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A Short-Term Decrease in Nitrogenase Activity (C_2H_2 Reduction) Is Induced by Exposure of Soybean Shoots to Their CO_2 Compensation Point[1]

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Photosynthesis and nitrogenase acetylene-reducing activity (ARA) were measured in soybeans (*Glycine max* [L.] Merr.) in which the shoots were exposed for 48 h to 60 μL L^{-1} CO_2, a value corresponding to their CO_2 compensation point. Six hours after the beginning of the light period at low CO_2, the ARA started to decrease, reaching a rate of 50% of the control rate in 14 to 24 h and 20% of the control rate in 34 to 38 h after the beginning of the CO_2 treatment. At these times, there was no net photosynthesis, and the transpiration rate was 20% lower than that in the control plants. An increase in the partial pressure of O_2 around the nodules alleviated this inhibition of ARA. The maximal ARA achieved at 40 kPa O_2 was 3 times higher than that at 20 kPa O_2 and similar to the maximal ARA of the control plants. It was argued that the decrease in ARA of soybean exposed to the CO_2 compensation point was due to a decrease in the nodule's permeability to O_2 diffusion.

Since N_2 fixation in legumes relies on plant carbohydrate, numerous studies have examined the effects of photosynthesis and photosynthate translocation on the ARA of legume nodules (Streeter, 1991; Vance and Heichel, 1991). In soybean (*Glycine max* [L.] Merr.), it is generally agreed that after 2 weeks at above-ambient pCO_2, there is an increase in both nitrogenase activity and nodule mass (Finn and Brun, 1982). However, high CO_2 had no short-term, i.e. within 48 h, effect on nitrogenase activity (Finn and Brun, 1982; Williams et al., 1982).

In white clover, Silsbury (1981) observed a short-term increase in nitrogenase activity when plants were transferred to high light irradiance. However, in soybean Williams and Phillips (1980) and Drevon et al. (1991) reported that exposure to light irradiance above that applied during plant cultivation decreased nitrogenase activity. Alternatively, decreasing light irradiance had no effect on nitrogenase activity within 24 h, even though this treatment caused a decrease in the *P* of soybean (Drevon et al., 1991). Another way to decrease photosynthesis is to lower the pCO_2 in the shoot environment. To our knowledge, only Williams et al. (1982) have performed such experiments, and they showed that nitrogenase activity decreased to 50% of the initial value after 5 d of exposure to 90 μL L^{-1} CO_2.

The latter result and those of most other studies of the relationships between photosynthesis and nodule function were initially interpreted as a consequence of changes in photosynthate allocation. However, it has recently been suggested that nodule activity might be O_2 limited rather than carbon limited (Drevon et al., 1988; Hunt et al., 1989; Godfroy and Drevon, 1991). This interpretation is based on the concept that cortical changes can restrict O_2 entry into the nodule (Hunt and Layzell, 1993; Witty and Minchin, 1994; Serraj et al., 1995).

The present study was performed to determine the lag before nitrogenase activity decreased after shoot exposure to low CO_2 and to test whether this decrease was due to a decrease in the permeability of the nodule to O_2 diffusion. Nodulated soybean plants were exposed for 48 h to a pCO_2 corresponding to their compensation point, and when nitrogenase activity was decreased, the pO_2 was increased in the environment of the nodulated roots to determine whether the inhibition was due to an O_2 limitation.

MATERIALS AND METHODS

Plant Material

Seeds of *Glycine max* (L.) Merr. cv Kingsoy were sterilized, inoculated with *Bradyrhizobium japonicum* strain PJ17 (Drevon et al., 1982), and sown in perlite. Six days after germination, plantlets were transferred into a climate-controlled chamber where they were grown with a 16-h photoperiod (400 μmol m^{-2} s^{-1} photon flux density, 80% RH, 25/20°C day/night temperatures). The nodulated roots of the plants were maintained in an aerated nutrient solution (Drevon et al., 1988) supplemented with 2 mM urea. The hydroponic solution was provided at a flow rate of 20 mL h^{-1} and completely replaced every week.

