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Effects of Ambient Oxygen and of Fixed Nitrogen on Concentrations of Glutathione, Ascorbate, and Associated Enzymes in Soybean Root Nodules[1]

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ABSTRACT

Soybean (*Glycine max* [L.] Merr.) root nodules contain the enzymes of the ascorbate-glutathione cycle for defense against activated forms of oxygen. Nodulated roots of hydroponically grown soybean plants were exposed to atmospheres containing 2, 21, 50, or alternating 21 and 50 kilopascals of O_2. The activities of ascorbate (ASC) peroxidase, monodehydroascorbate (MDHA) reductase, dehydroascorbate (DHA) reductase, and glutathione (GSSG) reductase were higher in nodules exposed to high pO_2. Nodule contents of ascorbate and reduced glutathione were also greater in the high pO_2 treatments. Treatment of nodulated plants with fixed nitrogen (urea) led to concomitant decreases in acetylene reduction activity, in leghemoglobin content, and in activities of ASC peroxidase, DHA reductase, and GSSG reductase. Activity of MDHA reductase and glutathione concentrations in nodules were not affected by treatment with urea. The enzymes of the ascorbate-glutathione cycle were also detected in uninfected soybean roots, although at levels substantially below those in nodules. These observations indicate that the ascorbate-glutathione cycle can adjust to varying physiological conditions in nodules and that there is a key link between N_2 fixation and defenses against activated forms of oxygen.

The oxygen relations of legume root nodules are extremely important in maintaining normal nodule functions. There are many aspects to the relationship between oxygen and N_2 fixation in nodules. The essential role of leghemoglobin as a O_2-binding protein and the O_2 sensitivity of nitrogenase have long been recognized as fundamentally important factors in nodules. N_2 fixation is an energy-intensive process, requiring large amounts of ATP that must be supplied by oxidative phosphorylation. Nodules have a high rate of respiratory O_2 consumption, but measurements with O_2 microelectrodes indicate that the pO_2 in nodule interior spaces is very low (24). The inner cortex of nodules presents a major impediment to O_2 diffusion and is a primary factor in maintaining the low internal pO_2. Numerous studies utilizing flow-through gas systems for measurement of acetylene reduction and respiration have provided indirect evidence that this diffusion barrier is variable and capable of responding to gradual changes in external pO_2 without damage to nitrogenase (10, 15). Mathematical models have been developed that describe well the observed responses in gas (O_2, CO_2, N_2, and H_2) exchange and the variable nature of the diffusion barrier (13, 23). The entry of O_2 into nodules has been identified as a possible limitation to N_2 fixation, particularly during periods of environmental stress (15, 26).

Activated forms of oxygen (*e.g.* H_2O_2 and superoxide and hydroxyl free radicals) are another fundamental aspect of oxygen relations in nodules. Nodules have a high potential to produce these damaging oxygen species due to the strong reducing conditions required for N_2 fixation and to the action of numerous proteins such as ferredoxin, leghemoglobin, uricase, and hydrogenase (6). Leghemoglobin is probably the most important of these various sources of reactive oxygen species. Leghemoglobin is present in large amounts and is subject to autoxidation in which superoxide and H_2O_2 are released (20). Leghemoglobin is also capable of generating hydroxyl radicals, one of the most damaging forms of oxygen (19). Legume nodules contain the enzymes of the ascorbate-glutathione cycle as part of a defense mechanism against these reactive forms of oxygen (6). These enzymes are involved in the scavenging of H_2O_2 in an initial reaction catalyzed by ascorbate peroxidase (EC 1.11.1.11). Subsequent regeneration of ascorbate proceeds by either a NADH-dependent reaction catalyzed by monodehydroascorbate reductase (EC 1.6.5.4; ref 5) or by coupled reactions involving glutathione-dependent dehydroascorbate reductase (EC 1.8.5.1) and glutathione reductase (EC 1.6.4.2; ref 6).

In the present study, we investigate the response of nodule oxygen defense mechanisms to external pO_2 and to treatment with fixed nitrogen (urea). Parameters examined include concentrations of key antioxidants—ascorbate and glutathione—and the activities of nitrogenase and enzymes associated with scavenging of H_2O_2. Arguments are presented supporting the essential relationship between N_2 fixation and defense against toxic oxygen species.

