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# Effectiveness of ascorbate and ascorbate peroxidase in promoting nitrogen fixation in model systems

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**Abstract**

Ascorbate and ascorbate peroxidase are important antioxidants that are abundant in $N_2$-fixing legume root nodules. Antioxidants are especially critical in root nodules because leghemoglobin, which is present at high concentrations in nodules, is prone to autoxidation and production of activated oxygen species such as $O_2^{\cdot -}$ and $H_2O_2$. The merits of ascorbate and ascorbate peroxidase for maintaining conditions favorable for $N_2$ fixation were examined in two model systems containing oxygen-binding proteins (purified myoglobin or leghemoglobin) and $N_2$-fixing microorganisms (free-living *Azorhizobium* or bacteroids of *Bradyrhizobium japonicum*) in sealed vials. The inclusion of ascorbate alone to these systems led to enhanced oxygenation of hemeproteins, as well as to increases in nitrogenase (acetylene reduction) activity. The inclusion of both ascorbate and ascorbate peroxidase resulted in even greater positive responses, including increases of up to 4.5-fold in nitrogenase activity. In contrast, superoxide dismutase did not provide beneficial antioxidant action and catalase alone provided only very marginal benefit. Optimal concentrations were 2 mM for ascorbate and 200 μg/ml for ascorbate peroxidase. These concentrations are similar to those found in intact soybean nodules. These results support the conclusion that ascorbate and ascorbate peroxidase are beneficial for maintaining conditions favorable for $N_2$ fixation in nodules. 

*Keywords:* Azorhizobium; Bradyrhizobium; Nitrogen fixation; Antioxidants; Ascorbate; Ascorbate peroxidase; Hydrogen peroxide; Leghemoglobin

## 1. Introduction

$N_2$ fixation in legume root nodules is a complex process that requires many physiological reactions in addition to the reduction of $N_2$ to $NH_3$. One such reaction involves oxygen binding by leghemoglobin (Lb), which is similar to mammalian hemoglobin and myoglobin (Mb) (Gibson et al., 1989). Lb transports $O_2$ to the bacteroids at low concentrations that are compatible with bacteroid respiration and nitrogenase activity (Bergersen, 1982). Under normal atmospheric conditions, 20% of Lb is in its reduced, oxygenated state ($Lb^{2+}O_2$) (Appleby, 1984). This ensures that >99% of $O_2$ that diffuses into the bacteroid is carried by Lb (Layzell & Hunt, 1990). Lb is probably the most abundant producer of activated oxygen species (AOS) in the nodule (Dalton, 1995). $Lb^{2+}O_2$ will spontaneous autoxidize to its ferric form ($Lb^{3+}$), releasing the superoxide radical ($O_2^{\cdot -}$) in the process. This subsequently leads to $H_2O_2$ from superoxide dismutase-catalyzed disproportionation and ultimately to the extremely deleterious hydroxyl radical ($^{\cdot}OH$) (Puppo & Halliwell, 1988). Mb produces AOS in a similar fashion (Everse & Hsia, 1997; Giulivi & Cadenas, 1993). The removal of AOS is critical in preventing oxidative damage to a wide array of biological molecules.

Nodules contain abundant antioxidants to protect against AOS. Among these are superoxide dismutase (SOD) and the components of the ascorbate–gluta-

