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Drought Stress, Permeability to O_2 Diffusion, and the Respiratory Kinetics of Soybean Root Nodules[1]

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In legume nodules, treatments such as detopping or nitrate fertilization inhibit nodule metabolism and N_2 fixation by decreasing the nodule's permeability to O_2 diffusion, thereby decreasing the infected cell O_2 concentration (*Oi*) and increasing the degree to which nodule metabolism is limited by O_2 availability. In the present study we used nodule oximetry to assess and compare the role of O_2 limitation in soybean (*Glycine max* L. Merr) nodules inhibited by either drought or detopping. Compared to detopping, drought caused only minor decreases in *Oi*, and when the external O_2 concentration was increased to raise *Oi*, the infected cell respiration rate in the drought-stressed plants was not stimulated as much as it was in the nodules of the detopped plants. Unlike those in detopped plants, nodules exposed to moderate drought stress displayed an O_2-sufficient respiration rate that was significantly lower than that in control nodules. Despite possible side effects of oximetry in altering nodule metabolism, these results provided direct evidence that, compared to detopping, O_2 limitation plays a minor role in the inhibition of nodule metabolism during drought stress and changes in nodule permeability are the effect, not the cause, of a drought-induced inhibition of nodule metabolism and the O_2-suffiecient rate of respiration.

N fixation in legumes is very sensitive to inhibition by drought stress. A number of studies (Pankhurst and Sprent, 1975b; Weisz et al., 1985; Durand et al., 1987) have provided evidence to suggest that the mechanism of this inhibition is similar to that occurring during other inhibitory treatments such as nitrate fertilization, photosynthate deprivation, prolonged exposure to $Ar:O_2$ or C_2H_2, and plant disturbance. These treatments cause a decrease in the permeability of the nodule to O_2 diffusion that, in turn, causes increased O_2 limitation of nodule respiration and N fixation (Hunt and Layzell, 1993). Increased O_2 limitation in drought-stressed nodules has been attributed to a decrease of nodule permeability resulting from collapse of lenticels and outer cortical cell layers (Pankhurst and Sprent, 1975a) or to a decrease in the concentration of Lb in nodules (Khanna-Chopra et al., 1984; Swaraj et al., 1986; Guerin et al., 1990, 1991; Irigoyen et al., 1992). In other forms of inhibition, such as that caused by photosynthate deprivation, or when nodules are acclimated to increases in atmospheric pO_2, decreases in nodule permeability have been attributed to decreases in the number or volume of gas-filled intercellular spaces in the inner cortex of the nodule (Witty et al., 1987; Layzell and Hunt, 1990). In addition, a recent mathematical model of O_2 diffusion in bacteria-infected cells has identified the possibility that these cells may have an innate ability to regulate their *P* by virtue of their geometry and a collapse in Lb-facilitated diffusion near the intercellular spaces (Thumfort et al., 1994).

In a companion study (Diaz del Castillo et al., 1994), an open-flow gas exchange system was used to measure the degree to which nitrogenase activity was limited by O_2 supply in soybean (*Glycine max* L.) nodules exposed to mild, moderate, or severe drought stress. Although nitrogenase activity was inhibited to 52% (mild stress) or 9% (moderate stress) of that observed in control plants, the nodules of the drought-stressed plants had O_2-limitation coefficients (a measure of the degree of O_2 sufficiency) that were similar to or only slightly less than that in the control nodules. This observation was inconsistent with the conclusion of many previous studies of drought stress (Pankhurst and Sprent, 1975b; Weisz et al., 1985; Durand et al., 1987) in that it showed that increases in nodule O_2 limitation played a very minor role in reducing nitrogenase activity under drought stress. It was proposed that drought stress reduced the capacity of the nodule to consume O_2 and fix N_2 and that the observed decrease in nodule *P* was a response to the changes in the nodule O_2 status rather than the cause of the decline in nodule respiration and N_2 fixation.

To test this hypothesis, the present study used a noninvasive spectroscopic method to measure the effects of three levels of drought stress on *FOL*, *Oi*, the nodule's *P*, the apparent $K_s(O_2)$ and V_{max} of respiration, and OLC_R (Layzell et al., 1990; Denison and Layzell, 1991). If the changes

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Abbreviations: *FOL*, fractional oxygenation of leghemoglobin; Lb, leghemoglobin; *Oi*, concentration of O_2 in the infected cells of the nodule; OLC_N, the O_2-limitation coefficient of nitrogenase; OLC_R, the O_2-limitation coefficient of nodule respiration; *PNA*, potential nitrogenase activity; *P*, permeability to O_2 diffusion; pO_2, partial pressure of O_2; *TNA*, total nitrogenase activity (H_2 production rate in $Ar:O_2$); V_{max}, maximum volume-specific respiration rate in the infected cell at the optimal O_2 concentration; V_{20}, volume-specific respiration rate in the infected cell in nodules exposed to air (20% O_2).

in nodule permeability are the effect and not the cause of the drought-induced decline in nitrogenase activity, drought stress should have only minor effects on *FOL*, *Oi*, and OLC_R. However, it should result in a significant decline in the O_2-sufficient rate of respiration (V_{max}). For comparative purposes, nodule permeability was also measured by the C_2H_2 lag-phase technique (Weisz and Sinclair, 1988) and oximetry measurements were carried out on plants that were detopped, a treatment known to reduce nitrogenase activity through increased O_2 limitation of nodule metabolism.