MATERIALS AND METHODS

Growth of Plants

Soybean (*Glycine max* [L.] Merr. cv Williams) plants were inoculated with *Bradyrhizobium japonicum* 122DES and grown in trays filled to a depth of 10 cm with Perlite. Plants were supplied daily with a half-strength, nitrogen-free nutrient solution containing 1 mM Mes at pH 6.2 (11). Plants were grown in a greenhouse with a temperature regime of 30°C

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days and 25°C nights. High pressure sodium lighting provided a 16 h photoperiod with a PPFD of 400 μmol·m^{-2}·s^{-1}. After 12 d, intact plants were carefully transferred to acrylic Plexiglas hydroponic growth chambers measuring 5 × 5 × 15 cm (Fig. 1). The chambers were wrapped with aluminum foil to exclude light. A split rubber septum was applied around the crown of each plant, and the roots were inserted through a 13-mm hole in the top panel (5 × 5 cm) of the chamber. Each chamber contained four plants. Plastic adhesive ("Holdit") from a local office supply store was used to provide a flexible, airtight seal around the stem. The chambers were filled with nutrient solution as described above to a depth of 10 cm. Continuous aeration was provided initially by filtered, compressed air at a flow rate of 100 mL·min^{-1}·chamber^{-1} through a 3-mm diameter glass tube which entered the top plate and extended to the bottom of the chamber. Even though most nodules were not submerged, they appeared to remain moist but with no apparent film of surface moisture. Each experiment consisted of 30 chambers (120 plants). The level of the nutrient solution was maintained daily by addition of fresh nutrient solution. Each chamber was emptied and refilled with fresh nutrient solution at 4-d intervals. After 10 d under these conditions (22 d after planting), aeration was switched to premixed certified standard grade compressed gas (Union Carbide, San Francisco) containing either 2, 21, or 50 kPa O_2. Each mixture contained 0.1 kPa CO_2 with the balance being N_2. Each experiment consisted of 20 chambers exposed to one of three gas treatments: (a) 2 kPa O_2; (b) 50 kPa O_2; or (c) 21 kPa O_2 alternating with 50 kPa O_2 at 30-min intervals. In the third treatment, solenoid valves were used to automatically control switching of gas mixtures. In each experiment, an additional 10 chambers were used as a control group and were exposed to 21 kPa O_2 from premixed cylinders. Gas was distributed to the chambers through 3 mm (i.d.) Tygon tubing at a flow rate of 50 mL·min^{-1}·chamber^{-1}. The rates to the individual chambers were equalized by adjusting plastic gang valves. Plants were grown under these conditions for an additional 5 d at which time respiration and acetylene reduction activity were measured and the nodules harvested for analysis of enzyme activities and content of antioxidants. Plants were 27 d old at the termination of the experiment.

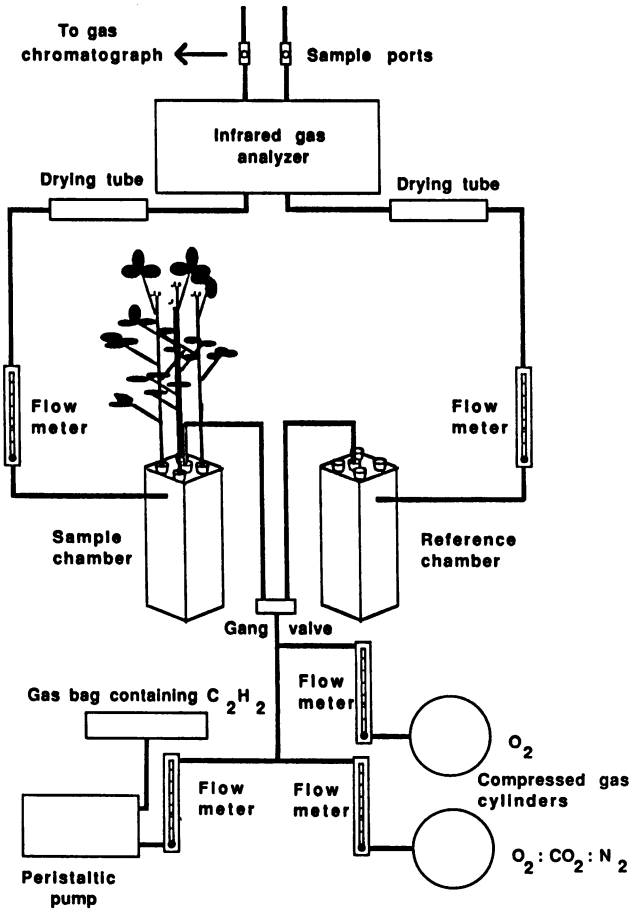

Figure 1. Schematic diagram of the flow-through gas system used for measurement of acetylene reduction and respiration of intact, nodulated soybean roots.

