The role of oxygen diffusion from the shoots and nodule roots in nitrogen fixation by root nodules of *Myrica gale*

JOHN TJEPKEMA
Harvard Forest, Harvard University, Petersham, MA, U.S.A. 01366
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Nitrogenase activity (acetylene reduction) and oxygen uptake by root nodules of *Myrica gale* L. were measured before and after removal of nodule roots. There was no significant effect of nodule root removal when respiration was measured in the gas phase (0.05–0.2 atm pO_2 (1 atm = 101.325 kPa)) or acetylene reduction in a stirred aqueous phase at 0.2 atm pO_2. However, when acetylene reduction was measured in 0.05 atm pO_2 in an unstirred aqueous phase, there was a 66 to 76% reduction in activity. These results indicate that nodule roots are important for oxygen uptake when the nodules are present in an aqueous phase at low pO_2, which is probably the normal environmental conditions for many of the nodules. Other measurements showed that diffusion of oxygen from the shoot to the root nodules is not important for nitrogen fixation. These measurements were done on whole plants with the shoots in air (0.20 atm pO_2) and the roots in water at the desired pO_2 value. With 0.0 atm pO_2 in the root environment, the rate of acetylene reduction was only 4% of the rate at 0.2 atm pO_2. Thus, only small amounts of oxygen are transported from the shoot to the nodules.

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L'auteur a mesuré l'activité de la nitrogénase (par réduction de l'acétylène) et l'absorption d'oxygène par les nodules racinaires du *Myrica gale* L. avant et après l'ablation des racines nodulaires. L'ablation des racines nodulaires ne provoque aucun effet significatif sur la respiration mesurée en phase gazeuse (0.05–0.2 atm pO_2 (1 atm = 101.325 kPa)) ni sur la réduction de l'acétylène mesurée à 0.2 atm pO_2 en phase aqueuse agitée. Cependant, il y a une réduction de l'activité de 66 à 76% lorsque la réduction de l'acétylène est mesurée à 0.5 atm pO_2 en phase aqueuse non-agitée. Ces résultats montrent que les racines nodulaires sont importantes pour l'absorption d'oxygène lorsque les nodules se trouvent en phase aqueuse à une faible pression d'O_2, ce qui est probablement le milieu normal pour plusieurs des nodules. D'autres mesures montrent que la diffusion d'oxygène de la tige vers les nodules racinaires n'est pas importante pour la fixation d'azote. Ces mesures ont été prises sur des plantes entières avec les tiges dans l'air (0.20 atm pO_2) et les racines dans l'eau à la valeur désirée de pO_2. A 0.0 atm pO_2 dans l'environnement racinaire, le taux de réduction de l'acétylène est seulement de 4% du taux à 0.2 atm pO_2. Par conséquent, seules de faibles quantités d'oxygène sont transportées de la tige vers les nodules.

[Traduit par le journal]

Introduction

Myrica gale L. is a dominant plant in some wetlands in Canada, northern United States, and northern Europe (1, 2, 3). These soils are often deficient in nitrogen and the ability of *Myrica gale* L. to fix nitrogen in its root nodules is probably an important factor in its ability to grow in these habitats (4). However, nitrogen fixation by the nodules requires oxygen (5), and the soils in which *Myrica gale* L. grows are usually very wet or submerged. Such soils are often low in oxygen content and the source of oxygen for nitrogen fixation is not clear.

Several possibilities exist. One is that the nodules absorb oxygen only through the nodule surfaces and are thus restricted to environments of relatively high oxygen concentration. Another is that oxygen diffuses from the shoot to the roots and nodules, and evidence has been presented that this can occur in *Myrica gale* L., although only non-nodulated roots were investigated (6). The most interesting possibility is the suggestion by Bond that oxygen is absorbed by the nodule roots, which are specialized roots that grow upward out of the nodule lobes (7, 8). Nodule roots contain very large air spaces (7, 8) which could serve as diffusion pathways to the nodule interior for oxygen absorbed at the surface of the nodule root. Bond has also found that the rate of nitrogen fixation is decreased when the nodule roots are removed (5). However, there have been no experiments which directly show that the nodule roots affect oxygen uptake by *Myrica gale* L., nor are there any data that indicate whether oxygen from the shoot can diffuse to the root nodules. In this paper, it is shown that nodule roots enhance oxygen uptake by the

nodules at low oxygen tensions and that oxygen diffusion from the shoot to the root is not significant.

