Bacteroid Proline Catabolism Affects N\textsubscript{2} Fixation Rate of Drought-Stressed Soybeans\textsuperscript{1}

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In prior work, we observed that soybean (\textit{Glycine max} L. cv Merr.) seeds inoculated with a mutant \textit{Bradyrhizobium japonicum} strain unable to catabolize Pro (Pro dehydrogenase\textsuperscript{-} [ProDH\textsuperscript{-}]) resulted in plants that, when forced to depend on N\textsubscript{2} fixation as the sole source of nitrogen and subjected to mild drought stress, suffered twice as large a loss in seed yield as did plants inoculated with the parental strain. Here, we used a continuous gas flow system to measure H\textsubscript{2} evolution as a function of time in five replicate experiments, the slope in inoculated with the parental strain. Here, we used a continuous gas flow system to measure H\textsubscript{2} evolution as a function of time and leaf water potential (\( \Psi_l \)). Since one H\textsubscript{2} is produced for every N\textsubscript{2} fixed as an obligate part of the mechanism of N\textsubscript{2} fixation, these measurements serve as the basis for continuous monitoring of the N\textsubscript{2} fixation rate. In five replicate experiments, the slope of the decline in N\textsubscript{2} fixation rate in response to water stress was always greater for plants inoculated with the mutant strain unable to catabolize Pro or take up H\textsubscript{2} (ProDH\textsuperscript{-}, \textit{hup}) than it was for plants inoculated with the parental strain (ProDH\textsuperscript{+}, \textit{hup}). In aggregate, the probability that this difference occurred by chance alone was 0.005. In combination with the earlier result, this is consistent with bacteroid catabolism of Pro synthesized in response to mild drought stress having a positive impact on N\textsubscript{2} fixation rate and seed yield.

Evidence supporting a role for Pro in protecting the N\textsubscript{2}-fixing machinery from injury during mild drought stress has been reviewed by Kohl et al. (1994) and is briefly outlined below. (1) Bacteroids in 55-d-old soybean (\textit{Glycine max} L. cv Merr.) plants subjected to short-term drought stress showed a 4-fold increase in Pro concentration and a 1.6-fold increase in bacteroid Pro dehydrogenase (ProDH) activity (Kohl et al., 1991). (2) In a large greenhouse experiment (30 pots per treatment), soybean plants were forced to depend on atmospheric N\textsubscript{2} and subjected to repeated mild drought stress. Seed yields of mature plants inoculated with a mutant \textit{Bradyrhizobium japonicum} strain unable to catabolize Pro (ProDH\textsuperscript{-}) decreased twice as much as did plants inoculated with the parental strain (ProDH\textsuperscript{+}; Straub et al., 1997). (3) Pro supplied to greenhouse-grown soybean plants stimulated acetylene-reducing activity (the conventional method for measuring nitrogenase activity) to the same extent as did the same quantity of succinate or Glu (Zhu et al., 1992), indicating that Pro is able to supply reduced carbon to support N\textsubscript{2} fixation as well as did succinate or Glu. (4) Twenty-four hours after irrigation with Pro, bacteroids from Pro-treated plants had about 8 times the amount of free Pro as did plants supplied with only water (Zhu et al., 1992). This occurred despite lack of evidence of active transport of Pro across the peribacteroid membrane.

RESULTS

Preliminary Experiments

H\textsubscript{2} evolution rate was measured continuously during imposition of drought and during recovery after rewetering. In order to relate N\textsubscript{2} fixation rate to water status, the following preliminary experiments were necessary. (1) Since we wanted to determine N\textsubscript{2} fixation rates by measuring rates of H\textsubscript{2} evolution, and since nitrogenase can reduce both H\textsuperscript{2} and N\textsubscript{2}, it was necessary to measure the relationship between H\textsubscript{2} evolution rate and N\textsubscript{2} fixation rate. In five replicate experiments, the slope of the decline in N\textsubscript{2} fixation rate in response to water stress was always greater for plants inoculated with the mutant strain unable to catabolize Pro or take up H\textsubscript{2} (ProDH\textsuperscript{-}, \textit{hup}) than it was for plants inoculated with the parental strain (ProDH\textsuperscript{+}, \textit{hup}). In aggregate, the probability that this difference occurred by chance alone was 0.005. In combination with the earlier result, this is consistent with bacteroid catabolism of Pro synthesized in response to mild drought stress having a positive impact on N\textsubscript{2} fixation rate and seed yield.