Plants for experiments on the effects of fixed N were grown in 20-cm diameter plastic pots filled with Perlite and supplied daily with nitrogen-free nutrient solution (6). The plants were maintained under greenhouse conditions as described above. Beginning at 15 d after planting, the experimental plants were supplied daily with nutrient solution amended with 5.5 mM urea. Control plants continued to receive nitrogen-free nutrient solution. All plants received excess water in lieu of nutrient solution 1 d per week.

Acetylene Reduction and Respiration

A flow-through gas system was used to monitor nitrogenase activity and nodule respiration directly in the hydroponic growth chambers (Fig. 1). Immediately prior to the assay, the level of nutrient solution in the chamber was reduced to 1 cm. The nodules remained moist throughout the assay, but no moisture film was visible. Each chamber was paired with a reference chamber lacking plants but with an equal level of nutrient solution. The gas mixture entering each chamber was then supplemented with calcium carbide-generated acetylene such that the partial pressure of acetylene was 10 kPa. The acetylene was stored in a polyvinyl fluoride gas bag and added to the flow by a peristaltic pump. Additional O_2 was also added from a separate cylinder of pure O_2 to compensate for the dilution caused by addition of acetylene. Thus, the partial pressure of O_2 during the acetylene reduction assay was the same as the level during the preceding 5-d treatment period. Total flow rate during the assay was 375 mL·min^{-1}·chamber^{-1}. The rate was measured both before and after passing through the assay chambers with Cole-Parmer series L-03217 variable area flow meters. Gas samples were removed from the outlet stream of each chamber at 1-min intervals for 10 min total with a 1 mL syringe. The samples were analyzed for ethylene with a Varian model 3300 gas chromatograph equipped with a 0.318 cm × 76 cm stainless steel column of Poropak N and a flame ionization detector. Gas samples removed from the paired reference chamber were used to determine background levels of ethylene. The rate of acetylene reduction was calculated on the basis of the largest amount of ethylene detected in any one sample. This value was usually

highest in the 6 to 9 min sample and is equivalent to the maximum, predecline rate as defined by Minchin *et al.* (14).

CO_2 evolution was simultaneously measured with a Li-Cor model Ll-6251 infrared gas analyzer that was standardized with gas from a cylinder of primary standard grade CO_2 (Union Carbide). The flow through the empty, paired chamber provided the reference gas for the IRGA. Humidity was removed from the sample and reference flows prior to the IRGA with in-line $CaSO_4$ cartridges. The CO_2 values were recorded at 1-min intervals for a total of 10 min. Rates of respiration declined as the assay proceeded, and the values reported here are the maximal rates which in all cases was observed 3 min after the assay began.

Acetylene reduction in the experiments with urea was measured by removing plants from pots and gently shaking and rinsing off loose Perlite with distilled water. Roots from four intact plants were placed in a 500-mL Erlenmeyer flask containing 250 mL of nutrient solution. The flasks were then sealed with a rubber stopper through which the shoots of all four plants passed. The nutrient solution was aerated with filtered air at a flow rate of 100 $mL \cdot min^{-1} \cdot chamber^{-1}$ for 15 h overnight to allow for plants to recover from disturbance effects. At midday of the following day, the amount of nutrient solution was reduced to 100 mL. Acetylene and O_2 were introduced as described above, and gas samples from the outflow were withdrawn at 1-min intervals for analysis of ethylene.