Materials and Methods

Seeds of *Myrica gale* L. were germinated in sand after cold treatment and were transferred either to water culture in foil-covered glass jars or to aeroponic tanks (9) and inoculated with nodule suspensions prepared from mature nodules taken from older, greenhouse-grown plants. Measurements were made on 8- to 10-week-old plants.

Oxygen uptake was measured volumetrically in respirometers as described previously (10). The respirometer vials had a volume of 9.0 ml and contained filter paper moistened with KOH to absorb the CO_2 evolved. The vials were placed in a constant temperature bath at 22°C, and oxygen uptake was measured by changes in the position of a drop of kerosene in the 0.6-mm bore of the capillary tubing attached to them. In Fig. 3, all measurements were made on the same nodules, with measurements being made in the sequence of 0.05, 0.12, and 0.20 atm pO_2 (1 atm = 101.325 kPa) twice before removing the nodule roots and twice afterwards. The gas mixtures were composed of O_2 and N_2. To measure the respiration rate of the roots and stems of a whole plant, the leaves were cut off and a section of rubber stopper was sealed to the junction of the root and stem with modeling clay. Respirometer vials were then slipped over the roots and shoots so that both vials were sealed by the single rubber stopper.

Nitrogen fixation was measured by the acetylene reduction method (11). Acetylene reduction by intact plants was measured in the apparatus illustrated in Fig. 1, which was placed in the growth chamber where the plants had been kept previously (16 h light, 8 h dark), at a temperature of 25°C. The nutrient solution was equilibrated with 0.1 atm acetylene and various concentrations of oxygen and nitrogen by sealing 400 ml of the gas mixture and 305 ml of nutrient solution into glass canning jars. The jars were shaken vigorously by hand for 1.0 min to equilibrate the gas and liquid phases. The nutrient solution was then poured into the assay containers with as little agitation as possible and the stopper and plant were placed in the container. After 10 min of incubation, the plant and stopper were transferred directly to a second container of nutrient solution equilibrated with acetylene at the next desired oxygen concentration. The contents of the first container were then poured into another canning jar of 705-ml volume with minimum agitation and the jar was sealed and then shaken vigorously for 1.0 min to equilibrate the ethylene between the gas phase (air) and the water phase. Gas samples were taken through rubber septa pressed into the lids of the jars and were analyzed for ethylene by gas chromatography. The bottles contained approximately 470 ml of gas phase and 235 ml of nutrient solution. From the value for the solubility of ethylene in water (12), it was calculated that 5.7% of the ethylene was dissolved in the nutrient solution at 25°C, and this was included in the calculation of the rate of acetylene reduction. Disposable 1-ml syringes were used to take gas samples and to inject them (0.5 ml) into the gas chromatograph. The gas chromatograph was a Carle model 9500 with a flame ionization detector. The column was 180 cm × 1.7 mm ID, filled with 50- to 80-mesh Porapak T, and was kept at 75°C.

To measure acetylene reduction in the gas phase, the whole plants were placed in a 140-ml flask with the desired gas mixture (including 0.1 atm pC_2H_2) and gas samples were taken and analyzed for C_2H_4 every 10 min until a constant rate was established (about 30 min). Then the next gas mixture was flushed through the flask and the rate of acetylene reduction was again measured until a constant rate was found.

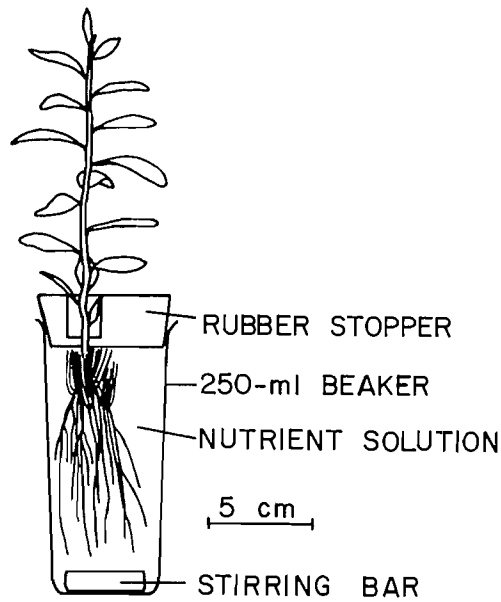

FIG. 1. Apparatus for measuring acetylene reduction with roots in a nutrient solution.