MATERIALS AND METHODS

Plant Culture

Seeds of soybean (*Glycine max* L. Merr cv Maple Arrow) were inoculated at the time of planting with *Bradyrhizobium japonicum* USDA 16, a strain lacking uptake hydrogenase activity. Plants to be used for the C_2H_2 lag-phase assay (Weisz and Sinclair, 1988) were grown in cylindrical pots (15 cm high × 7 cm in diameter) that could be connected to a flow-through gas exchange system. Plants to be used for nodule oximetry were grown in similar pots, but these pots had sides that could be removed to expose the nodule without uprooting the plant (Kuzma et al., 1993). All pots contained small stones to a depth of 4 cm. Pots containing plants to be used for the C_2H_2 lag-phase assay were then filled to the top with silica sand (grade 16). Those pots with plants destined to experience moderate drought stress (and their respective controls) were filled with low-nutrient soil (Sunshine Mix, Basic No. 2; Fisons Horticulture, Vancouver, British Columbia, Canada), whereas those that were to experience mild drought stress or to be detopped (and their respective controls) were provided with a 7-cm layer of silica sand followed by a layer of vermiculite:perlite (80:20) mix in the region where the crown nodules would eventually form.

All plants were grown in a growth chamber (model PGV 36; Controlled Environments Ltd., Winnipeg, Manitoba, Canada) at a constant temperature of 20°C and 80% RH, with a photon flux density of 500 μmol m^{-2} s^{-1} photosynthetically active radiation and a 16-h photoperiod. They were watered with a nutrient solution (Walsh et al., 1987) containing 0.5 mM KNO_3 until 1 week after germination and then with the same solution lacking nitrate for the remainder of the growth period. The plants grown for C_2H_2 reduction were watered twice daily, and the plants grown for oximetry were watered once a day in the morning.

Experimental Treatments

One day before the beginning of the stress treatments, 35-d-old plants were transferred to a growth chamber in the proximity of the nodule oximeter and the gas exchange system and watered to field capacity. During the subsequent 8 d, water was withheld completely from some of the plants growing in pots containing soil (moderate stress treatment) and control plants were watered the same as before. Some of the plants grown in pots containing vermiculite:perlite and in pots containing sand were exposed to mild drought stress by watering at the base of the pot, using a syringe, with sufficient nutrient solution to compensate for the plant's daily water loss (this was determined daily by weighing the plant before and after watering). This latter watering regime was assumed to stress the nodules without imposing severe water limitation on the shoot or roots. Control plants were watered the same as before. Mildly stressed plants grown in sand were used for the C_2H_2 lag-phase assay on the 4th and 5th d of stress. Mildly stressed nodules grown in vermiculite:perlite were used for oximetry on the 4th and 7th d of stress, whereas moderately stressed nodules were assayed on the 4th and 8th d of stress.

Finally, some of the plants grown in pots containing the vermiculite:perlite mixture, and having been watered daily, were used for the detopping treatment. The shoots of these plants were removed, and nodules were selected for oximetry measurements 3 to 5 h after detopping.

Nodule Oximetry

To minimize the inhibition of nitrogenase activity by plant disturbance (Minchin et al., 1986), the pots were removed from the growth chamber and placed on their sides, and one side of the pot was carefully removed as described previously (Kuzma et al., 1993). The rooting medium was gently removed from the region of a nodule and an oximeter probe containing two optical fibers and a gas supply tube was placed against the nodule surface. A single-channel, light-emitting diode-based nodule oximeter, similar to that described previously (Layzell et al., 1990), was used to provide an analog voltage signal proportional to the transmittance of 660 nm of light through the legume nodule. A microcomputer equipped with an analog-to-digital converter was used to monitor the voltage and to save a value to disk at a rate of one sample per s.

The nodule was exposed to a humidified gas stream (1 L min^{-1}) that was initially composed of synthetic air (20% O_2 in N_2) (stage A, Fig. 1). When a stable signal had been obtained, the gas stream was changed to pure N_2 until transmittance declined to a steady level (approximately 25–35 s) (stage B, Fig. 1). The gas stream was then changed to pure O_2 (stage C, Fig. 1), and when transmittance reached a plateau (approximately 20–35 s), it was returned to pure N_2 (stage D, Fig. 1) until nodule transmittance had declined to a steady level (approximately 50–80 s). The nodule was then harvested and its fresh weight measured; it was then dried for 76 h at 90°C and its dry weight was determined. The fresh weight:dry weight ratio was used to obtain an estimate of the nodule water potential as described previously by Diaz del Castillo et al. (1994).