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evolution and N₂ fixation under all of the experimental conditions to be used. (2) Because of the length of time (≥24 h) required for well-watered plants to become stressed and the marked decrease of N₂ fixation in the dark, it was necessary to determine whether there was an entrained diurnal effect that would influence the N₂ fixation rate of plants being exposed to continuous light. (3) We were interested in the N₂ fixation rate as a function of plant water status. The best measure of water status would have been to measure nodule water potential (ψᵣ). However, since the plants were to be monitored continuously during imposition of stress, it was not possible to measure ψᵣ during this period, since, in order to do this, it would have been necessary to uproot plants during the experiment. Rather, we measured leaf water potential (ψₛ) and determined the relationship between ψᵣ and ψₛ.

Relationship between H₂ Evolution Rate and N₂ Fixation Rate

The electron allocation coefficient (EAC) is a convenient reflection of the partitioning of electrons between protons and N₂, both of which can serve as substrates for nitrogenase (Edie and Phillips, 1983; Hunt et al., 1987).

\[
\text{EAC} = \frac{\text{electron flow to N}_2 \text{ fixation}}{\text{total electron flow}} = \frac{(\text{H}_2 \text{ produced in Ar}:\text{O}_2) - (\text{H}_2 \text{ produced in air})}{\text{H}_2 \text{ produced in Ar}:\text{O}_2}, \quad (1)
\]

where H₂ produced in air is the rate of H₂ production in atmosphere (21% O₂:79% N₂) and H₂ production in Ar:O₂ is the rate of H₂ production in 21% O₂:79% Ar. Taking into account that the reduction of N₂ to NH₃ requires six electrons while reduction of 2H⁺ to H₂ requires two electrons, that the numerator is 3 times the rate of N₂ production and that the denominator is 3 times the rate of N₂ production plus the rate of production of H₂ in air, Equation 1 can be transformed into Equation 2.

\[
\text{N}_2 \text{ fixation rate} = \frac{\text{H}_2 \text{ evolution rate} \times \text{EAC}}{3(1 - \text{EAC})} \quad (2)
\]

The maximum value of EAC is 0.75 since of the eight e⁻ required, only six are used to reduce N₂ to two NH₃. EAC values for soybean have been found to lie between 0.6 and 0.7 (Hunt and Layzell, 1993). The measurement of EAC is described in “Materials and Methods.”

Figure 1 shows that EAC did not vary with ψₛ. Therefore, H₂ evolution rate is proportional to N₂ fixation rate under the conditions of our experiments. This greatly simplified the experiments since determination of EAC is a time-consuming process (see “Materials and Methods”).

Diurnal Effect on N₂ Fixation

Figure 2 shows no entrained diurnal effect on N₂ fixation.

The Relationship between ψₛ and ψᵣ

While the water status of nodules (ψᵣ) is the relevant parameter, we measured ψₛ in order to avoid sacrificing plants during the course of the experiments. Figure 3 shows that the relationship between ψₛ and ψᵣ is linear over the entire time during which water was withheld and as plants recovered after rewatering. Thus, ψₛ can serve as a proxy for ψᵣ. The relationship between ψₛ and ψᵣ is essentially the same whether plants are undergoing dehydration or recovery.

ψₛ was measured up to six times during imposition of drought. While ψₛ can vary significantly from node to node, we found no significant difference in leaflets from the oldest nonsenescent node versus those from the second oldest nonsenescent node, nor from one leaflet to another at a single node (data not shown). This permitted six measurements per plant during an experiment: one leaflet from a single node three...
different times and one leaflet from a second node three different times.

Effect of Symbiont Strain on the Relationship between \( \Psi_L \) and N\(_2\) Fixation Rate

Figure 4 shows the results of a typical experiment. \( \Psi_L \) are indicated by rectangles. The average value for well-watered plants was \(-0.63 \pm 0.01 \) MPa (\( n = 55 \)). The N\(_2\) fixation rate for the well-watered control was constant over the course of the experiment (for example, see Fig. 2). In the example shown in Figure 4, N\(_2\) fixation rates began to drop 27 and 34 h after the last watering. In other experiments, the onset of the decline varied over a wide range of time since the last watering, depending on plant size, packing of support medium, etc. In the experiments that produced the data shown in Figure 4, the N\(_2\)-fixing rate of the well-watered plants was about the same (36–38 \( \mu \)mol N\(_2\) hr\(^{-1}\) g dry weight nodule\(^{-1}\)). However, in other experiments the rate varied up to 2-fold depending on plant size, density of nodules, etc. Leaves were visibly wilted when plants were rewatered (data not shown). After rewatering, plants returned to the prestress level of N\(_2\) fixation rate. Because of plant-to-plant variation in N\(_2\) fixation rate of well-watered plants, N\(_2\) fixation rates were expressed as percent of the well-watered value for the same plant. It is clear from Figure 4 that N\(_2\) fixation rate and \( \Psi_L \) both declined with time, as was expected, a reassuring verification that we were measuring a physiological response.