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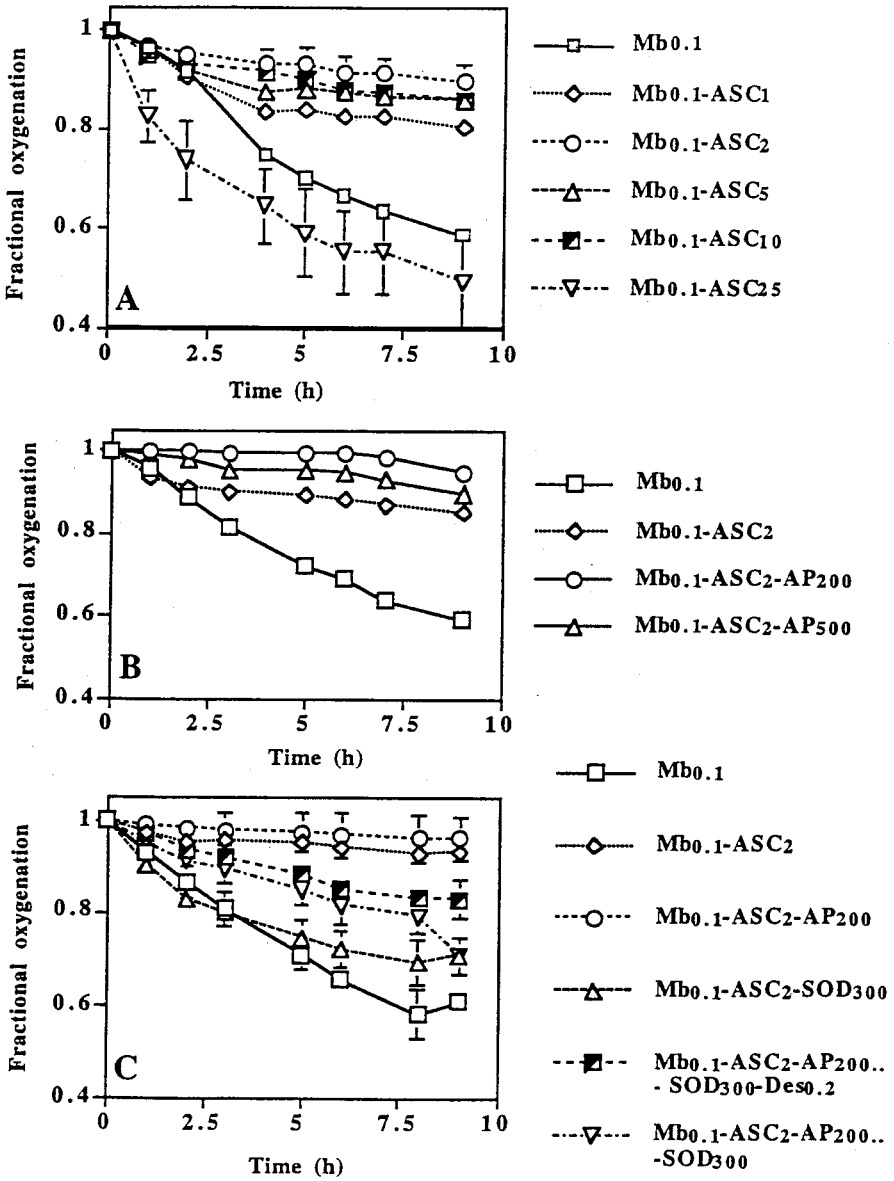


Fig. 1. Fractional oxygenation of Mb in model system 1 without bacteria. Each treatment contained 0.1 mM Mb and antioxidants as follows: ASC only (A), ASC and AP (B), and ASC, AP, SOD, and Des (C). Legend subscripts indicate concentrations as described in *Experimental*. Each value is the mean of five replicates ± S.E.M. When no error bar is present, the S.E.M. was smaller than the symbol for that data point.

thione (ASC–GSH) pathway (Dalton, 1995). In this pathway, the reducing power of ASC is used to convert $H_2O_2$ to water in a reaction catalyzed by ascorbate peroxidase (AP, reaction (1)) (Dalton, 1995; Dalton et al., 1986; Asada, 1992).

$$2\text{ASC} + H_2O_2 \xrightarrow{\text{AP}} 2\text{Monodehydroascorbate} + 2H_2O \quad (1)$$

Monodehydroascorbate may subsequently be reduced to ASC by monodehydroascorbate reductase or it may spontaneously disproportionate to ASC and dehydroascorbate. The regeneration of ASC in the latter case is achieved by coupled reactions involving dehydroascorbate reductase and glutathione reductase. The effectiveness of the nodule in fixing $N_2$ is strongly correlated with high concentrations and activities of the components in the ASC–GSH cycle (Dalton, Langberg & Treneman, 1993).