Enzyme Assays

Nodule extracts for determination of ascorbate peroxidase were prepared by grinding 0.5 g fresh weight of nodules with a mortar and pestle in the presence of liquid N_2. The liquid N_2 was allowed to boil dry, and the powder was quickly added to 2 mL of ice-cold, 50 mM K phosphate buffer (pH 7.8), containing 0.125 g of insoluble polyvinylpolypyrrolidone. This preparation was stirred gently and then centrifuged at 12,000*g* for 5 min. The supernatant was used for determination of ascorbate peroxidase activity in an assay based on the decrease in absorbance at 290 nm due to ascorbate peroxidation (16). Activities of other enzymes were measured in a separate crude extract that was prepared similarly except that the buffer was 50 mM K phosphate (pH 7.8), containing 0.2 mM EDTA and 10 mM β-mercaptoethanol. Activities of dehydroascorbate reductase and glutathione reductase were determined as described previously (6). Monodehydroascorbate reductase activity was assayed by following the decrease in A_{340} due to the oxidation of NADH (8). Crude extracts of roots were prepared as described for nodule extracts except that 1 g fresh weight of roots was ground in liquid N_2 and added to 3 mL of buffer containing 0.25 g polyvinylpolyrrolidone.

Other Assays

Extracts for glutathione determinations were prepared by grinding 0.25 g fresh weight of nodules in 1.5 mL of ice-cold, 5% (w/v) 5-sulfosalicylic acid with a mortar and pestle. The macerate was centrifuged at 12,000*g* for 5 min. Aliquots (20 μL) of the supernatant were analyzed for total glutathione equivalents (GSH and GSSG)[2] with a spectrophotometric assay based on the reduction of 5,5′-dithio-bis(2-nitrobenzoic acid) in the presence of excess glutathione reductase (7). GSSG content was determined after derivitization of 250 μL of the above extract with 5 μL of 2-vinyl pyridine and 20 μL of 50% (v/v) triethanolamine. Leghemoglobin was determined by measuring the absorbance values of the oxidized *versus* reduced difference spectrum of pyridine hemochrome (2). Total protein was determined by Coomassie blue G-250 binding with BSA as the standard (3).

RESULTS

Oxygen Treatments

O_2 partial pressure had numerous effects on nodule parameters relevant to N_2 fixation and oxygen defense mechanisms as summarized in Table I. Acetylene reduction activity was very low in the 2 kPa treatment and increased markedly in response to higher pO_2. Acetylene reduction activity in nodules exposed to alternating 21 and 50 kPa O_2 was less than half of the rate observed in either the constant 21 kPa or constant 50 kPa treatment. This suggests that the use of alternating pO_2s effectively introduced oxidative stress to the N_2-fixing (interior) regions of nodules and that the ability of the variable diffusion barrier to regulate internal O_2 tension may have been at least partially circumvented.

Nodules from the 2 kPa treatment had lower leghemoglobin content than any of the other treatments ($P \leq 0.05$ by *t* test compared to either 21, 50, or 21/50 kPa treatments), but there were no significant differences in leghemoglobin content among the other treatments. Although the total protein content of nodules and roots was slightly lower in the 2 and 21/50 kPa treatments, there were no statistically significant differences when analyzed by *t* tests. GSH content showed a strong proportional increase with increasing pO_2, but there was no consistent trend in GSSG content. Most of the total glutathione pool was present in the reduced form, as is usual for plant and animal samples. The low amount of GSH in the 2 kPa-treated nodules resulted in a very low GSH:GSSG ratio of 3.6:1 compared to values of 8.9:1 and 6.8:1 in the 21 and 50 kPa-treated nodules, respectively. Nodules from the 2 kPa treatment had significantly lower ascorbate content and rates of respiration, but there were no differences in ascorbate content or respiration among the 21, 50, and 21/50 kPa-treated nodules.

Increasing pO_2 led to very substantial increases in the activities of ascorbate peroxidase in nodules (Table II). MDHA reductase and DHA reductase activities increased slightly in response to O_2 treatments. MDHA reductase activity in 21/50 kPa-treated nodules was significantly higher than activity in any of the other treatments. Elevated levels of DHA reductase activity were apparent only in the 21/50 kPa-treated nodules when compared to the 2 kPa-treated nodules ($P \leq 0.01$). Activity of glutathione reductase in nodules was significantly lower in the 2 kPa treatment when compared to the other treatments ($P \leq 0.01$).

[2] Abbreviations: GSH, reduced glutathione; DHA, dehydroascorbate; MDHA, monodehydroascorbate.

Table I. *Effects of pO_2 on Acetylene Reduction, Respiration, and Concentrations of Leghemoglobin, Total Protein, Glutathione, and Ascorbic Acid in Nodulated Soybean Plants*

Soybean plants were grown in hydroponic chambers for 5 d with aeration by gas mixtures with various partial pressures of O_2. Acetylene reduction and respiration were determined with a flow-through gas system. Each value is the mean ± SEM.