Results

The results of Table 1 indicate that the stems of *Myrica gale* L. (with leaves removed) are capable of supplying the roots with oxygen for only a small part of their respiration. In the first measurement, the respiration of both stems and roots were measured in 0.2 atm pO_2. The roots had a higher respiration rate than the stems, indicating that at atmospheric oxygen concentrations, they are capable of absorbing much or all of their oxygen requirement through their surfaces rather than by diffusion from the stems. In the second measurement, the stems remained at 0.2 atm pO_2, but root respiration was measured in 0.0 atm pO_2. No oxygen uptake should have been observed in the root respirometers, but the small amount that did occur may have been due to failure to flush all oxygen from the respirometers. The apparent oxygen uptake may also have been due to transport of N_2 from the root respirometer to the shoot respirometer in response to the pN_2 gradient. If high rates of oxygen diffusion from the stems to the roots were possible, one would have expected a large increase in oxygen uptake by the stems in response to lowering the pO_2 in the root respirometers. This did not occur at all in experiment 1 and only to a small degree in experiments 2 and 3. From this we conclude that roots of *Myrica gale* L. growing in anaerobic soils are not able to maintain high aerobic respiration rates.

The results of Fig. 2 show that nitrogen fixation

TABLE 1. Effect of oxygen partial pressure on respiration by stems and nodulated roots. Measurements on whole plants, with roots and stems (leaves removed) sealed into separate vials

Organ	pO_2, atm*	Respiration rate, μmol O_2 h^{-1} Experiment 1	Experiment 2	Experiment 3
		First measurement		
Stem	0.2	1.1	0.5	1.7
Roots	0.2	4.3	4.2	16.6
		Second measurement		
Stem	0.2	1.1	1.0	1.9
Roots	0.0	0.7	0.1	0.3
		Fresh weight, g		
Stem	—	0.102	0.087	0.184
Roots	—	0.181	0.136	0.287

*1 atm = 101.325 kPa.

