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The Role of Oxygen in the Regulation of Nitrogenase Activity in Drought-Stressed Soybean Nodules[1]

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The aim of this study was to investigate the mechanism of nitrogenase inhibition in drought-stressed soybean (*Glycine max* L.) nodules to determine whether this stress was similar to other inhibitory treatments (e.g. detopping) known to cause an O_2 limitation of nodule metabolism. Nodulated soybean plants were either detopped or subjected to mild, moderate, or severe drought stress by growth in different media and by withholding water for different periods. All treatments caused a decline in nitrogenase activity, and in the drought-stressed nodules, the decline was correlated with more negative nodule water potentials. Increases in rhizosphere O_2 concentration stimulated nitrogenase activity much more in detopped plants than in drought-stressed plants, reflecting a greater degree of O_2 limitation with the detopped treatment than with the drought-stressed treatment. These results indicated that drought stress differs from many other inhibitory treatments, such as detopping, in that its primary cause is not a decrease in nodule permeability and a greater O_2 limitation of nodule metabolism. Rather, drought stress seems to cause a decrease in the maximum O_2-sufficient rate of nodule respiration or nitrogenase activity, and the changes in nodule permeability reported to occur in drought-stressed nodules may be a response to elevated O_2 concentrations in the infected cell that may occur as nodule respiration declines.

Nitrogenase activity in intact legume nodules is inhibited by changes in many environmental variables. These include the reduction of phloem sap supply by defoliation (Hartwig et al., 1987), detopping (Diaz del Castillo et al., 1992) or nodule excision (Sung et al., 1991), nitrate fertilization (Vessey et al., 1988), and exposure of the nodulated roots to gaseous environments in which N_2 fixation is prevented, such as 10% acetylene (Minchin et al., 1983) or Ar:O_2 (King and Layzell, 1991). In each of these cases, nitrogenase inhibition is associated with a decrease in the permeability of the nodule to gas diffusion. This causes a reduction in *Oi* (Layzell et al., 1990; King and Layzell, 1991), resulting in an O_2 limitation of nitrogenase-linked respiration. After each inhibitory treatment, nitrogenase activity can be partially recovered by increasing rhizosphere pO_2 (Witty et al., 1984; Hartwig et al., 1987; Vessey et al., 1988; King and Layzell, 1991; Diaz del Castillo et al., 1992).

The inhibition of nitrogenase activity that occurs during drought stress has also been linked to O_2 limitation of nitrogenase-linked respiration. In an early study, Pankhurst and Sprent (1975) suggested that during drought lenticels on the surface of soybean (*Glycine max* L.) nodules may collapse and restrict O_2 diffusion to the nodule central zone. However, this study used detached nodules (now known to have low nodule permeability; Sung et al., 1991) under severe drought conditions. An alternative inhibitory mechanism was proposed by Huang et al. (1975), who suggested that a decline in photosynthate supply to nodules during drought would cause carbohydrate limitation of nitrogenase activity. Although declines in photosynthate supply have subsequently been shown to cause a decrease in nodule permeability (Vessey et al., 1988), the time courses of photosynthetic inhibition and nitrogenase inhibition during drought do not support a relationship between the two processes. For example, at the onset of drought stress, nitrogenase activity declines with a rapidity that cannot be accounted for by the relatively slow and small decline in photosynthetic rate (Durand et al., 1987; Djekoun and Planchon, 1991).

Experiments with field-grown soybeans showed that inhibition of acetylene reduction activity during drought was linearly correlated with a decline in the nodule's permeability to gas diffusion (Weisz et al., 1985). Also, when rhizosphere pO_2 was increased, acetylene reduction activity in the stressed plants recovered, leading to the conclusion that a decreased nodule permeability to O_2 was the major factor limiting nitrogenase activity under drought conditions. However, a later study showed that elevated pO_2 caused only a partial recovery of nitrogenase activity in drought-stressed broad bean plants (Guerin et al., 1990). Therefore, although most experimental evidence to date indicates that reduced O_2 flux to the bacteroids is the major factor inhibiting nitrogenase activity during drought stress, factors other than decreased nodule permeability to O_2 may be involved in the inhibitory mechanism. It is pertinent that reduced nodule permeability may not always be a cause of nitrogenase inhibition. In a recent study (Kuzma and Layzell, 1994) with attached soybean nodules, nitrogenase inhibition caused by a decline in temperature was associated with a decline in nodule permeability, but this was identified as a response to reduced nodule respiration rather than a cause of inhibition.