Parameter	Units	Oxygen Treatment				*n*
		2 kPa	21 kPa	50 kPa	21/50 kPa[a]	
Nodules						
Acetylene reduction	nmol·min^{-1}·g fresh wt^{-1}	43.2 ± 8	1,480 ± 119	1,880 ± 121	668 ± 69	3
Leghemoglobin	nmol·g fresh wt^{-1}	59.7 ± 12	106 ± 16	117 ± 8	115 ± 6	6
Total protein	mg·g fresh wt^{-1}	13.0 ± 1.1	15.3 ± 0.4	16.3 ± 0.6	12.7 ± 2.3	6
Reduced glutathione	nmol·g fresh wt^{-1}	608 ± 67	996 ± 70	1425 ± 104	921 ± 44	5
Oxidized glutathione	nmol·g fresh wt^{-1}	169 ± 22	112 ± 7	209 ± 26	77 ± 2	5
Ascorbic acid	nmol·g fresh wt^{-1}	339 ± 90	668 ± 115	518 ± 145	618 ± 29	4
Roots						
Total protein	mg·g fresh wt^{-1}	2.28 ± 0.2	2.63 ± 0.1	2.49 ± 0.1	2.37 ± 0.2	6
Intact nodulated roots						
Respiration	μmol CO_2·min^{-1}·plant^{-1}	0.66 ± 0.08	1.71 ± 0.14	1.91 ± 0.14	1.94 ± 0.23	4

[a] Alternating 21 and 50 kPa at 30 min intervals.

pO_2 also affected the activity levels of these enzymes in roots, but the responses were not identical to those in nodules (Table II). Ascorbate peroxidase activity increased with increasing pO_2, but 21/50 kPa-treated roots did not have elevated levels. DHA reductase activity increased markedly with increasing pO_2. MDHA activity was unaffected by O_2 levels except that activity was lower in the 21/50 kPa treatment when compared to any of the other treatments ($P \leq 0.01$). Activity of glutathione reductase in roots was extremely low regardless of O_2 treatment.

Urea Treatments

Treatment of nodulated roots with urea led to decreases in acetylene reduction activity (Fig. 2) and contents of leghemoglobin and total protein (Fig. 3). Urea-treated nodules harvested at 60 d after planting were in the advanced stages of senescence as evidenced by the lack of acetylene reduction activity and the bilious green appearance typical of degraded leghemoglobin. GSH content of nodules showed a steady increase with plant age, reaching a maximum at 50 d after planting (Fig. 3). Treatment with urea had no effect on GSH content until 60 d after planting at which time urea-treated nodules contained 30% less GSH than untreated nodules. Nodule GSSG content showed no consistent response with respect to plant age or urea treatment (Fig. 3). The GSH:GSSG ratio gradually increased from 7.3:1 at d 15 to maxima at d 50 of 38:1 (untreated nodules) and 39:1 (urea-treated nodules).

Activities of ascorbate peroxidase, DHA reductase, and glutathione reductase were all lower in urea-treated nodules when compared to untreated nodules (Fig. 4). These differences were especially striking for DHA reductase and glutathione reductase, with declines in activity evident as soon as d 25 (10 d after application of urea). By d 50, untreated nodules had a 3.0-fold higher activity of DHA reductase and a 2.2-fold higher activity of glutathione reductase. In untreated nodules, the activities of these enzymes increased with plant age then decreased as nodules entered senescence. In

Table II. *Activities of Enzymes of the Ascorbate-Glutathione Cycle in Extracts from Nodulated Soybean Plants Exposed to Various Partial Pressures of Oxygen for 5 d*

Source of Extract	Enzyme	Oxygen Treatment			
		2 kPa	21 kPa	50 kPa	21/50 kPa
		activity units·g fresh wt^{-1}[a]			
Nodules	Ascorbate peroxidase	7,410 ± 520	8,600 ± 990	13,400 ± 520	12,000 ± 450
	Monodehydroascorbate reductase	6,230 ± 260	6,550 ± 280	5,880 ± 100	7,440 ± 290
	Dehydroascorbate reductase	2,110 ± 80	2,520 ± 250	2,380 ± 140	3,120 ± 220
	Glutathione reductase	367 ± 29	653 ± 72	760 ± 59	764 ± 72
Roots	Ascorbate peroxidase	3,030 ± 110	3,550 ± 200	4,420 ± 390	2,480 ± 350
	Monodehydroascorbate reductase	3,220 ± 290	3,080 ± 230	3,160 ± 270	2,140 ± 120
	Dehydroascorbate reductase	238 ± 13	431 ± 43	467 ± 29	509 ± 25
	Glutathione reductase	11 ± 1	18 ± 3	18 ± 1	16 ± 3