Plants were used 45 to 60 d after sowing, which corresponded to early- and mid-flowering stages. Their mean dry weights were 16 ± 3 g shoot $plant^{-1}$, 10 ± 3 g root

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Abbreviations: ARA, acetylene reduction activity; *P*, photosynthesis rate; pCO_2, partial pressure of CO_2; pO_2, partial pressure of O_2; *Tr*, transpiration rate.

plant^{-1}, and 0.8 ± 0.3 g nodule plant^{-1}, and their leaf area was 0.35 m^2 plant^{-1}. Their *P* values ranged from 2 to 6 mmol CO_2 consumed plant^{-1} h^{-1}, their *Tr*s were from 0.4 to 1.5 mol H_2O plant^{-1} h^{-1}, and their ARA values were from 25 to 55 μmol C_2H_2 reduced plant^{-1} h^{-1}.

Measurement of Shoot Activities

For measurement of whole-plant activities, the plants were transferred 1 d before the experiments to air-tight environmental chambers previously described in detail by Vidal et al. (1994). The shoot and root compartments were separated by an air-tight seal of putty around the plant collar and ventilated with 20 L h^{-1} compressed air. Climatic conditions for shoot environment were the same as during cultivation.

For control plants, *P* was measured in a closed system from the record, at the frequency of one record every 15 min, of pulsed CO_2 injections that were monitored by a computer to maintain the pCO_2 in the shoot environment at 340 μL L^{-1} (Gerbaud, 1990). This pCO_2 was similar to that applied during plant cultivation.

In the absence of pulsed injection of CO_2, the pCO_2 in the shoot chamber rapidly decreased until a steady pCO_2 was reached at which there was no net uptake of CO_2, which therefore corresponds to the CO_2 compensation point. In the present work, this concentration was close to 60 μL L^{-1}, i.e. higher than the values of 35 to 45 μL L^{-1} corresponding to CO_2 compensation points of individual leaves for C_3 plants (Bauer and Martha, 1981). This discrepancy was probably due to the involvement of all shoot tissues in our determination of the CO_2 compensation point.

The experiments lasted 4 d. On d 1 and 4, the shoots were exposed to 340 μL L^{-1} CO_2, whereas during the light period of d 2 and 3, the pCO_2 was at the compensation point. During the dark period, the pCO_2 was maintained at 340 μL L^{-1} for the control and at 60 μL L^{-1} for the low-CO_2 treatment by means of a CO_2 trap that could be switched into the gas stream as described previously by Gerbaud (1990).

The water vapor produced by the plant was condensed on a cooler and then collected in a container placed on an electronic balance. The weight of water was computed every 15 min and used to calculate the *Tr*.

Measurements of Nodulated-Root Activities

The nodule's nitrogenase activity was assayed by measuring the ARA of intact nodulated roots with an open-flow system as previously described by Drevon et al. (1988) for hydroponically grown plants. However, in the present study, the nodulated roots remained submerged in the nutrient solution. Climatic conditions for the nodulated-root environment were the same as during cultivation, except that temperature was maintained during day and night at 20°C. ARA measurements during day/night cycles were performed for only 20 min every 4 h as recommended by Vidal et al. (1994), who observed in similar experimental conditions that continuous exposure of nodulated roots to 10% C_2H_2 for more than 4 h decreased *P* and ARA. Gas samples were introduced into a DI 200 gas chromatograph (Delsi, Suresnes, France) by a pneumatic valve for measurements of C_2H_4 and C_2H_2 concentrations. ARA was calculated as the product of C_2H_4 concentration and the flow-rate through the root chamber. The latter was 22.2 L h^{-1}, comprising 20 L h^{-1} air and 2.2 L h^{-1} C_2H_2, and the volume of the gas phase was 0.5 L including the volume of the condenser and the space between the surface of the nutrient solution and the seal around the plant collar. In the present study, there was no apparent C_2H_2-induced decline of nitrogenase activity.