Determination of *FOL*, *Oi*, Nodule Permeability, and Respiratory Kinetics

The transmittance voltages from each nodule oximetry assay were used to determine *FOL*, *Oi*, *P*, and apparent $K_s(O_2)$ and V_{max} of infected cell respiration. The calculations were adapted from those used in previous studies

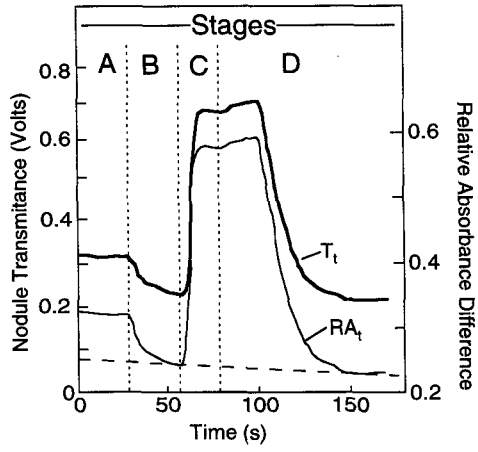

Figure 1. A standard nodule oximetry assay showing values for nodule transmittance at 660 nm (T_t, heavy line) and for the relative absorbance (RA_t) as calculated from Equation 1 (see text). The vertical, dotted lines denote times when there was a change in the gas composition surrounding the nodule: stage A, 20% O_2 in N_2; stage B, pure N_2; stage C, pure O_2; stage D, pure N_2. The linear regression denoted by the dashed line links the lowest absorbance points of stages B and D. When values from this regression were subtracted from the RA_t values at the same time t, the resulting drift-corrected RA'_t values (not shown) were used in subsequent calculations of nodule respiration and permeability (see text).

(King et al., 1988; Layzell et al., 1990; Denison and Layzell, 1991; Denison et al., 1992a). Briefly, this involved the following steps.

1. The voltage values were proportional to nodule transmittance (T_t) measured at each time t and were converted to relative absorbance values (RA_t) using Equation 1, modified from Layzell et al. (1990):

$$RA_t = \ln(10) - \ln\left[\frac{10}{1 + T_t}\right] \quad (1)$$

To correct for drift, a straight line was drawn between the lowest value in stage B and the lowest value in stage D (Fig. 1, dashed line). The zeroed value at each time t was subtracted from its corresponding RA_t value to obtain a drift-corrected relative absorbance value defined as RA'_t.

2. The RA'_t values for data in stage C and for data in stage D (Fig. 1) were each plotted against time and fitted to a third-order polynomial as a method to smooth the data. These best-fit curves were used for all subsequent calculations requiring values from stages C and D.

3. The *FOL* at each time t (FOL_t) was calculated from Equation 2 (King et al., 1988):

$$FOL_t = \left[\frac{RA'_t}{RA'_{Cmax}}\right] \quad (2)$$

where RA'_{Cmax} is the maximum RA'_t value in stage C (Fig. 1).

4. The *Oi* at time t (Oi_t, in nM) was calculated from Equation 3 (King et al., 1988):

$$Oi_t = \frac{FOL_t \times K_s^{Lb}}{1 - FOL_t} \quad (3)$$

where K_s^{Lb} is the ratio of the rate constants for Lb oxygenation and deoxygenation and assumed to be equal to 48 nm (Gibson et al., 1989). To obtain an estimate of the *Oi* in nodules under atmospheric conditions, the *FOL* value used in Equation 3 was the average of the values obtained during the last 5 s of stage A (Fig. 1).

5. The amount of LbO_2 in the nodule at each time t during stage C (LbO_t^C, in mol) and stage D (LbO_t^D, in mol) (Fig. 1) was calculated as:

$$LbO_t^C = FOL_t^C \times Lb^{total} \times FW_{nod} \quad (4a)$$

$$LbO_t^D = FOL_t^D \times Lb^{total} \times FW_{nod} \quad (4b)$$

where FOL_t^C and FOL_t^D were the *FOL* values measured at time t during stage C or D (Fig. 1), respectively, Lb^{total} was the total Lb concentration in the nodule (measured as 0.216 $\times 10^{-6}$ mol g^{-1} fresh weight, data not shown), and FW_{nod} was the nodule fresh weight (in g).

6. The respiration rate ($Resp_t^D$, in mol s^{-1}) of the nodule in the presence of various *Oi* was calculated from the rate of decline in LbO_t^D during stage D (Fig. 1). Therefore,

$$Resp_t^D = \frac{LbO_t^D - LbO_{t-1}^D}{\Delta t} \quad (5)$$

where Δt is the time interval(s) are between t and $t - 1$. This calculation ignores the contribution of free O_2 (i.e. *Oi*) to the total O_2 pool in the nodule (estimated to be less than 0.1%) and assumes that the decline in *Oi* and LbO_2 was due solely to respiration, not to diffusion out of the nodule (Denison and Layzell, 1991).