[a] Activity units are nmol ascorbate·min^{-1}·g fresh wt^{-1} for ascorbate peroxidase and dehydroascorbate reductase and nmol NAD(P)H·min^{-1}·g fresh wt^{-1} for monodehydroascorbate reductase and glutathione reductase. Each value is the mean of six replicates ± SEM.

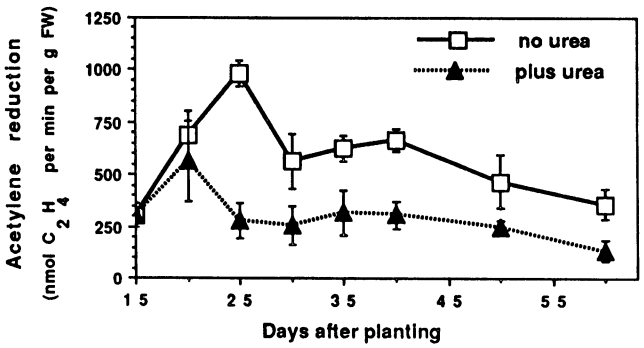

Figure 2. Effects of fixed N on N_2 fixation (acetylene reduction activity) of intact, nodulated soybean roots. All plants were provided daily with a nutrient solution lacking nitrogen until 15 d after planting at which time some plants began to receive nutrient solution containing 5.5 mM urea. Each value is the mean ±1 SEM. Replicate numbers are identical to those in Table I.

contrast, the activity of MDHA reductase was unaffected by urea treatment or by plant age between d 15 and 60 (Fig. 4B).

DISCUSSION

Elevated ambient pO_2 led to increases in concentrations of GSH and ascorbate and in activities of enzymes of the ascorbate-glutathione cycle. These observations suggest that this cycle plays an essential role in the oxygen relations of nodules. However, an unequivocal cause and effect relation between pO_2 and activities of the enzymes of the ascorbate-glutathione cycle has not been established. Although increases in defense enzyme activity are, in some cases, correlated with increases in pO_2, some of the responses in levels of defense enzymes could be a response to increases in N_2 fixation activity or to increases in metabolism associated with increased respiration. Even though the lowest pO_2 treatment (2 kPa) resulted in low activities of the defense enzymes, this may not be a direct consequence of low oxygen stress, since many secondary responses could result from O_2 limitation. The strongest case for response occurs in the 21/50 kPa treatment where oxidative stress was likely high. In this treatment, N_2 fixation activity was low compared to the 21 kPa treatment (Table I), but activities of defense enzyme activities were high (Table II).

The response of the ascorbate-glutathione cycle provides a means of self-regulation of oxygen relations beyond the regulation that results from the variable diffusion barrier. Vessey *et al.* (26) proposed that the ability of nodules to compensate for large increases in external pO_2 may involve two possible strategies: (a) higher rates of respiratory O_2 consumption and (b) increase in nodule resistance to gas diffusion. The diffusion barrier can respond within minutes to changes in pO_2 (10). Nitrogenase activity and respiration in nodules return to initial levels within 30 min following abrupt shifts in the pO_2 from 20 to 30 kPa. The evidence presented here indicates that a third mechanism—the ascorbate-glutathione cycle—should be added to the list of oxygen protection strategies that respond to external pO_2.