ASC is a widespread and versatile molecule in plants. In addition to its ability to scavenge $H_2O_2$, ASC also has the advantageous capacity to convert Lb from the ferryl ($Fe^{4+}$) to ferric ($Fe^{3+}$) state (Moreau, Puppo & Davies, 1995). A similar reaction can occur with Mb (Giulivi & Cadenas, 1993). Further reduction to the functional ferrous ($Fe^{2+}$) state may also be accomplished by ASC (Moreau et al., 1995), though it is likely that a NADH-dependent reductase is primarily responsible for this step (Ji et al., 1991).

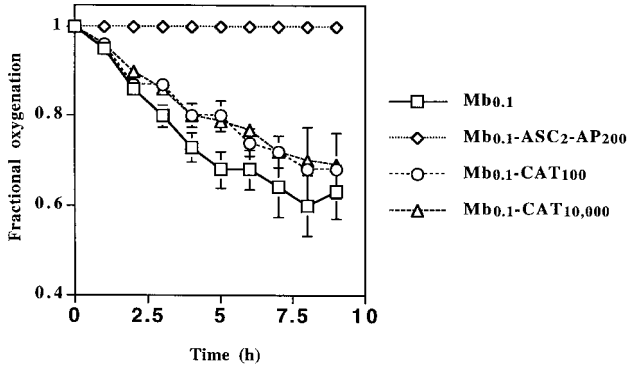


Fig. 2. Fractional oxygenation of Mb in model system 1 without bacteria. Each treatment contained 0.1 mM Mb and antioxidants as indicated. Further details are described in the legend for Fig. 1.

In the present study, two $N_2$-fixing in vitro model systems were constructed to mimic nodule function. The performance of these systems was examined in the absence or presence of antioxidants, chiefly ASC and AP, in order to examine the hypothesis that antioxidants have a beneficial effect on $N_2$ fixation in legume root nodules.

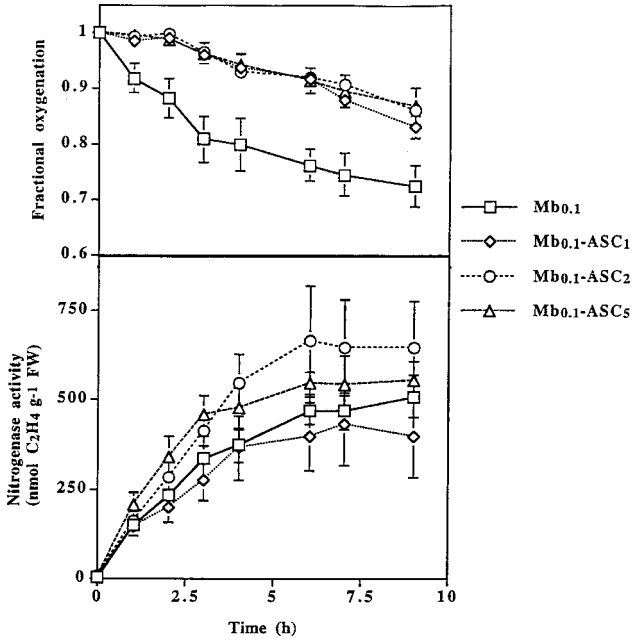


Fig. 3. Fractional oxygenation of Mb and nitrogenase activity (acetylene reduction) in model system 1 with bacteria (*Azorhizobium caulinodans*) in the presence of various concentrations of ASC. Further details are described in the legend for Fig. 1.