The response of nodule ARA to changes in external pO_2 was established after an initial measurement of ARA at 20 kPa O_2. Then, pO_2 in the gas stream flushing the root compartment was increased to 30 kPa O_2 in replacement of 10 kPa N_2 and maintained at this level for about 40 min. Within less than 20 min, the ARA stabilized at a steady state, during which the C_2H_4 concentration in the efflux was measured every 6 min. Then, the pO_2 in the gas mixture was increased to 40 kPa, by replacing 10 kPa N_2, and a new steady-state nitrogenase activity was attained within 20 min. The C_2H_4 concentration was again determined every 6 min during the next 20 min. This was followed by the same procedure for the determination of ARA at 50 kPa O_2 after which the pO_2 in the gas stream was returned to 20 kPa with a concomitant increase in partial pressure of N_2 to 70 kPa. The whole experiment lasted less than 4 h with continuous exposure to 10% C_2H_2.

Nodulated-root respiration was calculated from the CO_2 enrichment of outlet atmosphere measured with an IRGA CO_2 analyzer (Maihak, Hamburg, Germany).

Statistical Analyses

The data of ARA as a function of pO_2 and of ARA, nodulated-root respiration, *P*, and *Tr* as functions of time were fitted to linear or nonlinear regressions through covariance analyses. Differences between CO_2 treatments were statistically analyzed by Student's *t* test on the parameters of the regression models, as previously reported by Godfroy and Drevon (1991). For *P*, *Tr*, and respiration rate, values measured every 15 min were included in the statistical analyses. However, means and SDs are shown in the figures only for every 4th h, for similarity with the presentation of ARA.

RESULTS

Shoot Gas Exchanges

Figure 1A shows that the CO_2 assimilation of control plants during four day/night cycles in the assay chamber increased significantly, by about 11% every day. For each 16-h light period, *P* was maximal between 2 and 12 h after the beginning of the day and declined to about 80% of the day maximum at the end of the light period. Net photosynthesis was nil for plants exposed to 60 μL L^{-1} pCO_2, i.e. their CO_2 compensation point, during d 2 and 3 of the experiment (Fig. 1A). After the pCO_2 was returned to 340 μL L^{-1} at the beginning of the light period of d 4, *P* of the

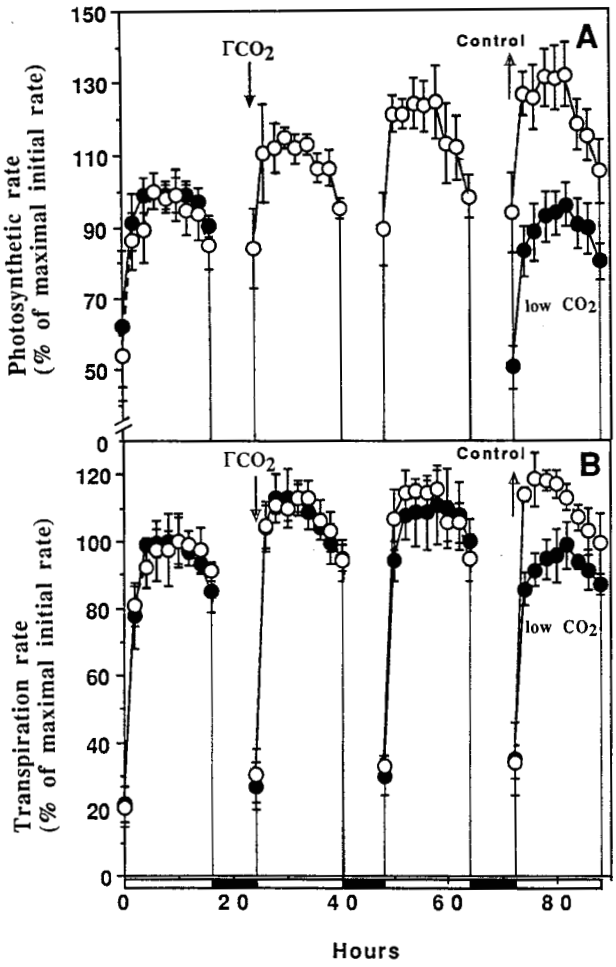

Figure 1. Changes in photosynthesis (A) and transpiration (B) during four day/night cycles of control soybean (○) and soybean exposed to the CO_2 compensation point (ΓCO_2) for 48 h (●). Results are the means ± SD of six replicates. Arrows indicate the beginning (▽) and the end (△) of the low-CO_2 treatment.

treated plants did not recover completely but reached 70% of the control *P* at the end of d 4 of the experiment (Fig. 1A).