Whereas changes in diffusion resistance can occur within minutes, the response time of the ascorbate-glutathione cycle probably is substantially longer, but this has not been examined in detail. Several studies have indicated that soybean nodules can compensate for long-term exposure to supra-ambient pO_2. Exposure of soybean nodules to 89 kPa O_2 leads to an initial and severe decrease in acetylene reduction activity, but continued exposure results in substantial recovery (4). Exposure of nodulated soybean roots to 30 or 40 kPa O_2 does

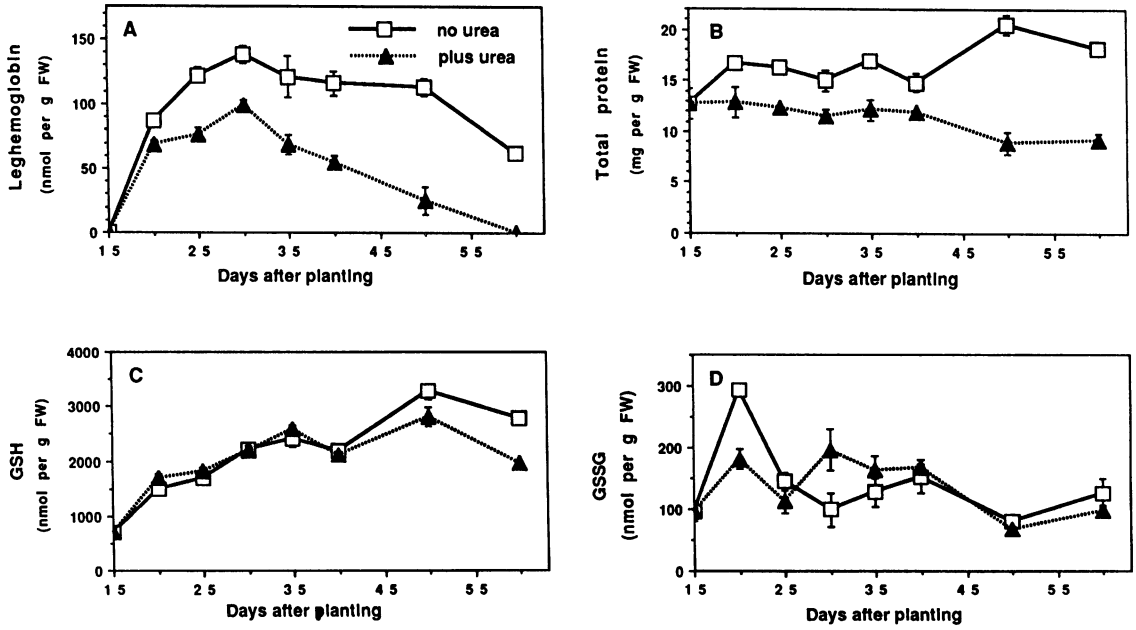

Figure 3. Effects of fixed N (5.5 mM urea) on leghemoglobin (A), total protein (B), GSH (C), and GSSG (D) content of soybean root nodules. See Figure 2 legend for further details.