The aim of the present study was to investigate the mech-

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Abbreviations: *ANA*, apparent nitrogenase activity (H_2 production rate in N_2:O_2); DW, dry weight; FW, fresh weight; *Oi*, concentration of O_2 in the infected cells of the nodule; OLC_N, the O_2 limitation coefficient of nitrogenase; *PNA*, potential nitrogenase activity; pO_2, partial pressure of O_2; *TNA*, total nitrogenase activity (H_2 production rate in Ar:O_2).

anism of nitrogenase inhibition during drought stress by monitoring nitrogenase activity in attached soybean nodules exposed to different degrees of stress during different periods and by measuring the degree to which elevated pO_2 may recover nitrogenase inhibition in each treatment. To determine whether or not the inhibitory mechanism may differ from that occurring during other environmental treatments, the data collected from drought-stressed plants were compared with data collected using plants in which nitrogenase activity was inhibited by shoot removal (detopping).

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* U.S. Department of Agriculture No. 16, producing a symbiosis deficient in uptake hydrogenase activity (Layzell et al., 1984). Seeds were planted in pots that could be sealed for gas exchange, and all pots contained 4 cm of small stones in the base. The planting medium overlaying the stones differed depending on the degree of drought stress desired during periods of imposed drought. To produce rapid inhibition of nitrogenase activity (severe stress treatment), plants were grown in silica sand; whereas to produce slower nitrogenase inhibition (moderate stress treatment), plants were grown in low nutrient soil (Sunshine Mix, Basic No. 2; Fisons Horticulture, Vancouver, British Columbia, Canada). Mild stress was achieved by growing plants in stratified media consisting of silica sand to a level just below the crown nodules and then vermiculite:perlite (80:20) to the top of the pot.

All plants were maintained in a growth chamber (Conviron, Manitoba, Canada) at a constant temperature of 20°C, 80% RH, and a photon flux density of 500 μmol m^{-2} s^{-1} photosynthetically active radiation during a 16-h photoperiod. Plants were watered with a modified Hoagland solution 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 in soil and those grown in vermiculite:perlite were watered once daily in the morning, whereas those grown in silica sand were watered in both the morning and evening. Plants were between 35 and 42 d old when drought-stress treatments were initiated.

Measurements of Respiration Rate and Nitrogenase Activity in Nodulated Roots

Respiration rate and nitrogenase activity in nodulated roots were measured by sealing the roots within their growth pots using a flexible sealant (Terostat IX; Teroson, GmbH, Heidelberg, Germany) and attaching the pots to a computer-controlled, open-flow gas-exchange system similar to that described previously (Hunt et al., 1989). Respiration rate was measured as CO_2 evolution using an IRGA (ADC 225 MK3; Analytical Development Corp., Hoddesdon, UK), and nitrogenase activity was measured as H_2 evolution using an H_2 gas analyzer (model 150; Morgan Instruments Ltd., Andover, MA). Three different aspects of nitrogenase activity were measured according to the methods of Diaz del Castillo et al. (1992). *ANA* was measured as the H_2 evolution rate in an atmosphere of N_2:O_2 (80:20), *TNA* was measured as the peak H_2 evolution rate in an atmosphere of Ar:O_2, and *PNA* was measured as the peak rate of H_2 evolution attained when pO_2 in Ar:O_2 was increased linearly from atmospheric to 100% during a 30-min period. The degree to which O_2 supply limited nitrogenase activity in any treatment was expressed in terms of the OLC_N (=*TNA*/*PNA*; Diaz del Castillo et al., 1992).

Time Course of Induced Drought Stress and Gas-Exchange Measurements

One day prior to the initiation of drought stress, plants were transferred to a growth chamber having conditions of temperature, humidity, light intensity, and photoperiod identical with those in the chamber in which the plants were grown. The plants were maintained in this chamber during the imposition of drought stress and during all gas-exchange measurements. Late in the evening, just prior to the end of the photoperiod, all plants were watered to field capacity, and in the afternoon of the following day measurements were made of the initial (prestress) *ANA* and *TNA*. Samples of these plants were also used for determination of *PNA*, and these were then harvested for the determination of nodule, root, leaf, and stem FW. The DW of each plant organ was measured after drying for 72 h at 92°C, and the FW:DW ratio was calculated.

Drought stress was imposed on the severely stressed and moderately stressed plants by withholding watering for the duration of the experimental period (3 and 8 d, respectively). In the mildly stressed treatment, the roots of the plant were watered twice daily for 9 d by injecting 20 to 30 mL of nutrient solution through a hole in the base of the pot. This was sufficient nutrient solution to maintain the pot at a constant daily weight as determined by weighing the pot before and after each watering. This method of watering ensured that the upper layer of vermiculite:perlite, containing the majority of the nodule mass, was kept dry. Control pots for each treatment were watered according to the prestress schedule.