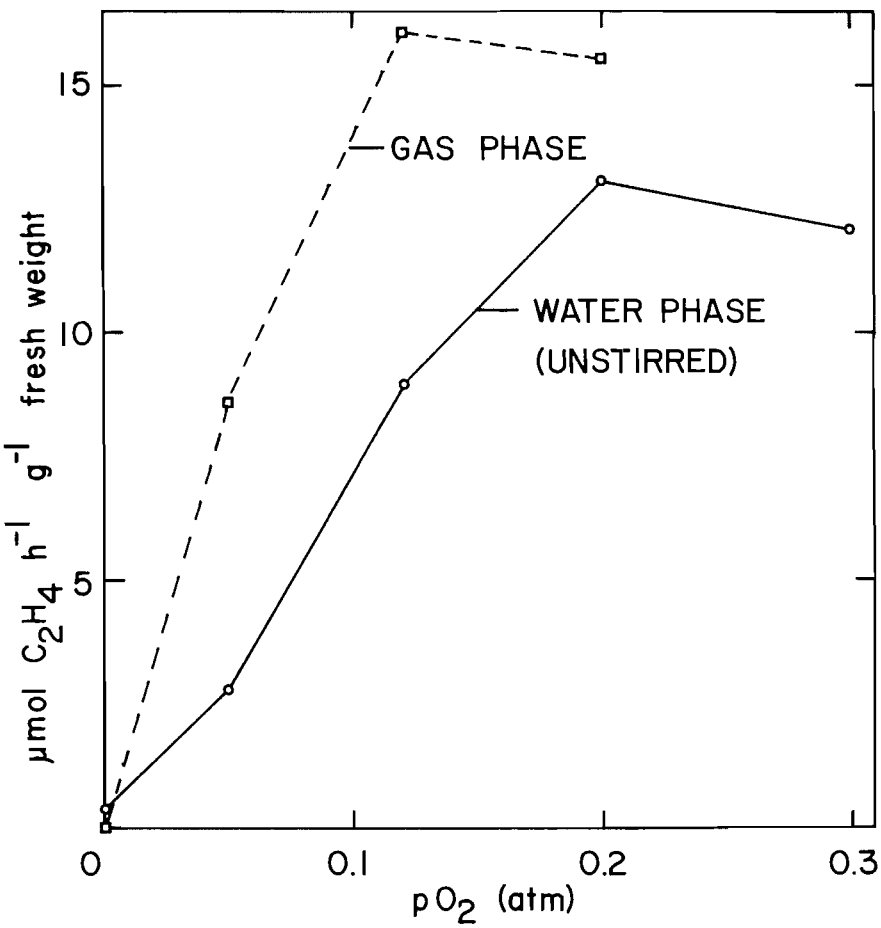

FIG. 2. Acetylene reduction by an individual plant of *Myrica gale* L. as a function of pO_2. In the first measurement (unbroken line), the shoots were in air and only the roots were in water at the indicated pO_2 values. In the second (broken line), the entire plant was in gas at the indicated pO_2 values. 1 atm = 101.325 kPa.

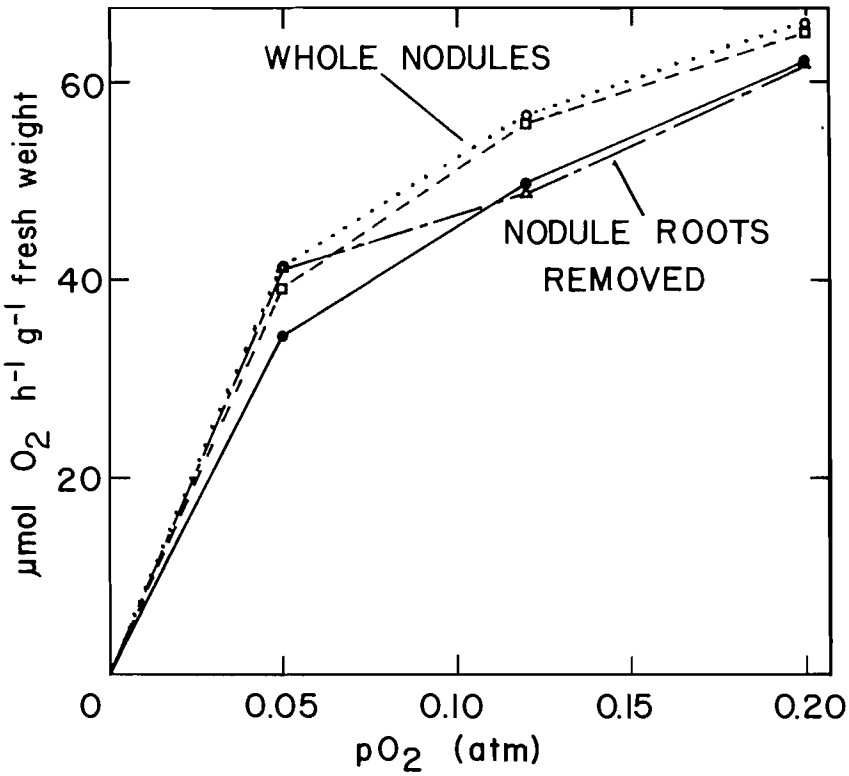

FIG. 3. Effect of the removal of nodule roots on the respiration of detached nodules in a gas phase. 1 atm = 101.325 kPa.