Figure 5 shows the relationship between N\(_2\) fixation rate and \( \Psi_L \) in the best of five experiments. This experiment was done 40 DAP. Results with plants inoculated with the parental strain (ProDH\(^+\)) are indicated by the dashed line, and those inoculated with the ProDH\(^-\) strain are indicated by the solid line. Values of \( \Psi_L \) at different times during the experiment are indicated in the boxes. The average value of \( \Psi_L \) for well-watered plants was \(-0.63 \pm 0.01 \) MPa (\( n = 55 \)).

During recovery, both \( \Psi_L \) and N\(_2\) fixation increased (Fig. 6). However they did not increase in synchrony. N\(_2\) fixation showed a transient decrease following rewatering, possibly due to temporary water logging. By the time N\(_2\) fixation rates showed any increase over the rate at the time of rewatering (3 h after rewatering), \( \Psi_L \) had recovered about 65% of full turgor. This is not surprising since restoration of \( \Psi_L \) is a physical process, while recovery of N\(_2\) fixation is physiological. Because rate of plants whose bacteroids were unable to catabolize Pro (ProDH\(^-\)) was greater than it was for plants inoculated with ProDH\(^+\) B. japonicum (\( P = 0.03 \)). Data for all five experiments are given in Table I below.

In five replicate experiments, the slope of the decline in N\(_2\) fixation rate as a function of \( \Psi_L \) of plants whose bacteroids were unable to catabolize Pro (ProDH\(^-\)) was always greater than it was for ProDH\(^+\) plants. The probability of a significant difference in four of the five replicate experiments was >0.05. However, since the difference was always in the same direction, very robust statistical differences emerge (\( P = 0.005 \); Table I).

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of the difference between \( \Psi_1 \) and \( \Psi_{\text{N}_2} \) fixation in timing of recovery, there is no basis for determining whether there is a difference between plants inoculated with ProDH\(^1\) or ProDH\(^2\) strains in recovery of \( \text{N}_2 \) fixation rate as a function of \( \Psi_1 \).

An alternative approach is to examine recovery as a function of time. However, even with curve-fitting techniques, we were sometimes unable to develop objective criteria for choosing the time at which to calculate the rate of change of \( \text{N}_2 \) fixation rate, the parameter we wanted to compare for the two plant types. In the remaining, valid data set, the plant-to-plant variation within replicates exceeded the differences between the strains. Thus, our data provide evidence neither for nor against a difference in recovery rates due to strain differences.

**DISCUSSION**

The following are among the interesting results reported here. The efficiency of \( \text{N}_2 \) fixation (EAC) did not decrease as plants became drought stressed (Fig. 1). Unlike many biological processes, the \( \text{N}_2 \) fixation rate did not exhibit an entrained diurnal rhythm (Fig. 2). Despite leaves receiving their water from the xylem and nodules from the phloem, \( \Psi_1 \) was an excellent proxy for \( \Psi_{\text{N}_2} \) (Fig. 3). Besides being interesting results in themselves, these results decreased the difficulty of testing the hypothesis stated in the introduction; namely, the inability to catabolize Pro might result in a steeper rate of decline of the \( \text{N}_2 \) fixation rate in response to mild drought stress or in its slower recovery after rewatering. The quality of the data did not allow us to test the relative rates of recovery from mild drought stress. However, the results clearly show that the ability of bacteroids to catabolize accumulated Pro resulted in a less steep decline in \( \text{N}_2 \) fixation rate when plants were subjected to mild drought stress (Table 1).

It has been suggested that the Pro accumulating in response to drought stress might serve to stabilize protein structure (Schobert and Tschesche, 1978) and/or as an osmoprotectant (Kauss, 1977). We have hypothesized that the energy derived from oxidation of Pro might be useful to bacteroids. If the former were the case and those effects were the dominant manifestation of Pro accumulation, then we would expect plants inoculated with ProDH\(^2\) *B. japonicum* to perform better than those inoculated with a ProDH\(^1\) strain.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>ProDH</th>
<th>Slope</th>
<th>( R^2 )</th>
<th>Difference in Slope between Strains</th>
<th>% Difference</th>
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<tr>
<td>1</td>
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</table>

**Table 1. Decline in \( \text{N}_2 \) fixation rate (as percent of well-watered control) with \( \Psi_1 \)**

Mean of values in column 5 = 14.44; \( t \) value for calculating whether 14.4 > 0, \( t = 4.57 \); \( P = 0.005 \).