At intervals during each experimental period, samples from each treatment were obtained for determination of respiration rate, *ANA*, *TNA*, *PNA*, FW:DW of nodules, roots, leaves, and stems, and nodule water potential as described below.

Values of *TNA*, *PNA*, and OLC_N in drought-stressed plants were compared to those in plants inhibited by detopping. The plants used in the detopping treatment were grown in either silica sand or soil and were watered twice daily. On the day that drought stress was initiated, *ANA* and *TNA* were measured in these plants, and then their shoots were excised. Steady-state *ANA* was attained between 3 and 5 h after detopping, at which point *TNA* and *PNA* were measured. The plants were then harvested, and the nodules were dried for 72 h at 92°C and weighed.

Determination of Nodule Water Potential

The water potentials of nodules sampled in each experiment were determined from a plot of nodule FW:DW ratio

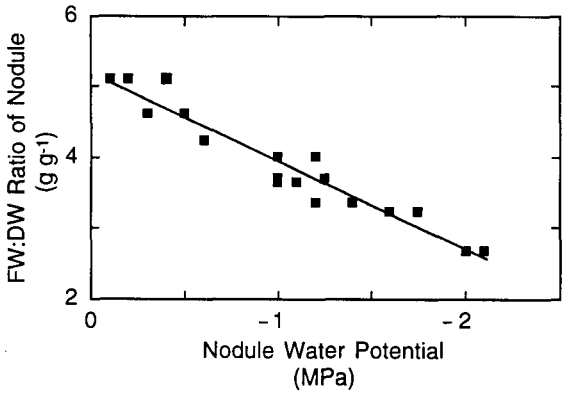

Figure 1. The relationship between the FW:DW ratio of nodules and their water potential as measured by Shardakov's method (Knipling, 1967). The line represents a linear regression through the points ($y = 1.25x + 5.20$; $r^2 = 0.92$).

against the water potential of nodules measured by Shardakov's method as described by Knipling (1967). Briefly, this involved the preparation of 11 mannitol solutions having water potentials of −0.05 to −2.0 MPa. A 1.8-mL aliquot of each solution was placed in a vial with five to seven excised nodules and incubated for 40 min at room temperature. Then, a single drop of a dyed mannitol solution (containing approximately 0.0007% [w/v] methylene blue) having the same water potential as that in the initial solution was released into the middle of the incubated solution using a pipette. The water potential of the nodule tissue was assumed to be equivalent to that of the solution equilibrated with the nodules in which the dyed mannitol drop remained stationary, neither sinking nor rising to the top of the cuvette. The water potential of nodules measured in this way was correlated with the FW:DW ratio of nodules from the same population. To obtain nodules that varied in their FW:DW ratio and water potential, the nodules were excised from the roots and left for various lengths of time to dry in air before FW:DW ratio and water potential were measured.

RESULTS

Relationship between Nodule FW:DW Ratio and Nodule Water Potential

When the nodule FW/DW ratio was plotted against the nodule water potential as measured by the Shardakov method, the data could be fitted to a linear regression (Fig. 1). This relationship was used to determine water potential in all experiments in which the nodule FW:DW ratio was measured. A previous study suggested that this approach results in estimates of water potential that are more negative than those made with a psychrometer (Pankhurst and Sprent, 1975). However, the method was assumed to reflect accurately relative changes in water potential of nodules subjected to different treatments.

Indications of Plant and Nodule Stress during Drought Treatments

In each treatment, the total DW of nodules increased throughout the course of the experiment. A similar increase was observed in the control nodules for each treatment and no significant differences were observed between the drought-stressed nodules and their respective controls at the end of each experimental period. Very little difference was observed between the final DWs of nodules grown in different media (Fig. 2A).

The FW:DW ratios of the control nodules in each treatment were similar and did not change during the course of the experiments, whereas the FW:DW ratios of nodules in all drought treatments declined (Fig. 2B). The most rapid drying of nodules occurred in the severely stressed plants, the FW:DW ratio declining to 60 ± 4% of its control value during the 3-d experimental period (Fig. 2B). This corresponded to a decline in nodule water potential from −0.44 ± 0.05 to −1.92 ± 0.19 MPa (Fig. 2B). Rapid dehydration of the severely stressed plants was also indicated by a sharp decline in the FW:DW ratio of the leaves and the roots, with the ratios declining to 46 ± 8 and 20 ± 1% of their control values,