The *Tr* of the control plants during the experiment increased by 10, 2, and 6% at d 2, 3, and 4, respectively (Fig. 1B). For each day, *Tr* was maximal between 4 and 12 h after the beginning of the light period and then decreased at the end of the light period (Fig. 1B). The day/night cycle of *Tr* of the treated plants was similar to that of the control at d 2, i.e. the 1st d with low CO_2. However at d 3, the treated plant *Tr* was significantly lower ($P < 0.01$) than that of the control, and it was only a few hours before the end of the light period that *Tr* of the treated plant reached that of the control. At d 4, when the pCO_2 was returned to 340 $\mu L\ L^{-1}$, *Tr* of the previously treated plants increased until it reached 80% of the maximum *Tr* of the control (Fig. 1B).

Nodulated-Root ARA and Respiration

According to the data shown in Figure 2A and the statistical analysis by regression models, the ARA of control plants increased significantly ($P < 0.01$) by about 10% every day during the four day/night cycles of the experiment, which shows similarities with the simultaneous increase in *P* (Fig. 1A). The maximal ARA was reached between 6 and 16 h after the beginning of the light period and then decreased by about 20% during the dark period, although the temperature of the nodulated-root chamber was maintained.

There was no significant difference in ARA between control and treated plants during d 1 or during the first 6 h of d 2 after the pCO_2 was decreased for the treated plants. Between 6 and 14 h of d 2, the ARA of the treated plants decreased drastically to about 40% of the control ARA and maintained this level between 14 and 24 h. During d 3, steady-state ARA values as low as 20% of the control ARA were observed between 34 and 38 h after the beginning of the CO_2 treatment. After the pCO_2 was returned to 340 $\mu L\ L^{-1}$ at the beginning of d 4, the ARA of the treated plants increased rapidly, although it did not recover completely, since it was only 50% of the control ARA values at the end of the light period of d 4 (Fig. 2A).

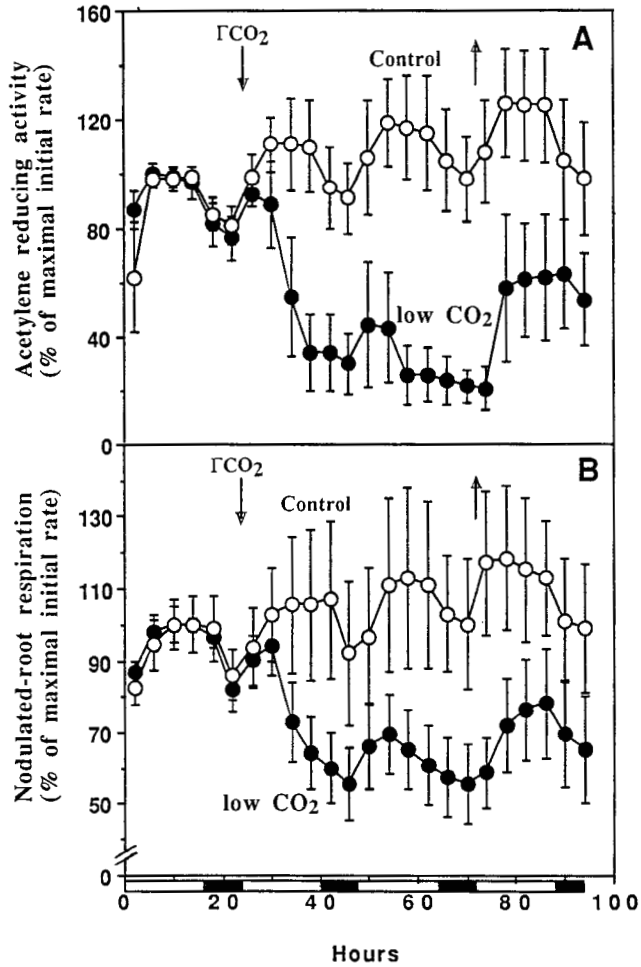

Figure 2. Changes in ARA (A) and nodulated-root respiration (B) during four day/night cycles of control soybean (○) and soybean exposed to the CO_2 compensation point (ΓCO_2) for 48 h (●). Results are the means ± SD of six replicates. Arrows indicate the beginning (▽) and the end (△) of the low-CO_2 treatment.