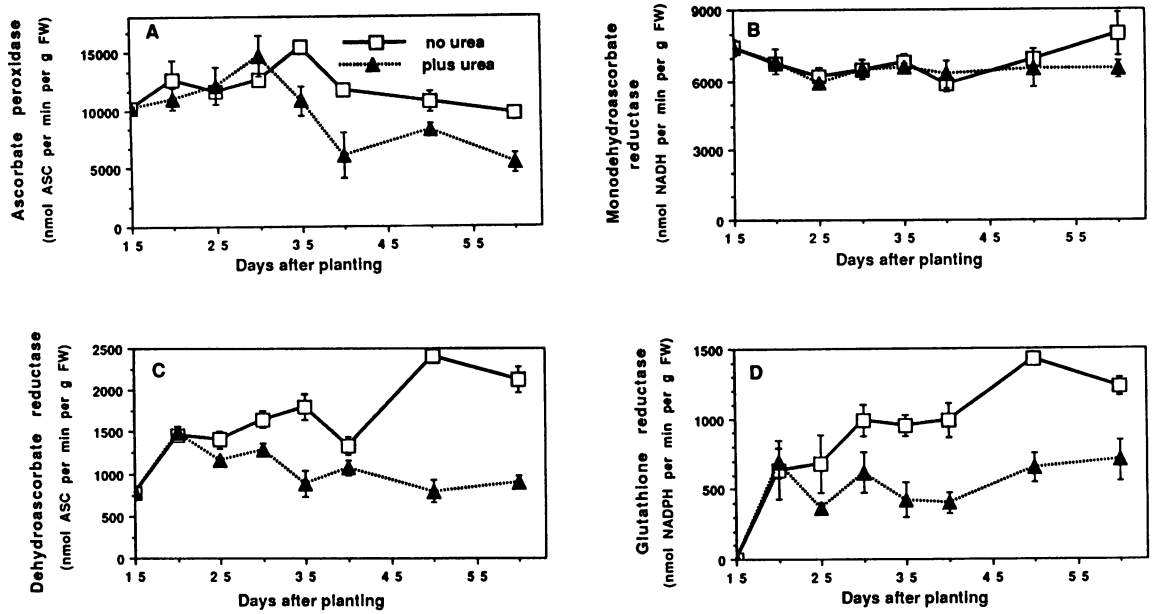

Figure 4. Effects of fixed N (5.5 mM urea) on activities of ascorbate peroxidase (A), MDHA reductase (B), DHA reductase (C), and glutathione reductase (D) in soybean root nodules. Each value is the mean of six replicates ±1 SEM. See Figure 2 legend for further details.

not substantially affect acetylene reduction activity over a period up to 6 d when compared to rates under ambient pO_2 (27). Nitrogenase activity can actually increase with pO_2 up to 100 kPa provided the pO_2 is raised gradually (9). Responses of the ascorbate-glutathione cycle may partially explain the ability of soybean nodules to adjust to long-term exposure to supra-ambient pO_2, although the variable diffusion barrier also plays a critical role. In cases where nitrogenase activity (H_2 evolution) increases with elevated pO_2 (9), the possible protective advantage of increased O_2 uptake via the Knallgas reaction ($2\ H_2 + O_2 \rightarrow 2\ H_2O$) in hydrogenase-positive rhizobia strains must also be considered.

A supply of fixed N in the form of urea led to decreases in nitrogenase activity and in activities of three of the four enzymes of the ascorbate-glutathione cycle. Numerous studies have established the inhibitory effect of fixed N on N_2 fixation and leghemoglobin content in nodules (17, 22). The concomitant decrease in activities of enzymes of the ascorbate-glutathione cycle suggests a key link between the process of N_2 fixation and mechanisms for protection against oxygen. The short-term responses (≤20 d after urea application) are more revealing, since long-term responses could be complicated by nonspecific declines related to senescence. Similar arguments have been put forward for the relationship between N_2 fixation and superoxide dismutase (18). A decrease in N_2 fixation activity could be expected to lead to a decrease in production of activated forms of oxygen, since concentrations of leghemoglobin—probably the main source of damaging oxygen species in nodules—decline in nodules in which N_2 fixation is inhibited. Furthermore, decreased nitrogenase activity may directly result in fewer free radicals being produced, since there is strong, though indirect, evidence that nitrogenase may directly produce superoxide or H_2O_2 (21).

The use of urea, as opposed to other forms of fixed N such as nitrate, has considerable advantages in the demonstration of the relationship between oxygen defense and N_2 fixation. Although nitrate is clearly inhibitory to N_2 fixation in nodules, it presents complications involving oxygen relations. Nitrate is reduced to nitrite which then reacts with leghemoglobin to form nitrosylleghemoglobin (12). This severely interferes with nodule function, because nitrosylleghemoglobin can not bind O_2. Nitrite accumulates in nitrate-treated nodules and may oxidize leghemoglobin to the nonfunctional ferric form (1). Further oxygen-related complications can arise from nitrate treatment, since nitrate in some cases leads to increases in H_2O_2 content and lipid peroxidation of nodules (1). In contrast, urea avoids these complications. Urea is converted to NH_4^+ by urease which is present in high levels in soybean roots, nodules, and isolated bacteroids (DA Dalton, unpublished observations). The NH_4^+ can then be readily assimilated into organic forms.

The considerably higher levels of activity of the enzymes of the ascorbate-glutathione cycle in nodules when compared to levels in uninfected roots provides further evidence for the critical link between N_2 fixation and oxygen defense. The increased levels in nodules were especially pronounced for DHA reductase activity, which was 5.8-fold higher in nodules, and glutathione reductase activity, which was 36-fold higher in nodules (21 kPa O_2 treatment, Table II). Similar strikingly higher activity in nodules as compared to roots has led to suggestions that enzymes such as phosphoenolpyruvate carboxylase, aspartate aminotransferase, asparagine synthetase, and others may be nodulins (nodule-specific proteins) (25). In view of the low, but detectable, levels of enzymes of the ascorbate-glutathione cycle in uninfected roots, it is probably more appropriate to consider them as nodule-enhanced rather than nodule-specific, but further investigations are required to clarify this.

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