**Figure 6.** Relationship between \( \text{N}_2 \) fixation rate and \( \Psi_1 \) versus time after rewatering drought-stressed plants. Soybean plants (42 DAP) were inoculated with either ProDH\(^+\) or ProDH\(^-\) strains of *B. japonicum*. \( \text{N}_2 \) fixation rates are indicated by black symbols (circles for ProDH\(^+\) and triangles for ProDH\(^-\)), and \( \Psi_1 \) are indicated by white symbols (squares for ProDH\(^+\) and triangles for ProDH\(^-\)).

**Figure 7.** Cartoon of gas flow system used for this work, including gases for measuring \( \text{H}_2 \) evolution as well as for EAC determinations.
However, in our experiments, the opposite is the case. It is also possible that the enhanced respiration resulting from the catabolism of Pro might have affected the O$_2$ diffusion barrier (Denison and Layzell, 1991), which in turn could have affected N$_2$ fixation rate. Other indirect effects are also possible. We are unable to specify the mechanism responsible for the better performance of plants whose nodule bacteroids were able to catabolize Pro.

The results reported in this paper, together with prior results described in the introduction, support the proposition that the ability of B. japonicum bacteroids to catabolize Pro imparts benefit to the N$_2$-fixing machinery as the plants become drought stressed. This conclusion is not in conflict with the absence of evidence for a Pro porter in the PBM. By whatever mechanism, Pro can move from the cytosol through the PBM and into bacteroids as discussed in the introduction. The rate of Pro uptake can be substantial compared with that of malate. For example, Pedersen et al. (1996) investigated the rate of uptake of malate and Pro into bacteroids within intact, peribacteroid units (symbiosomes) isolated in subambient oxygen. The rate of Pro uptake was 3 times that reported by Udvardi et al. (1990) for uptake into intact symbiosomes that were isolated in ambient oxygen. Pedersen et al. (1996) reported an approximately equal rate of uptake of malate (1 mm) versus Pro (2 mm). There may well be twice or more the amount of Pro versus malate in the cytosol of drought-stressed infected nodule cells. The photosynthetic rate (and presumably the quantity of malate in the nodules) declines sharply under drought-stressed conditions, while the amount of nodular Pro increases sharply (4-fold; Kohl et al., 1991).

While the importation of tricarboxylic acids from the host to the bacteroids provides the main reduced carbon source supporting N$_2$ fixation when photosynthesis is vigorous, under conditions of water stress, photosynthesize supplies decrease. In that circumstance, an alternative reduced carbon source might be useful to bacteroids, as discussed above. Pedersen et al. (1996) showed that nitrogenase activity supported by Pro was 8-fold higher in bacteroids from drought-stressed nodules than in bacteroids from control nodules, while there was no significant response to drought stress in the rate of nitrogenase activity supported by malate.

Soybeans growing in the United States Midwest suffer mild drought stress on most days during the growing season. By 2 PM these leaves are often visibly wilted. Since the catabolism of stress-induced Pro appears to provide some benefit to the N$_2$-fixing machinery, it would be interesting to be able to direct more Pro into bacteroids. Engineering a Pro porter into the PBM and/or overexpressing it there might accomplish this. The sequences for a PBM-specific targeting transporter (Delauney et al., 1990) and for an Escherichia coli Pro porter (Culham et al., 1992) are known. This information should make it possible to accomplish this goal.

### MATERIALS AND METHODS

#### Bacterial Inoculant and Plant Material

Two strains of Bradyrhizobium japonicum were used in the study. JH47 (JH47::hup::TriS KM$^+$ into hup locus) was able to catabolize Pro. KLI (JH47[prn::pkiI-Prn::pyrAT locus]) was made from this parent (Straub et al., 1996). KLI was unable to catabolize Pro. Neither had a functional H$_2$ uptake system (hup$^-$). Strains were grown in yeast extract/manitol (Dalton, 1980) medium in the presence of the appropriate antibiotics (100 µg/ml rifampicin and 100 µg/ml kanamycin for both strains and, in addition, 100 µg/ml of spectinomycin for KLI). Cultures were immobilized on peat (materials and protocol were provided by John Koslaski, Liphatech, Nitrogen Division, Milwaukee, WI).

