Soil and nodule water potentials (nodule water potentials were determined from the relationship of Fig. 4); B: net photosynthesis, transpiration, acetylene reduction, and dark respiration of the shoot.

combining with carbon compounds as NH_3 does. Hence, it reflects the availability of reductant and ATP, but not changes in the availability of the carbon skeletons that combine with NH_3. It is probable, however, that the effects of desiccation on N_2 fixation would be at least as severe as those for acetylene reduction, since both require reductant and ATP (3, 12).

Gas diffusion appeared to limit acetylene reduction until soil water potentials decreased to -2 bars (matric potentials of -0.4 bar). Acetylene reduction increased with desiccation until this soil water potential was reached, but decreased markedly thereafter. Thus, acetylene reduction displayed a sharp optimum which was controlled by the simultaneous requirements for rapid gas exchange and adequate water availability. Shoot activities did not display a similar optimum. Either aeration and water availability were adequate for the roots of the host plant over a wide range of soil water potentials, or root activities had little influence on the activities of the shoot.

Although improved rates of gas diffusion can explain the rise in rates of acetylene reduction in the early phases of soil desiccation, they cannot account for the inhibition in soil having water potentials lower than -2 bars. Desiccation to soil water potentials of -19.5 bars eliminated acetylene reduction. Rates of gas diffusion would have improved as desiccation took place, and therefore the inhibitory effect of soil desiccation must have been caused either by inhibitory effects of a gas or some more direct effect of desiccation on the nodules or host plant or both. Oxygen has been shown to be inhibitory at high concentrations (3, 9, 12). However, the highest O_2 partial pressure in the root zone was 0.19 bar and the inhibitory effects of O_2 have only been demonstrated above that concentration (3, 9, 12). Of the other gases, increased acetylene (N_2) and CO_2 should have enhanced the rate. Thus, inhibitory effects of soil gases are unlikely to account for the inhibitory effects of desiccation below soil water potentials of -2 bars.

Since it is likely that more direct effects on the Rhizobium-host relationship are involved, there are several possibilities that might account for the results. First, there could be direct effects of the low soil water potentials on the activity of the nodules, perhaps mediated by decreases in nodule respiration (11, 29, 30). Second, the decrease in photosynthesis and transpiration (19) could affect N_2 fixation, either because of a decrease in reductant, ATP, or carbon compounds, or a decrease in the rate at which amino acids are transported away from the nodules in the transpiration stream (26-28).

Of these possibilities, effects on dark respiration are the least likely to account for the inhibition of acetylene reduction. Dark respiration continued in the shoot at water potentials that completely inhibited acetylene reduction. Although these measurements were made on shoots, similar effects probably occurred in the roots which would have been at higher water potentials than the shoots. This result is supported by the data of Sprent (29), who showed that nodule respiration decreased only 40% when acetylene reduction decreased 95% due to desiccation in soybean. Our results indicate that dark respiration of the shoot decreased 48% when acetylene reduction decreased 95% (Fig. 5). Of course, there is the possibility that dark respiration could not supply the intermediates for acetylene reduction in spite of reasonable rates of respiration. This possibility could not be tested in the present study.

While respiration of the nodules probably was not involved, other direct effects on the nodules or indirect effects of photosynthesis or transpiration might explain the inhibition of acetylene reduction at low water potentials. The correlation between transpiration, photosynthesis, and acetylene reduction favors but does not prove that acetylene reduction was limited by one or the other.

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