Nodulated-root respiration for the control plants during the four day/night cycles of the experiment increased by about 5% every day and was maximal between 6 and 16 h, which shows similarities with the day/night cycle of ARA. During d 2 and 3, nodulated-root respiration for the treated plants decreased to about 59 and 48%, respectively, of the maximal value for the control (Fig. 2B). The decrease in nodulated-root respiration paralleled that of ARA during the first hours with low CO_2, although it was more drastic between 6 and 24 h of this treatment. Moreover, nodulated-root respiration of the treated plants continued to decrease during the dark period, although nodulated-root respiration of the control plants and ARA for both treatments remained constant. At d 4, when the pCO_2 was returned to 340 μL L^{-1}, nodulated-root respiration recovered faster than ARA from the low-CO_2 treatment, since nodulated-root respiration of the previously treated plants increased to 60% of the maximal respiration of the control (Fig. 2B).

Effect of External pO_2 in the Nodulated-Root Environment

The effect of external pO_2 on ARA was tested between 34 and 38 h after the application of the CO_2 treatment, since the ARA was relatively stable during this period (Fig. 2A). The objective of increasing pO_2 was to address whether or not the decrease in ARA due to low CO_2 was caused by a decrease in nodule permeability to O_2 diffusion. If this were the case, the stimulation of ARA by increasing pO_2 in the nodulated-root environment would be greater in the treated than in the control plants and would compensate for the decrease in ARA due to low CO_2.

The typical responses of ARA to increases in pO_2 for control and treated plants are shown in Figure 3. The control plants were little affected by increasing external pO_2. In contrast, the ARA of the treated plants increased by 208% after pO_2 was increased from 20 to 30 kPa O_2. Increasing pO_2 from 30 to 40 or to 50 kPa O_2 increased ARA of these plants by 334 and 290%, respectively. After these successive incubations at increased pO_2, the return to 20 kPa O_2 induced a rapid decrease in ARA of both treated and control plants. However, a major difference was that the final level of ARA of the treated plants was similar to the initial ARA before pO_2 was increased, although the final level of ARA of the control plants was only 28% of the initial ARA.

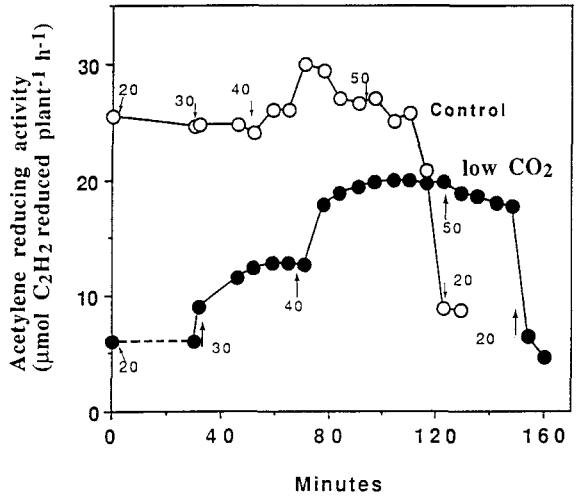

Figure 3. Time-course response of ARA to external pO_2 in the nodulated-root environment of control soybean (○) and soybean exposed to the CO_2 compensation point (●). Example of one experiment performed between 34 and 38 h after the beginning of the low-CO_2 treatment.

This experiment was repeated five times. This confirmed that the external pO_2 had no significant effect on the ARA of control plants and that ARA of the treated plants was stimulated significantly by increasing the pO_2 in their nodulated-root environment (Fig. 4). The maximum stimulation of ARA by pO_2 was observed with 40 kPa O_2, for which the ARA was 300% of the initial ARA and equal to the maximum ARA of the control plant. Therefore, the increase in pO_2 compensated for the inhibition of the ARA associated with the low CO_2 concentration in the shoot environment.