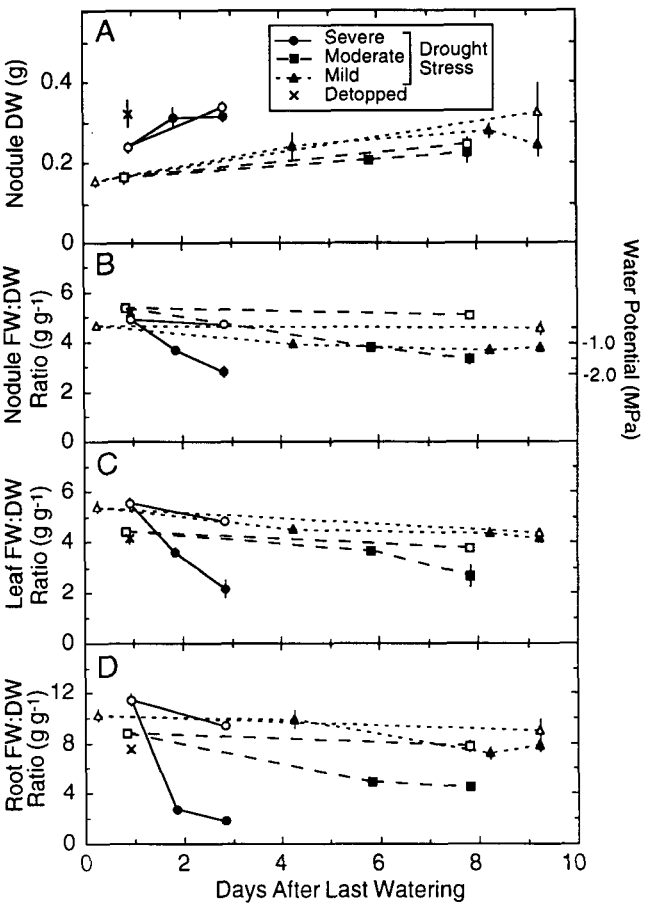

Figure 2. Time course of changes in total nodule DW (A); nodule FW:DW ratio and water potential (B); leaf FW:DW ratio (C); and root FW:DW ratio (D) in intact, attached soybean nodules subjected to mild (▲), moderate (■), and severe (●) drought stress and in nodules on detopped plants (X). Each symbol represents the mean ± SE of four replicate samples. Open symbols represent the control values for mild (Δ), moderate (□), and severe (○) drought-stress treatments.

respectively, during the experimental period (Fig. 2, C and D). Wilting of the plants was observed on the 2nd d after water was withheld.

Drying of nodules occurred more slowly in the moderately stressed plants, with the FW:DW ratio declining to 66 ± 4% of its control value during the 8-d period of the experiment (Fig. 2B). This was associated with a decline in nodule water potential from −0.24 ± 0.05 to −1.45 ± 0.18 MPa (Fig. 2B). FW:DW ratios of leaves and roots declined to 72 ± 13 and 58 ± 2% of their control values, respectively (Fig. 2, C and D), and wilting was apparent on the 5th d without watering.

The mildly stressed plants showed the slowest and smallest change in nodule FW:DW ratio, with the value declining to 83 ± 9% of the control value during the 9-d experimental period (Fig. 2B). This was associated with a decline in nodule water potential from −0.56 ± 0.19 to −1.10 ± 0.12 MPa (Fig. 2B). Leaf and root FW:DW ratios did not change with respect to controls during the experimental period (Fig. 2, C and D), and no wilting of the plants was observed. This indicates that watering the pot from the base had the desired effect of inducing drought stress in the nodules while maintaining a normal water balance in the tissues of the root and shoot.

The total DW of nodules in the detopped plants and their FW:DW ratios were similar to those in the control plants for each drought treatment (Fig. 2, A and B). The FW:DW ratios of the leaves and roots of the detopped plants were also similar to the ratios measured in control plants before detopping (Fig. 2, C and D).

Changes in Nitrogenase and Respiration Rate during Drought Treatments

Initial values of *TNA* in control plants of the severely, moderately, and mildly stressed treatments were 347 ± 26, 255 ± 21, and 273 ± 23 μmol g^{-1} DW nodule h^{-1}, respectively (Fig. 3). These values were within the range of those reported previously for uninhibited soybean-*Bradyrhizobium* symbioses grown in sand culture (Hunt and Layzell, 1993). *TNA* in control nodules of the severely and moderately stressed plants declined slightly during the course of the experiment, whereas *TNA* in the control nodules of the mildly stressed plants increased (Fig. 3). *TNA* values in all stressed nodules declined relative to controls during the experimental period, with the most rapid inhibition being observed in the severely stressed nodules in which *TNA* declined to 17 ± 7% of its control value after 3 d without watering (Fig. 3C). Severe inhibition of nitrogenase activity (to 9 ± 3% of control *TNA*) was also observed in the moderately stressed plants, but this occurred gradually during the 8-d time course of the experiment (Fig. 3B). The mildly stressed plants showed the smallest and slowest inhibition of nitrogenase activity, with *TNA* declining to 52 ± 15% of *TNA* in the control nodules 9 d after the treatment was initiated (Fig. 3A).