7. To provide values for volume-specific respiration, $Resp_t^D$(units of mol m^{-3} s^{-1}) was divided by [(FW_{nod}/*Den*) $\times$ *CZvol*], where FW_{nod} was the nodule fresh weight (in g), *Den* was the nodule density (measured as 1.04×10^6 g m^{-3}, in both control and drought-stressed nodules, data not shown), and *CZvol* was the proportion of total nodule volume present in the central zone (0.55, Lin et al., 1988). In a plot of the volume-specific respiration rate versus Oi_t, the maximum respiration rate was identified as V_{max} and the half-saturation constant for respiration [$K_s(O_2)$] was defined as the Oi_t at which the volume-specific respiration rate was 0.5 $\times$ V_{max}.

8. Nodule permeability of O_2 diffusion (P, in m s^{-1}) was calculated from the rate of change in the amount of LbO_2 present in the nodule during stage C (Fig. 1) using an equation adapted from that described previously (Denison and Layzell, 1991; Denison et al., 1992a, 1992b).

$$P = \left[\left(\frac{LbO_t^C - LbO_{t-1}^C}{\Delta t}\right) + Resp_{Oi}^D\right] / [A \times (Oe - Oi)] \quad (6)$$

where A is the surface area (in m^2) of the central zone, *Oe* is the external O_2 concentration (40.9 mol m^{-3}), and *Oi* is the infected cell O_2 concentration during that time interval (in

mol m^{-3}). Finally $Resp^{D}_{Oi}$ is the infected cell respiration rate (in mol s^{-1}) estimated to exist at each *Oi* value. This was determined from the data of stage D (Fig. 1) as described in item 6 above.

9. The degree to which the respiration rate of the legume nodule was limited by O_2 was defined as the O_2-limitation coefficient for nodule respiration (OLC_R) and was calculated as:

$$OLC_R = V_{20}/V_{max} \quad (7)$$

where V_{20} was the respiration rate calculated from the data of stage D (Fig. 1) ($Resp^{D}_{Oi}$) when the *Oi* was identical with that during stage A (Fig. 1) (i.e. approximately 20 kPa).

Determination of Nodule Permeability using the C_2H_2 Lag-Phase Method

Nodule permeability in control and drought-stressed plants was also measured using the C_2H_2 lag-phase technique described by Weisz and Sinclair (1988). Prior to the assay, the void volume of each pot was minimized by completely filling the pot with silica sand. At the time of the assay, the nodulated roots of intact plants were sealed within their growth pots and flushed with 10% C_2H_2 in N_2:O_2 (70:20) at a flow rate of 3000 mL min^{-1}. C_2H_4 production from the nodulated roots was measured by removing samples from the effluent gas stream every few seconds and analyzing them for C_2H_4 using a gas chromatograph (model GC-8A; Shimadzu, Columbia, MD) equipped with a Poropak N column (100–120 mesh; 2 m × 1/8 inch o.d. stainless steel). After each assay, the void volume of each pot was determined by measuring the volume of water required to completely fill the pot containing the nodulated root and silica sand. Finally, the plants were harvested into nodules, leaves, stems, and roots for fresh weight and dry weight determinations.

RESULTS

Water Potential and *FOL* in Control and Treated Nodules

Nodules from control and detopped plants had mean water potentials that were less negative (−0.50 to −0.74 MPa) than those observed in nodules of plants exposed to the mild drought-stress treatment for 4 to 7 d (−1.28 ± 0.03 MPa) or to the moderate drought stress for 4 to 8 d (−1.49 ± 0.06 MPa) (Fig. 2). In the control nodules under atmospheric pO_2, the average *FOL* ranged from 0.26 to 0.29. Detopping caused a significant decrease in *FOL* to 34% of control or 0.10 ± 0.02 (Fig. 2). In contrast, the *FOL* in mildly stressed nodules was 80% of control, or 0.23 ± 0.02 (not significantly different from its control value), whereas in moderately stressed nodules, *FOL* was reduced to 69% of the control, or 0.18 ± 0.02 (significantly different at $P < 0.05$). The *Oi* equivalent to these *FOL* values ranged from a low of 5.2 ± 1.1 nM (detopped treatment) to a high of 20.2 ± 2.9 nM (control treatment) (Fig. 2).