Soybean Glycine max L. cv. Merr. seeds (Williams 82) were inoculated with either JH47 or KLI. Five seeds per pot were planted in Hi Dry (Sud Chemie Absorbents, Munich), a calcined clay containing less than 0.06 mg N g$^{-1}$, and able to hold 60% of its dry weight as water (data not shown). The pots were fabricated from 6-inch PVC pipe. Pots were 6 inches tall with a drainage tube near the bottom of the pot, which served as well for gas flow. After germination, plants were thinned to one per pot. Supplemental illumination (800 photons m$^{-2}$ s$^{-1}$ as measured with a LI-COR model LI6500B detector) at plant height) was provided. Plants were fertilized with a nitrogen-free mineral medium (Fishebeck et al., 1973). One week prior to the start of each experiment, plants were moved to the growth chamber (CMP 3244; Conviron, Winnipeg, Canada) used for gas exchange measurements.

#### Determination of the Rate of N$_2$ Fixation

All of the experiments reported here depend upon measurements of the H$_2$ evolved by nodules infected with hup$^+$ strains of B. japonicum, either with or without the ability to catabolize Pro. The N$_2$ fixation rate was calculated from the rate of H$_2$ evolution (Layzell et al., 1984) as given in Equations 1 and 2 in “Results.”

N$_2$ fixation rate is a function of H$_2$ fixation rate and EAC (Eq. 2). H$_2$ evolution rate was measured as described below. EAC was calculated based on Equation 1 in “Results.”

When measuring H$_2$ evolution rate in Ar:O$_2$ as required by Equation 1, care was taken to limit exposure to Ar to the briefest time necessary to make the measurement (6 mins), since nitrogenase activity is inhibited by Ar and to wait until H$_2$ evolution rates had returned to their pre-Ar exposure levels (approximately 6 h) before using H$_2$ evolution rate data.

Measurement of H$_2$ was done in a gas flow system similar to that described by Layzell et al. (1989). The system was fabricated by Qubit Systems, Kingston, Ontario. A diagram of the system is shown in Figure 7. It has eight ports accommodating six plants, the other two ports being used for reference gas (air) and for calibration. It includes an O$_3$ sensor in order to make sure that the O$_3$ concentration in the N$_2$:O$_2$ gas mixture was the same as that of the Ar:O$_2$ mixture, since (1) nitrogenase activity is dependent on O$_3$ concentration and (2) the output of the H$_2$ detector is dependent on the composition of the gas mixture. The system also includes an infrared CO$_2$ analyzer, which allows determination of the effect of stress on root respiration and nodule conductance. The H$_2$ and CO$_2$ analyzers were calibrated with gasses of known composition and concentrations. Several concentrations of H$_2$ with both air and Ar:O$_2$ are required since the output of the detector depends on the gas composition.

#### Preparation of Plants for Gas Exchange Measurements

One week prior to the start of each experiment, plants were moved to a growth chamber used for gas exchange measurements held at a constant temperature (22°C). The sharp decrease in N$_2$ fixation during dark periods (data not shown) complicated interpretation of the decrease due to drought, since experiments typically lasted about 72 h. For this reason, we acclimated the plants in 24 h of light (300 photons m$^{-2}$ s$^{-1}$ at plant height). Preliminary experiments established that there was no entrained diurnal effect on N$_2$ fixation.

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output of the $H_2$ detector varies with relative humidity. The rate of $H_2$ evolution from each plant was measured 6 min before cycling to the next port. The system can accommodate up to six plants (Fig. 7). Initially, we used four ports of the system for two drought-stressed plants inoculated with the ProDH$^+$ strain and two ports for well-watered controls. Once it was established that the $N_2$ fixation rate of the well-watered controls was constant, three plants inoculated with the ProDH$^+$ strain and three plants inoculated with the ProDH$^-$ strain were connected to the six ports. After a stable $H_2$ evolution was established in well-watered plants, drought was imposed by withholding water.

Estimation of the Degree of Water Stress

$\Psi_f$ and $\Psi_s$ were measured by dew point psychrometry (HR 33Y Dew point microvolt meter with thermocouple psychrometers: Wescor, Logan, UT). Preliminary experiments showed a very high correlation between $\Psi_f$ and $\Psi_s$ (Fig. 3). Therefore, $\Psi_f$ was used as a proxy for water status of the nodule. $\Psi_s$ measurements would have required uprooting the plant, making it impossible to make measurements of $H_2$ evolution over the course of the experiment. $\Psi_f$ was measured on one leaflet from the lowest two nonsenescent nodes. Since there were no significant differences between $\Psi_f$ for the three leaflets on either node or for the average between the two nodes, we made up to six measurements of $\Psi_f$ per plant during an experiment.

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