At all stages of the experiment, very little stimulation of *TNA* was observed in control nodules of each treatment as external pO_2 was increased gradually to 100% O_2. Consequently, *PNA* was very similar to *TNA*, the mean initial OLC_N of the control nodules in all treatments was 0.99 ± 0.01, and the mean final OLC_N was 0.98 ± 0.01 (Fig. 3). Very little stimulation of *TNA* by O_2 was observed in any of the drought-

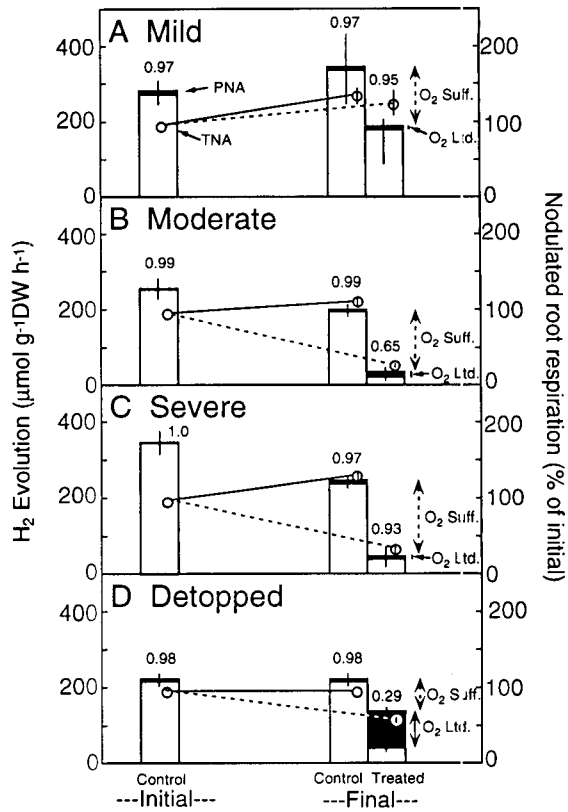

Figure 3. Changes in *TNA* (top of open bar), *PNA* (top of closed bar), and nodulated root respiration (line plots) in intact nodulated soybean roots subjected to mild (A), moderate (B), or severe (C) drought-stress treatments or in plants that were detopped (D). The numbers associated with each bar graph are values for OLC_N (=*TNA*/*PNA*). SE values for *TNA* and *PNA* are represented by lines extending below and above, respectively, the overlayed bar graphs. SE values (n = 4) for initial and final respiration rates are presented as bars passing through the symbols associated with the solid lines (control plants) and dashed lines (treated plants). Initial respiration rates (i.e. 100% value) for the mild, moderate, severe, and detopped treatments were 193 ± 19, 245 ± 7, 262 ± 8, and 244 ± 20 μmol CO_2 g^{-1} $DW_{nodulated\ root}$ h^{-1}, respectively. The times between initial and final measurements were 9, 8, and 3 d for the mild, moderate, and severe drought-stressed treatments, respectively, and 3 to 5 h for the detopped treatments. The vertical lines on the right side of the figure indicate the proportion of the total decline in nitrogenase activity that was attributed to either O_2-limited nodule metabolism (O_2 Ltd.) or to some other inhibitory mechanism that occurs under O_2 sufficient (O_2 Suff.) conditions.

stressed nodules, although the degree of stimulation differed depending on the treatment and its duration. Within 2 d of imposition of the severe stress treatment, *TNA* declined to a greater degree than *PNA*, resulting in an OLC_N value of 0.75 ± 0.04 (time course not shown, but see Fig. 5B). However, after 3 d of this treatment, when both *TNA* and *PNA* had declined to very low values relative to controls, OLC_N (0.93 ± 0.03) was similar to that in the control nodules (Fig. 3C). In the moderately stressed nodules, OLC_N declined from the initial control value of 0.99 ± 0.01 to 0.67 ± 0.10 within 6 d and to 0.64 ± 0.10 within 8 d of initiation of the treatment