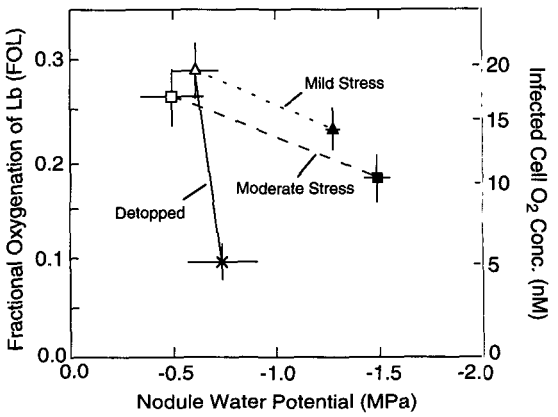

Figure 2. The effects of detopping (X), mild drought-stress (triangles), and moderate drought-stress (squares) treatments on *FOL* in soybean nodules. The open symbols represent the mean of the control values for each treatment. *Oi* values were calculated from the measured *FOL* values using Equation 3. Lines associated with each symbol represent SE values. Values of *n* for each treatment are the same as those shown in Table I.

Nodule Respiration, O_2-Limitation Coefficient, and $K_s(O_2)$ in Control Nodules

The rate of decline in *FOL*, when the atmosphere around the nodule was changed from pure O_2 to pure N_2 (i.e. Fig. 1, stage D), provided a measure of nodule respiration at various *Oi* values. The averaged measured relationship between nodule-specific respiration and *Oi* is shown in Figure 3. O_2 saturation of respiration was reached in all treatments when *FOL* was between 0.73 and 0.86 (equivalent to *Oi* values between 170 and 352 nM). When the respiration rates ($Resp^{D}_{i}$) calculated from Equation 5 were expressed per central zone volume, the V_{max} in nodules of the control plants ranged from 16.5 ± 1.0 (Fig. 3B) to 21.0 ± 2.0 mmol m^{-3} s^{-1} (Fig. 3, A and C). In these same nodules, the V_{20} ranged from 8.8 ± 1.1 to 11.2 ± 1.0 mmol m^{-3} s^{-1} (Fig. 3). The ratio of V_{20} to V_{max} provided a measure of OLC_R as described previously (Denison et al., 1992a). In control nodules, mean OLC_R values ranged from 0.53 ± 0.05 to 0.56 ± 0.05 (Table I). The concentration of O_2 that supported 50% of the V_{max} [i.e. the $K_s(O_2)$] was 15 ± 2 and 17 ± 2 nM in the control nodules from the various treatments (Fig. 3).

Treatment Effects on Respiration, OLC_R, and $K_s(O_2)$

In the mildly stressed, moderately stressed, and detopped treatments, the mean value for the V_{max} of nodule respiration ranged from 13.1 to 18.9 mmol m^{-3} s^{-1} (Fig. 3). These values were 80 to 90% of that observed in control nodules. Only the V_{max} of moderately drought-stressed nodules was significantly lower than that of controls ($P < 0.05$).

V_{20} in stressed nodules ranged from 4.6 to 9.5 mmol m^{-3} s^{-1} (Fig. 3). These values were, in mild, moderate, and detopped treatments, 85, 59, and 41%, respectively, of the V_{20} values measured in nodules from the respective control treat-

ment. Only the V_{20} values from the nodules of moderately drought-stressed plants and detopped plants were significantly different from their respective mean control values ($P < 0.05$). OLC_R values were 0.57 ± 0.04 and 0.41 ± 0.05 in the mildly and moderately stressed nodules, respectively, and 0.31 ± 0.09 in the nodules from the detopped plants (Table I). Only the OLC_R value in the detopped treatment was significantly different from the control ($P < 0.05$).

Finally, the concentration of O_2 that supported 50% of the V_{max} [i.e. $K_s(O_2)$] declined from an average value of 17 ± 2 nm in control plants to 10 ± 2 and 10 ± 3 nM in the mildly stressed and detopped plants, respectively (significantly different at $P < 0.05$) (Fig. 3). However, the $K_s(O_2)$ value in the moderately stressed nodules (17 ± 3 nm) was not significantly different from its control (15 ± 2 nM).

Treatment Effects on Nodule Permeability

The rate of increase in *FOL* when the atmosphere around the nodule was changed from pure N_2 to pure O_2 (i.e. Fig. 1, stage C) provided a measure of the nodule's *P* as described in Equation 6. In control nodules, mean values for nodule permeability measured by oximetry ranged from 0.34 to 0.66 $\mu m\ s^{-1}$ (Table II). The lower permeability in the nodules of the control plants used in the moderate stress treatment than in those control plants with the other treatments was attributed, in part, to a smaller nodule size in the former population of plants.