DISCUSSION

Our data show that exposure of soybean shoots to a low CO_2 concentration was associated with a decrease in the in vivo nitrogenase activity (ARA) as soon as 6 h after the beginning of the treatment. After 2 d under low CO_2, ARA and nodulated-root respiration of the treated plants were 20 and 50%, respectively, of the control values.

In this study, there was no evidence of a C_2H_2-induced decline of nitrogenase activity as previously described by Minchin et al. (1986). In other experiments in which a gas-exchange system was used with a lower time constant and nodules that were not submerged, a C_2H_2-induced decline was found for similar hydroponically grown symbiotic pairs (Ribet and Drevon, 1993): a minimum ARA was reached after 40 min and was 70% of the apparent maximum ARA, and this percentage was lower for symbiotic pairs with higher permeability associated with *P* deficiency (Ribet and Drevon, 1993). However, in the present study, the relatively high time constant of 1.35 min and the submersion of nodules might have prevented the resolution of C_2H_2-induced decline during the first few minutes of the assay. Another possibility is that the nodule permeability of the hydroponically grown soybean was lower in this experiment than in others. This would explain the relatively low ARA per nodule mass of approximately 50 μmol C_2H_4 g^{-1} nodule dry weight h^{-1} compared to levels close to 300 μmol C_2H_4 g^{-1} nodule dry weight h^{-1} that are generally reported for sand/vermiculite-grown soybeans with open-flow systems (Hunt and Layzell, 1993). Such discrepancies may be due to abundant nodulation of soybean in hydroponic cultivation and to artificial light in the chamber, which may partially limit the potential of nodule activity via a low permeability (Drevon et al., 1991). Moreover, in the present study, nodulated soybeans were grown with 2 mmol urea $week^{-1}$, in contrast to the studies of Ribet and Drevon (1993) in which this complement of N was not maintained after nodulation.

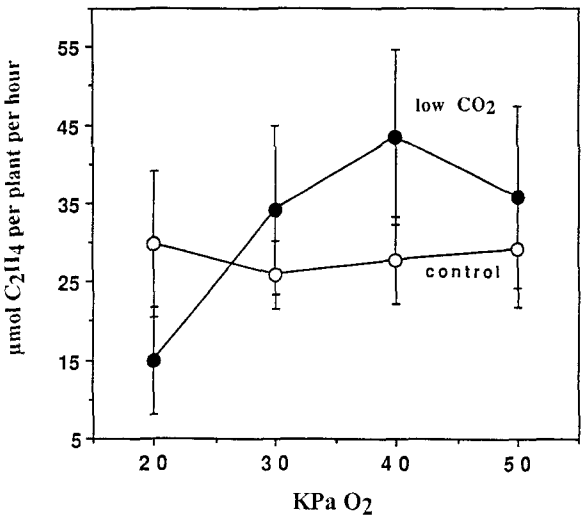

Figure 4. Effect of external pO_2 on ARA of control soybean (○) and soybean exposed to the CO_2 compensation point for 48 h (●). Results are the means ± SD of five replicates. Experiments were performed between 34 and 38 h after the beginning of the low-CO_2 treatment.

However, our methodology made it possible to detect, for the first time to our knowledge, a rapid decrease in nodule nitrogenase activity during the light period after the pCO_2 in the shoot environment was lowered. This result is consistent with the previous observations of Williams et al. (1982), although the latter were performed after a 5-d exposure of soybean shoot to 90 $\mu L\ L^{-1}$ CO_2. Therefore, our work further substantiates that the low-CO_2, i.e. compensation point, treatment decreases nitrogenase activity within a few hours and that 2 d of this treatment does not alter the nodule's ability to regulate its permeability. Indeed, the nodule activity recovered without any apparent lag after the pCO_2 was returned to 340 $\mu L\ L^{-1}$, although the recovery was to only 50% of the control ARA after 16 h. Other short-term decreases in nodule activity were previously associated with shoot exposure to increases in light intensity (Williams and Phillips, 1980; Drevon et al., 1991), with decapitation or stem girdling (Walsh et al., 1987; Vessey et al., 1988), and with exposure to dark during the day period, the latter being compensated by increasing external pO_2 (Carroll et al., 1987).