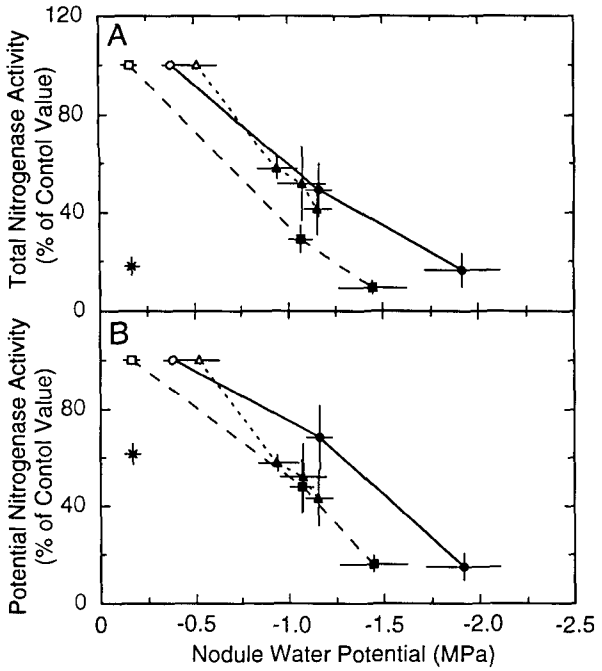

Figure 4. The relationship between nodule water potential and *TNA* (A) and *PNA* (B) in intact, attached soybean nodules subjected to mild (▲), moderate (■), and severe (●) drought stress and in nodules on detopped plants (X). Each symbol represents the mean ± SE of four replicate samples for each parameter. Initial control values (open symbols) for *TNA* were 273 ± 23, 255 ± 21, 347 ± 27, and 218 ± 9 μmol g^{-1} DW_{nodule} h^{-1} and for *PNA* were 282 ± 22, 257 ± 21, 347 ± 27, and 222 ± 10 μmol g^{-1} DW_{nodule} h^{-1} in the mild, moderate, severe, and detopped treatments, respectively.

(Fig. 3B, and see Fig. 5B). In the mildly stressed nodules, OLC_N did not change significantly from control values throughout the course of the experiment (Fig. 3A and see Fig. 5B).

The changes in OLC_N observed in the severely and moderately stressed nodules were much smaller than those which occurred in nodules after plants were detopped. Within 3 to 5 h of detopping, *TNA* had declined to 18 ± 3% of its initial value (Fig. 3D), a degree of inhibition that was observed in severely drought-stressed nodules after 3 d, and in moderately drought-stressed nodules after 8 d, without watering. However, in contrast to the drought-stressed nodules, increasing pO_2 in the rhizosphere of the detopped plants caused a recovery of *TNA* to 62 ± 4% of the control value, resulting in a mean OLC_N value of 0.29 ± 0.04 (Fig. 3D).

Respiration rates of nodulated roots in the severely and moderately stressed plants showed trends similar to nitrogenase activities, declining to 28 ± 5 and 28 ± 2%, respectively, of initial values during the course of the treatments, whereas rates of respiration of their respective control nodules did not change during the experiment (Fig. 3, B and C). In the mildly stressed treatment, respiration rates of nodulated roots in both the control and treated plants increased slightly during the 9-d experimental treatment (Fig. 3A). Respiration rates of plants prior to detopping were similar to those of the control plants in the drought treatments (Fig. 3D). After detopping, respiration rate declined gradually to reach a value that was 49 ± 3% of the initial value after 3 to 5 h.

The Relationship between Nodule Water Potential and Nitrogenase Activity

When *TNA* measured in all treatments was plotted against nodule water potential, a positive correlation was obtained, i.e. a lower *TNA* was associated with a more negative water potential (Fig. 4A). A very similar relationship was observed when *PNA* was plotted against nodule water potential, indicating that *TNA* and *PNA* declined in tandem as drought stress was imposed. Water potential in the nodules of detopped plants remained similar to that in control nodules as nitrogenase activity was inhibited (Fig. 4A), and there was no change in nodule water potential as *TNA* increased during measurement of *PNA* (Fig. 4B).

O_2 Limitation of Nitrogenase Activity in Drought-Stressed Nodules

No apparent relationship was observed between the OLC_N and nodule water potential (Fig. 5A). The degree of O_2 limitation of nitrogenase activity appeared to depend more on the manner in which the stress was imposed than on the magnitude of the decline in water potential or nitrogenase activity. For example, OLC_N values of mildly stressed nodules at a water potential of −1.16 ± 0.06 MPa did not differ from those of control nodules (water potential = −0.47 ± 0.04), whereas at a similar nodule water potential, OLC_N values in both the severely and moderately stressed nodules were significantly lower than in control nodules. Also, OLC_N in the