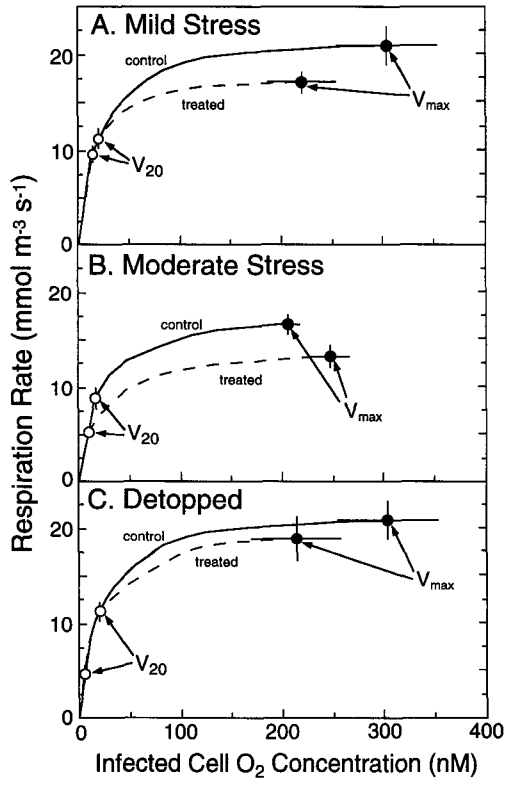

Figure 3. The relationship between the volume-specific central zone respiration rate and the O_2 concentration in the infected cells as determined from the changes in LbO_2 concentration following a change in the atmosphere surrounding a nodule from high pO_2 to pure N_2 (stage D, Fig. 1). Control (solid line) and treatment (dashed line) values are presented for the mild drought-stress treatment (A), moderate drought-stress treatment (B), and detopped treatment (C). For each relationship, values are provided for V_{max} (●) and for V_{20} (○). The V_{max} and V_{20} values are presented ± 1 SE for respiration, and the V_{max} value is also provided with ± 1 SE to depict the *Oi* at which the V_{max} was measured. Values of *n* for each treatment are the same as those shown on Table I.

Table I. *The effect of mild drought stress, moderate drought stress, or detopping on the OLC_R in soybean nodules*

OLC_R was calculated from the data of Figure 3 using Equation 7. Values are presented as means ± SE (sample size).

Treatment	OLC_R	
	Control	Treated
Mild drought stress	0.56 ± 0.05 (11)	0.57 ± 0.04 (13)
Moderate drought stress	0.53 ± 0.05 (7)	0.41 ± 0.05 (6)
Detopping	0.56 ± 0.05 (11)	0.31 ± 0.09 (6)[a]

[a] Significantly different at $P < 0.05$.

In the mildly and moderately drought-stressed nodules, permeability was 74% (not significantly different at $P < 0.05$) and 62% (significantly different at $P < 0.05$), respectively, of that measured in the control plants. In contrast, the detopping treatment displayed nodule permeabilities that were only 39% of that in the control plants (significantly different at $P < 0.05$) (Table II). For comparative purposes, the C_2H_2 lag-phase technique was also used to measure permeability in control (water potential −0.69 ± 0.06 MPa) and mildly stressed nodules (water potential −1.04 ± 0.09 MPa). As in the oximetry measurements, the permeability of mildly drought-stressed nodules was 76% of control, but the values were not significantly different ($P < 0.05$) (Table II).

It was interesting to note that the absolute values obtained for nodule permeability were 35 to 68 times higher in the C_2H_2 lag-phase assay than in the oximetry assay. This was attributed to the fact that the C_2H_2 lag-phase assay measures permeability to C_2H_2 and C_2H_4 diffusion, whereas the oximetry assay measures *P*. C_2H_2 is about 30 times more soluble than O_2, and both C_2H_2 and C_2H_4 have higher diffusion coefficients than O_2. These factors would explain the higher permeability values obtained with the C_2H_2 lag-phase assay compared to the ones obtained by oximetry. In addition, the C_2H_2 lag-phase assay would not provide a measure of the contribution of Lb to nodule permeability, and recent studies (Thumfort et al., 1994) have suggested that this may be an important component of the nodule's diffusion barrier.

DISCUSSION

The oximetry measurements made in the present study were carried out to test a hypothesis put forward in a previous study of nodule gas exchange (Diaz del Castillo et al., 1994). It was proposed that the primary cause for the inhibition of nitrogenase activity by drought stress was not due to O_2 limitation caused by a decrease in nodule per-

Table II. *The effect of mild drought stress, moderate drought stress, or detopping on nodule permeability to gas diffusion as measured by either nodule oximetry (O_2 diffusion) or the C_2H_2 lag-phase assay (C_2H_2 and C_2H_4 diffusion) (Weisz and Sinclair, 1988)*

The oximetry measurements were from the same nodules as those used to obtain the data in Figures 2 and 3. The nodules from the plants used in the C_2H_2 lag-phase assay had water potentials of −0.69 ± 0.06 and −1.04 ± 0.03 MPa in the control and treated plants, respectively. Values are presented as means ± SE (sample size).