The nodule O_2 concentration may be a limiting factor of in vivo nitrogenase activity (Drevon et al., 1988; Hunt et al., 1989; Godfroy and Drevon, 1991). In the present study, increasing external pO_2 in the nodule environment of the treated plants alleviated the decrease in nodule activity due to low CO_2, since the ARA under 40 kPa O_2 was 3-fold higher than under 20 kPa O_2 and similar to the maximal ARA of the control. In contrast, increasing pO_2 had no effect on the ARA of control plants, as previously observed by Heckmann et al. (1989) on similar symbiotic pairs treated with 3 mM NO_3^-. Since ARA at 20 kPa O_2 was higher before than after exposure of control plants to supra-ambient pO_2 (Fig. 3), their nodule permeability probably decreased in response to excess O_2 as shown in several reports recently reviewed by Hunt and Layzell (1993). In contrast, the treated plants had similar ARA at 20 kPa O_2 before and after exposure to supra-ambient pO_2, which suggests that the saturating O_2 concentration for their nodule activity was not exceeded by the range of pO_2 tested (Fig. 3). Collectively, our observations agree with the concept that the decrease in ARA due to low CO_2 was caused by a decrease in the nodule's permeability to O_2 diffusion.

Decreases in nodule permeability have been previously associated with glycoprotein accumulation in intercellular space of the mid-cortex (James et al., 1991) and with cell contraction in the inner cortex (Serraj et al., 1995), the kinetics of which are consistent with the low-CO_2 effect reported here. Since both cortical tissues are located near the vascular bundles of the nodule, transport in or out of the nodule may be linked to the control of nodule permeability. Consequently, exposure to the CO_2 compensation point may be one way to search for plant mechanisms and signals involved in this control.

One possibility raised by this work might be an alteration in photosynthate allocation to nodules concomitantly with the absence of net CO_2 uptake under CO_2 compensation point conditions. However, decreases in photosynthesis due to lowering shoot illumination had no short-term effect on soybean nodule ARA (Bethlenfalway and Phillips, 1977; Schweitzer and Harper, 1980; Drevon et al., 1991), whereas in the present study, a decrease in ARA was observed within 6 h of exposure to low CO_2. Moreover, Walsh et al. (1987) concluded that large carbon stores in the leaves allow soybean nodules to maintain their nitrogenase activity for about 20 h under continuous dark. The latter is consistent with the maintenance of ARA at a high level during dark periods of our 4-d experiments, although we observed a slight but significant day/night cycle in ARA at steady nodulated-root temperature, in agreement with previous observations of Mederski and Streeter (1977) and Drevon et al. (1991). Moreover, extra carbon resources must have been available in the nodules of the plants exposed to low CO_2 to support their increase in ARA in response to increasing external pO_2. Therefore, the decrease in nodule permeability due to low CO_2 was probably not due to a deficiency in carbon reserves. Alternatively, Streeter (1993) hypothesized that a decrease in permeability might be due to limited water export from the nodule. This would not be the case in our experiment, since the *Tr* was not affected during the 1st d with low CO_2, although nodule permeability decreased. Moreover, the *Tr* is expected to increase in response to CO_2 concentrations at the CO_2 compensation point, although it is not known why, in this study, *Tr* did not increase with low CO_2.

Parsons et al. (1993) speculated that changes in nodule permeability might be effected through nitrogen compounds, such as Asn, Gln, glutamate, or Ser, arriving in the phloem of vascular bundles and moving into the surrounding cells of the inner cortex or mid-cortex. Low CO_2 concentration in the shoot environment is known to increase photorespiration in C_3 plants (Gerbaud and André, 1980), and photorespiration is sometimes associated with an increase in Ser content because of the increased activity of the glycolate cycle (Hitz and Stewart, 1980), which might be

the cause of the decrease in nodule permeability shown here. Moreover, Bedmar and Olivares (1980) increased nodule ARA by inhibiting photorespiration in alfalfa, whereas nodule permeability was not measured. Therefore, more work would be required to address whether low CO_2 and other manipulations of photorespiration would affect concomitantly the permeability of soybean nodule and the composition in amino acids of the phloem sap arriving in the nodule.

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