cannot be supported at a significant rate in the absence of oxygen in the root zone. At 0.00 atm pO_2 in the aqueous phase, the acetylene reduction rate was only 4% of the rate in 0.2 atm pO_2, and this small amount may have been due to residual oxygen in the nutrient solution. The rate of acetylene reduction was almost directly proportional to oxygen partial pressure in the range of 0.0 to 0.2 atm pO_2, which was presumably due to a corresponding increase in respiration rate that supplies the ATP required for nitrogen fixation (13). The measurements in the gas phase were made after those in the water phase, with the whole plant being placed in the same assay flask. Thus the results in the gas phase have no bearing on the possible diffusion of oxygen from the stem to the roots. In the gas phase, acetylene reduction reaches its maximum rate at 0.12 atm pO_2, rather than the value of 0.2 atm observed in the water phase. The reason for this is probably that the boundary layer of water at the nodule surface is a substantial part of the total diffusion resistance between the site of respiration and the source of oxygen. If so, the diffusion resistance would be lower when the water was absent and respiration and acetylene reduction would be oxygen saturated at lower pO_2 values in the gas phase than in the water phase.

The role of nodule roots in oxygen uptake by nodules in air was investigated in Fig. 3. The results indicate that the only effect of removal of the nodule roots is a slight reduction in respiration rate at all pO_2 values, which is probably due to the respiration of the nodule roots that were removed rather than to changes in the respiration of the nodules themselves.

However, the possibility remained that there might be an effect on oxygen uptake if the experiments were done in a water phase where the results of Fig. 2 indicate that there is greater diffusion resistance to oxygen uptake. Equipment was not available to measure oxygen uptake in an aqueous phase so the effect of removal of nodule roots on acetylene reduction rate was measured instead. The results of Fig. 4 show that at 0.05 atm pO_2, the acetylene reduction rate is dependent on the presence or absence of stirring. This supports the hypothesis that there is substantial resistance to oxygen diffusion in the water at the surface of the nodule. However, no difference in rate was observed between stirring rates of approximately 250 and 500 rpm with a magnetic stirrer. After the nodule roots were removed, there was a large decrease in acetylene reduction rate. With stirring, the rate after nodule root removal was 60% of the