Assay Method	Treatment	Permeability	
		Control	Treated
		μm/s	
Oximetry	Mild drought stress	0.66 ± 0.08 (11)	0.49 ± 0.05 (13)
Oximetry	Moderate drought stress	0.34 ± 0.04 (7)	0.21 ± 0.02 (6)[a]
Oximetry	Detopping	0.66 ± 0.08 (11)	0.26 ± 0.04 (6)[a]
C_2H_2 lag phase	Mild drought stress	23 ± 3.8 (3)	17.5 ± 2.5 (3)

[a] Significantly different at $P < 0.05$.

meability but to a decline in the capacity of the nodule to support O_2-saturated respiration and nitrogenase activity. This hypothesis differed from the conclusions of earlier studies (Pankhurst and Sprent, 1975b; Weisz et al., 1985; Durand et al., 1987) and identified drought stress as an inhibitory treatment that differs in a fundamental way from nodule inhibition by detopping (Minchin et al., 1986; Hartwig et al., 1987; Denison et al., 1992a), stem girdling (Vessey et al., 1988; Layzell et al., 1990), nitrate fertilization (Schuller et al., 1988; Vessey et al., 1988; Faurie and Soussana, 1993), or Ar:O_2 exposure (Minchin et al., 1983; King and Layzell, 1991). The results of the present study provide significant support to this new hypothesis, but the support is not unequivocal. As discussed in the introduction, the hypothesis offered predictions on the effects of drought stress on various parameters that could be measured by nodule oximetry. These will be considered individually in the following three sections.

FOL and *Oi*

Previous studies have shown that inhibitory treatments such as detopping, stem girdling, nitrate fertilization, or Ar:O_2 exposure cause a significant decline in *FOL* and *Oi* as measured by a nodule oximeter (Layzell et al., 1990; King and Layzell, 1991; Denison et al., 1992a; Kuzma et al., 1993), an observation consistent with a greater O_2 limitation of nodule respiration. Indeed, the detopping treatment in the present study reduced *Oi* from 20 to about 5 nM (Fig. 2). In contrast, *Oi* in the mild drought-stress treatment was similar to its control value and in moderate drought stress it was only reduced to 69% of that of its control (Fig. 2). The differences between the *Oi* in the detopped and drought treatments could not be attributed to differences in the magnitude of the inhibition because the previous study (Diaz del Castillo et al., 1994) showed that, at the nodule water potentials measured here (Fig. 2), total nitrogenase activity would have been inhibited in the mildly and moderately stressed nodules to values equivalent to 41 and 10% of control nodules, respectively (fig. 4 of Diaz del Castillo et al., 1994). In comparison, a detopping treatment similar to that used here inhibited total nitrogenase activity to 18% of control (fig. 4 of Diaz del Castillo et al., 1994). These data indicate that, despite similar levels of nitrogenase inhibition, drought-stressed nodules have infected cells that are more highly oxygenated than nodules on detopped plants. This observation supports the hypothesis being tested in the present study.

OLC_R

In legume nodules, the respiration rate and nitrogenase activity are known to be limited by O_2 supply. OLC_N and OLC_R have been defined to provide quantitative estimates of the degree to which these processes are O_2 limited (Denison et al., 1992a; Diaz del Castillo et al., 1992). The OLC_N measured by gas exchange is typically 0.95 to 0.99 in control soybean nodules but decreases to 0.29 in detopped plants (Diaz del Castillo et al., 1994), to 0.32 following Ar:O_2 exposure (King and Layzell, 1991), and to 0.52 following nitrate fertilization (Vessey et al., 1988), indicating a higher O_2 limitation in these treated nodules. However, in drought-stressed nodules, OLC_N was only reduced to 0.65 to 0.95 (Diaz del Castillo et al., 1994), suggesting a diminished role for O_2 limitation in this treatment compared to other inhibitory treatments that have been studied.

In the present study, values for OLC_R were measured by oximetry and found to be 0.53 to 0.56 in control nodules but decreased to 54% of control in detopped plants compared with 77 to 101% of control in drought-stressed plants (Table I). These data for OLC_R were consistent with those for OLC_N and with the hypothesis being tested here, in that O_2 limitation appears to have a lesser role in the inhibition of nodule metabolism by drought stress than other inhibitory treatments such as detopping.

However, it is important to note that in the control nodules the values obtained for OLC_R (0.53–0.56) were much lower than those previously obtained for OLC_N[r] (0.95–0.99) (Diaz del Castillo et al., 1994). Possible reasons for these differences will be discussed in a subsequent section.

The V_{max} of Infected Cell Respiration

The term "potential nitrogenase activity" or *PNA* has been used to describe the maximum rate of electron flow through nitrogenase that can be supported at an optimal *Oi* and is measured by gas exchange as the peak of *TNA* observed in a nodule exposed to an increase in the external pO_2 from 20 to 100 kPa O_2 ((Diaz del Castillo et al., 1992).

Previous studies (Diaz del Castillo et al., 1994) have shown that detopping inhibits *PNA* to a lesser degree (*PNA* inhibited to 62% of control; fig. 4 of Diaz del Castillo et al., 1994) than drought stress (*PNA* inhibited to 18–61% of control; fig. 4 of Diaz del Castillo et al., 1994). These data were an important factor in the hypothesis that drought stress differs from other treatments that inhibit nitrogenase activity in that it decreases the capacity of the nodule to consume O_2 in support of nitrogenase activity.