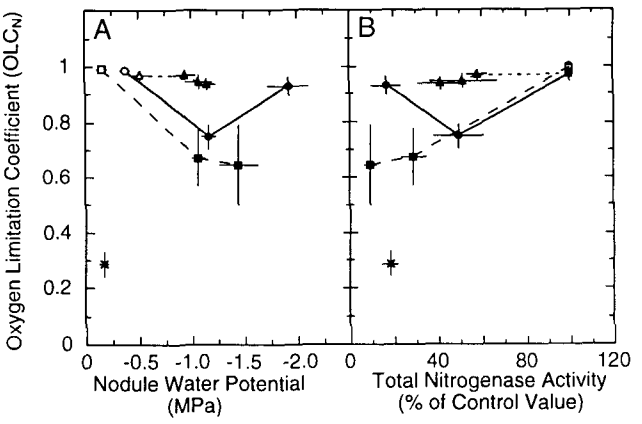

Figure 5. The relationship between the OLC_N of nitrogenase and either nodule water potential (A) or *TNA* (B) in intact attached nodules of soybean subjected to mild (▲), moderate (■), and severe (●) drought stress and in nodules on detopped plants (X). Each symbol represents the mean ± SE of four replicate samples for each parameter. Initial control values (open symbols) for *TNA* were the same as in Figure 4. Initial control values for nodule water potential were −0.52 ± 0.01, −0.16 ± 0.04, −0.38 ± 0.04, and −0.17 ± 0.04 MPa in the mild, moderate, severe, and detopped treatments, respectively.

severely stressed nodules increased again as water potential reached its most negative value.

Similarly, no relationship was observed between the degree of O_2 limitation of nitrogenase activity and *TNA* in the drought-stressed nodules (Fig. 5B). The mildly stressed nodules showed no change in OLC_N as their *TNA* values declined, whereas OLC_N declined slightly in the moderately stressed nodules and remained stable at this reduced value as *TNA* declined further. In the severely stressed nodules OLC_N declined initially with *TNA* and then increased again as *TNA* reached its lowest value. The single value of OLC_N measured in the detopped plants was significantly lower than any OLC_N in the drought-stressed nodules at any nodule water potential or *TNA* value.

DISCUSSION

O_2 Limitation Is Not the Primary Cause of Nitrogenase Inhibition during Drought Stress

Many environmental factors that inhibit nitrogenase activity do so by causing a decrease in nodule permeability to O_2, resulting in an O_2 limitation of nitrogenase-linked respiration (Hunt and Layzell, 1993). This "O_2-limited" inhibition is recoverable by elevating rhizosphere pO_2 and accounts for approximately 62% of the total inhibition of nitrogenase that occurs when plants are detopped (Fig. 3D). The remaining "O_2-sufficient" inhibition is defined as that component of inhibition that is not recoverable by elevating pO_2 and, therefore, due to a decrease in the maximum O_2-sufficient rate of nodule respiration or nitrogenase activity. In contrast to previous studies (Pankhurst and Sprent, 1975; Weisz et al., 1985; Durand et al., 1987), our results show that O_2-sufficient, rather than O_2-limited, inhibition was predominant in nodules under drought conditions (Fig. 3, A–C). Irrespective of the manner in which drought stress was imposed or the duration and severity of the drought treatment, O_2-limited inhibition never exceeded 36% of the total nitrogenase inhibition observed. Therefore, a decline in the maximum O_2-sufficient rate of nodule respiration or nitrogenase activity, rather than a direct O_2 limitation of nitrogenase-linked respiration, was determined to be the major cause of nitrogenase inhibition in the drought-stressed nodules.

Some stimulation of nitrogenase activity by O_2 was observed in the moderately and severely stressed nodules during the initial decline in nodule water potential and nitrogenase activity as indicated by their reduced values of OLC_N (Fig. 5A). However, OLC_N in the mildly stressed nodules did not change from control values even though water potential and nitrogenase activity declined to an extent similar to that in the other two treatments (Figs. 4A and 5A). This lack of a correlation between OLC_N and nodule water potential supports the conclusion that reduced nodule permeability to O_2 was not the major cause of nitrogenase inhibition during the drought treatments. A reason why this conclusion differs from those reached in previous studies may be that nitrogenase activity in the previous studies was measured by the acetylene reduction assay in either detached nodules (Pankhurst and Sprent, 1975) or in intact nodulated roots (Weisz et al., 1985; Durand et al., 1987). Exposure to 10% acetylene has been shown to cause nitrogenase inhibition in soybean nodules by reducing nodule permeability to O_2 (Minchin et al., 1983; Witty et al., 1984), and nodule detachment has been shown to have a similar inhibitory mechanism (Hunt et al., 1987; Sung et al., 1991). Therefore, the O_2-limited inhibition of nitrogenase reported in previous studies of the effect of drought may have been caused by the experimental methods used rather than by the imposed drought treatments.