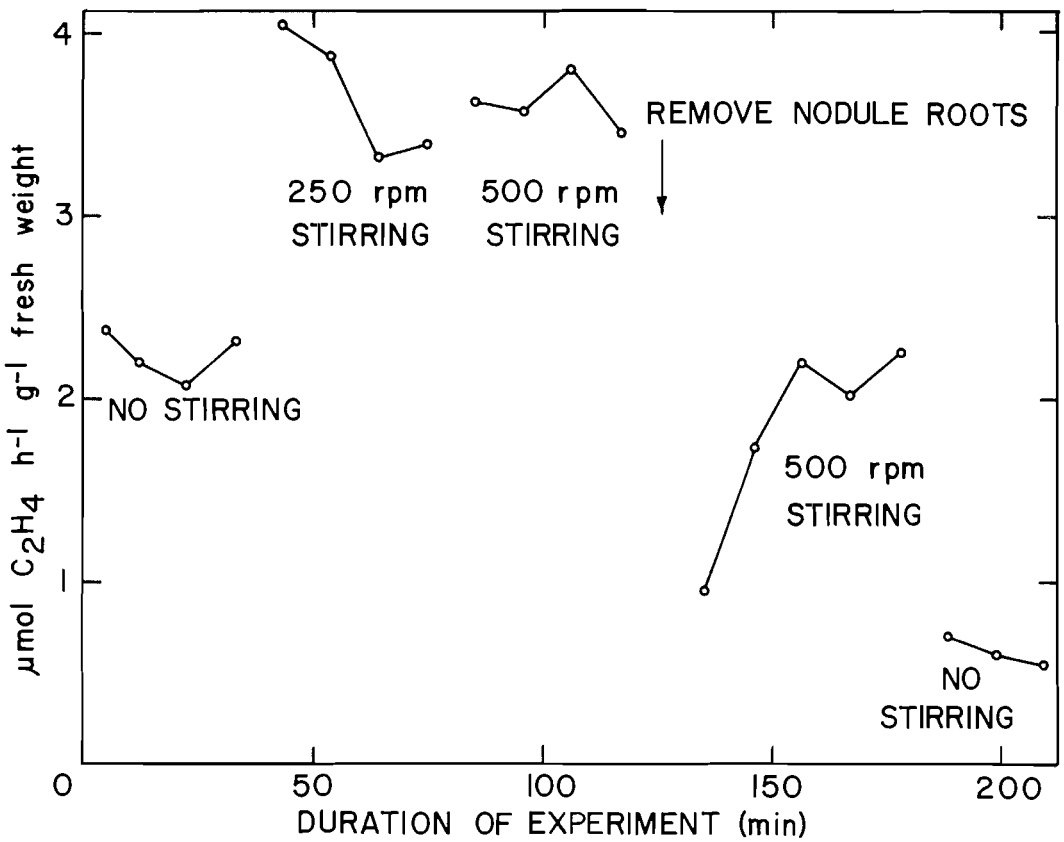

FIG. 4. Effect of stirring rate and nodule root removal on acetylene reduction by whole plants. The shoots were in air and the roots were in a nutrient solution equilibrated with 0.05 atm pO_2 and 0.1 atm pC_2H_2. 1 atm = 101.325 kPa.

original rate, while without stirring, the rate after removal was 24% of the original rate.

Figure 5 shows that there was no measurable effect of nodule root removal on acetylene reduction rate when the measurements were made in 0.2 atm pO_2 with stirring. The same result was observed in several other experiments, but as before, there was a large effect in 0.05 atm pO_2 without stirring, with the rate after nodule root removal being 34% of the original rate. Thus it appears that nodule roots are only important for oxygen uptake when the nodules are immersed in water at low oxygen tensions. Figure 5 also shows that even a brief exposure of the nodules to a lower pO_2 value results in a lag of about 30 min before the original rate of acetylene reduction at 0.2 atm pO_2 is restored. However, when the pO_2 is changed from a high to a low level, the new lower rate is immediately linear with respect to time. This behavior must be allowed for in the experimental design.

Discussion

The experimental results indicate that most of the oxygen for respiration and nitrogen fixation by root nodules of *Myrica gale* L. is supplied from the medium surrounding the roots rather than from the shoots. If a large quantity of oxygen could diffuse from the shoots to the roots, one would expect substantial oxygen uptake from the shoots and acetylene reduction by the root nodules when the roots are placed under anaerobic conditions. This was not observed in Table 1 and Fig. 2, but the data do not exclude the possibility that a small amount of oxygen does diffuse from the shoots to the roots.

Since most of the uptake of oxygen by the nodules is probably through the nodule surfaces, Bond's suggestion that nodule roots serve to increase oxygen uptake and nitrogen fixation by increasing the surface area available for absorption of oxygen is very plausible (7). However, one must explain why there was no substantial effect of removing the nodule roots on oxygen uptake in the gas phase (Fig. 3) or on acetylene reduction in the water phase at 0.20 atm pO_2 with stirring (Fig. 5). For the latter, the explanation may be that respiration was saturated with an excess of oxygen so that reducing the surface area through which oxygen could be absorbed had little effect. In the gas phase