Assuming that nitrogenase activity and nodule respiration are tightly coupled processes in legume nodules (at least at low *Oi*; Kuzma et al., 1993), the *PNA* in gas exchange studies would be expected to be proportional to the measurements of V_{max} in studies of nodule oximetry. If this were the case, compared to control plants, drought stress should cause a greater reduction in V_{max} than the detopping treatment. In the present study, the V_{max} of the detopped treatment was 90% of the control, whereas V_{max} values observed in the nodules exposed to mild or moderate drought stress were 82 or 80%, respectively, of that in the control plants. However, the V_{max} was significantly different from the control only in the nodules from the moderately drought-stressed plants. These data provided additional support for the suggestion (Diaz del Castillo et al., 1994) that the decline in respiratory capacity plays a relatively more important role than an increased O_2 limitation in nodules inhibited by drought stress than in treatments such as detopping.

It was interesting to note that the magnitude of the decline in V_{max} measured in the present study was much less (approximately 80–90% of control) than the magnitude of the decline in *PNA* measured previously (approximately 18–62% of control, Diaz del Castillo et al., 1994). This discrepancy provides a challenge to the hypothesis that nitrogenase inhibition by drought stress is mediated primarily through a reduction in the respiratory capacity of the legume nodule.

The relatively small decline in V_{max} in the present study may be attributed to one, or both, of two reasons.

Changes in Lb Concentration

When the infected cell respiration rate is calculated, the oximetry method converts values of *FOL* into the amount of LbO_2 by multiplying *FOL* by the nodule Lb content. The total Lb concentration was measured only in the control nodules, and if it was significantly lower in the treated nodules, the respective rates of respiration shown in Figure 3 would overestimate actual rates. Previous studies have reported that the Lb content of legume nodules can decline during drought stress (Khanna-Chopra et al., 1984; Swaraj et al., 1986; Guerin et al., 1990, 1991; Irigoyen et al., 1992). For example, using drought-stressed soybean nodules, Swaraj et al. (1986) reported a minor decrease in Lb content in mildly stressed nodules but a decrease in nodule Lb content to 40% of the initial value under more severe stress. Although this explanation may account for the relatively high V_{max} (and V_{20}) values in the drought-stress treatments, it is not likely to be responsible for the higher-than-expected V_{max} value in the detopped treatment relative to control.

Side Effects of Oximetry on the Metabolism of Legume Nodules

Recent studies (Kuzma et al., 1993) have shown that nodules used in oximetry had *TNA* and *PNA* values (expressed per g fresh weight nodule) that were 16 to 48% and 30 to 57%, respectively, of that measured in undisturbed nodulated roots. This observation is consistent with the fact that the OLC_R values measured in control nodules in the present study (0.53–0.56, Table I) were much lower than the OLC_N values measured by gas exchange in undisturbed nodulated roots (0.99 ± 0.01, Diaz del Castillo et al., 1994). These results add to the mounting evidence (Kuzma et al., 1993) that nodule oximetry, as implemented in our laboratory, is not the noninvasive method it was once hoped to be (King et al., 1988; Denison and Layzell 1991), and therefore, the results obtained using this technique must be interpreted with caution. At least in soybean nodules, the use of a nodule oximeter appears to decrease nodule permeability resulting in a greater O_2 limitation of nodule metabolism (i.e. lower OLC_N and OLC_R values) and to decrease the maximum rate of respiration and electron flow through nitrogenase that can be achieved at optimal *Oi* (i.e. lower *PNA* and V_{max}). In this way, nodule oximetry has many of the same drawbacks for the study of nodule respiration and nodule O_2 status as do studies involving extended exposure to 10% C_2H_2 or to $Ar:O_2$ atmospheres for the measurement of nitrogenase activity (Minchin et al., 1983; Hunt and Layzell, 1993).

Permeability Change: Cause or Effect?

Even when the limitations of nodule oximetry are taken into consideration, the results of the present study support the hypothesis that, compared to detopping, nitrogenase inhibition by drought stress was more due to a decrease in respiratory capacity and less to a decrease in O_2 availability. Therefore, in the detopping treatment, the observed decrease in nodule permeability (Table II) had a causal role in the inhibition of metabolism by reducing *Oi* and limiting O_2 availability for nodule respiration (Hartwig et al., 1987; Vessey et al., 1988; Denison et al., 1992a). However, in the drought-stressed plants, the changes in permeability, *Oi*, and V_{20} were less pronounced and the decline in V_{max} was more important than in nodules from the detopped plants. These observations support the suggestion that in drought-stressed plants nodule permeability to O_2 changes in response to a changing respiratory demand to maintain a given *Oi*. If so, the lowered permeability is likely to be more the consequence of other changes in the nodule rather than being the primary factor that is used by the nodule to down-regulate metabolic activity.

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