This conclusion is supported by a re-evaluation of the data presented by Weisz and co-workers (1985). In that study, elevated pO_2 increased nitrogenase activity in drought-stressed plants to levels similar to those in control plants under normal atmospheric conditions, leading to the conclusion that drought stress inhibits nitrogenase activity by O_2 limitation. However, when rhizosphere pO_2 was increased in the control plants, nitrogenase activity was stimulated to a much greater extent than in the drought-stressed plants, indicating that the control plants themselves were O_2 limited and that the drought treatment actually induced a decline in the V_{max} of nitrogenase (i.e. O_2-sufficient inhibition). In this way, nitrogenase inhibition by drought stress seems to be less like detopping and more like low-temperature inhibition of nitrogenase activity; this is another treatment that has recently been shown to be associated with an apparent decrease in the V_{max} of nodule respiration (Kuzma and Layzell, 1994).

Treatment Effects on Nitrogenase Inhibition during Drought Stress

It was interesting that the degree of O_2 limitation of nitrogenase activity in the drought-stressed nodules differed according to the method used to impose the stress. In the mildly stressed treatment, the roots of the plants were watered daily from the base of the pot, with the result that the FW:DW ratio of the leaves remained close to control values and no plant wilting was observed (Fig. 2C). These plants showed no change in OLC_N as nitrogenase activity and nodule water potential declined (Fig. 5). Plants in the other two treatments did not receive any water during the course of the experiment and wilting was observed as the FW:DW ratio of the leaves declined. At the same nodule water potential of approximately −1.0 MPa, nitrogenase activity in the nodules of these plants was more O_2 limited than that in the mildly stressed plants (Fig. 5A). Also, the greatest inhibition of *TNA* and the greatest degree of O_2 limitation of nitrogenase activity occurred in the moderately stressed plants at a nodule water potential that was less negative than that in the severely stressed plants (Fig. 5A). Together, these data indicate that some factor(s) other than nodule water potential itself must be involved in the O_2-recoverable component of nitrogenase inhibition.

It is possible that the reversible component of inhibition in the moderately and severely stressed nodules resulted from a reduction of photosynthate allocation to the nodules as wilting occurred and is, therefore, similar to the inhibition that occurred in the detopped plants. Alternatively, the O_2-reversible inhibition may be due to a reduction in the leghemoglobin content of the nodules (Khanna-Chopra et al., 1984; Guerin et al., 1990, 1991), causing O_2 limitation of

nitrogenase activity by reduced facilitated diffusion of O_2 to the bacteroids. However, neither of these hypotheses accounts for the increase in OLC_N that occurred in the severely stressed nodules as nodule water potential and nitrogenase activity declined to their lowest values (Fig. 5B). One explanation is that at such low water potential the nodules lost their capacity to regulate internal O_2 concentration (*Oi*), with the result that *Oi* increased to a level at which nitrogenase activity was no longer O_2 limited or to a level causing irreversible nitrogenase inhibition. It is also pertinent that respiratory capacity may be reduced in isolated bacteroids subjected to drought stress (Guerin et al., 1990). If the bacteroids were affected in such a way in nodules subjected to severe dehydration, increased flux of O_2 to the sites of nitrogenase-linked respiration would not be expected to recover nitrogenase activity and OLC_N would remain close to unity.

Nodule Permeability and Irreversible Inhibition of Nitrogenase Activity during Drought Stress

Little is known about the mechanisms that may account for the decline in V_{max} of nitrogenase activity and nitrogenase-linked respiration during drought. However, one consequence of a reduced nodule water content would be an increase in cytoplasmic salt concentration leading to the inhibition of enzyme activity. This and the proteolytic breakdown of nodule enzymes that occurs under severe drought stress (Guerin et al., 1991) would cause a reduction in nodule respiratory rate with a consequent increase in *Oi*. During the period in which the nodule maintains its capacity to regulate *Oi*, a reduction in respiration rate would cause nodule permeability to decrease, thereby protecting nitrogenase from O_2 inactivation. Therefore, *Oi* might be expected to remain at a fairly constant level as nitrogenase activity and permeability declined. This sequence of events would account for the decrease in nodule permeability that has been measured in a previous study of drought stress (Weisz et al., 1985) but would attribute the decreased permeability as a consequence rather than a cause of reduced nodule metabolic activity. This hypothesis is currently being tested using nodule oximetry (Denison and Layzell, 1991) to measure *Oi* and nodule permeability as drought stress is imposed.

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