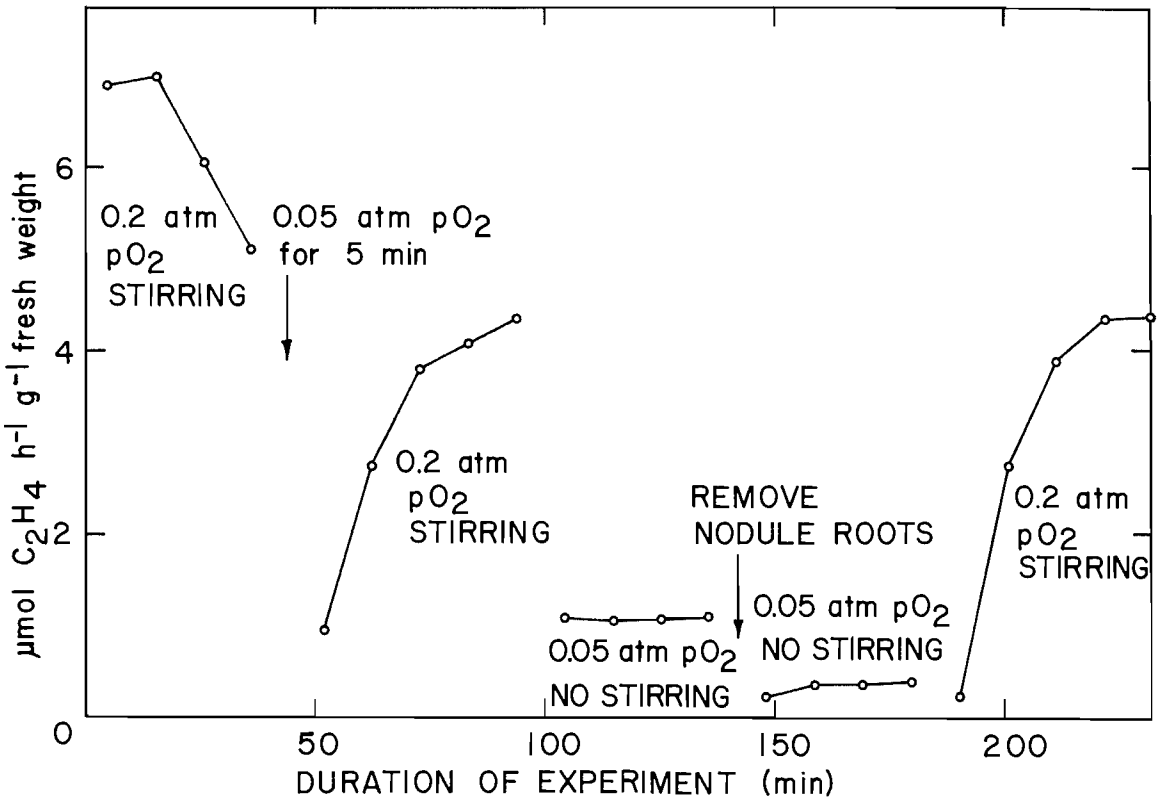

FIG. 5. Effect of pO_2, stirring, and nodule root removal on acetylene reduction by whole plants. The shoots were in air and the roots were in nutrient solutions at the indicated pO_2 values. Stirring was at 250 rpm. 1 atm = 101.325 kPa.

(Fig. 3), however, there was little effect of nodule root removal even at 0.05 atm pO_2 where respiration was unsaturated. In this case, most of the resistance to oxygen diffusion to the respiratory enzymes may have been within the nodules rather than at the surface. If so, removing the nodule roots and thus decreasing the surface area would have little effect. Then why would there be an effect of nodule root removal in the water phase? The explanation may be that as has been observed in other plant material (14), there may be a substantial boundary layer of water at the nodule surface. This boundary layer may have a high resistance to oxygen diffusion, which is large compared with the internal resistance to oxygen uptake within the nodules. If so, decreasing the surface area by cutting off the nodule roots would substantially decrease oxygen uptake and hence decrease the acetylene reduction rate. This was observed, so Bond's hypothesis (7) appears to be correct under these conditions of aqueous phase and low pO_2. Stirring the water would decrease the thickness of the boundary layer, thus decreasing the external resistance to oxygen diffusion and making the internal resistance more important. Consequently, removing the nodule roots should have a smaller effect with stirring than without, and this result was observed (Fig. 4).

Another possible function of the nodule roots might be in the uptake of N_2 for nitrogen fixation or of C_2H_2 for the acetylene reduction assay. This appears, however, to be unlikely since removing the nodule roots had no effect on nitrogenase activity when measured at 0.2 atm pO_2 (Fig. 5). Furthermore, in preliminary measurements, I have found that the rate of O_2 uptake by root nodules of *Myrica gale* is at least 10 times the rate of N_2 uptake. Thus, the rate of O_2 transport rather than N_2 transport is more likely to be limiting the rate of nitrogen fixation.

Root nodules of *Myrica gale* L. are often found submerged in waterlogged soils which are probably low in oxygen content. Under these conditions, the results of this paper show that the nodule roots would be very important in oxygen uptake. As suggested by Bond (7), the upward growth of the nodule roots, even though the length is a maximum of 4 cm, may be sufficient to give them access to the gas phase at the soil surface or at least to water of higher oxygen concentration. If so, their role in

oxygen uptake and nitrogen fixation may be even greater than that indicated by the results of Figs. 4 and 5.

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