## 2. Results

### 2.1. System 1 — without bacteria

In the absence of ASC, the fractional oxygenation of Mb declined from 1.0 to 0.59 during the 9-h incubation period even without any bacteria being present (Fig. 1A). The inclusion of ASC (1, 2, 5 or 10 mM) resulted in the maintenance of higher levels of fractional oxygenation of Mb (0.81–0.90 after 9 h). The improvement with ASC was first evident at h 3 ($p < 0.05$, unpaired $t$ test) and continued to become more pronounced with time. The highest oxygenation was achieved with 2 mM ASC so this concentration was chosen for use in further experiments. In contrast, there was a rapid decline in fractional oxygenation in treatments containing 25 mM ASC in which case the oxygenation remained below that observed in treatments containing no ASC throughout the 9-h duration of the experiment.

The inclusion of AP in addition to ASC provided further protection against decreases in fractional oxygenation of Mb as compared to reaction mixtures containing ASC alone (Fig. 1B). An AP concentration of 200 μg/ml provided nearly complete protection with the fractional oxygenation decreasing only from 1.0 to 0.948 after 9 h. This value was significantly different from the value obtained with ASC alone ($p < 0.0004$). A higher concentration of AP (500 μg/ml) did not provide additional protection.

The inclusion of SOD (to scavenge $O_2^{\cdot -}$) or desferrioxamine (to prevent Fe-catalyzed free radical formation) did not provide protection against declines in oxygenation as well as did treatments containing ASC and AP in combination (Fig. 1C). Treatments with ASC and SOD together resulted in declines in oxygenation that were equally as severe as those that occurred with Mb alone (no ASC or AP). SOD not only failed to provide antioxidant protection, but even appeared to act as a prooxidant as seen most clearly when comparing the $Mb_{0.1}$–$ASC_2$–$AP_{200}$ treatment to the $Mb_{0.1}$–$ASC_2$–$AP_{200}$–$SOD_{300}$ treatment ($p < 0.001$ at $t = 8$ or 9 h). Desferrioxamine alone provided no protection against deoxygenation (data not shown).

Catalase alone provided only marginal benefits in maintaining oxygenation even when included at a high concentration of 10,000 units/ml (Fig. 2). The protective benefits of catalase were statistically significant after 5 hours ($p = 0.0045$) but not at later times.

### 2.2. System 1 — with bacteria

Similar patterns of decline in fractional oxygenation of Mb were observed when bacteria (*Azorhizobium*) were included in the reaction mixtures (Fig. 3). In the absence of ASC, fractional oxygenation declined to 0.725 after 9 h whereas this value declined only to 0.862 when 2 mM ASC was included. ASC was equally effective at concentrations of 1, 2 or 5 mM.

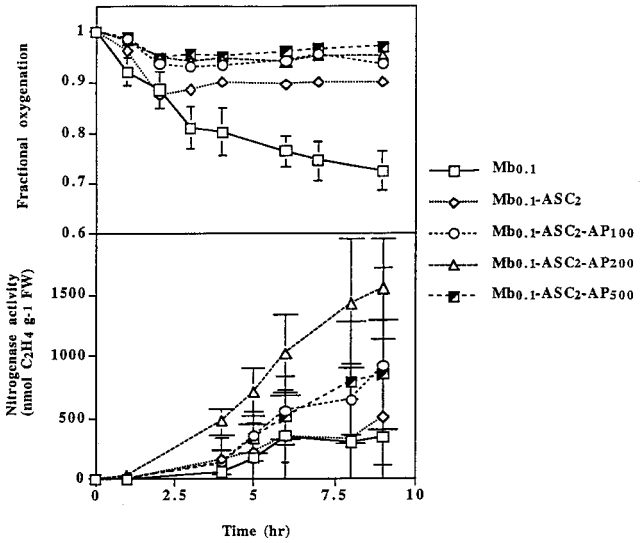


Fig. 4. Fractional oxygenation of Mb and nitrogenase (acetylene reduction) activity in model system 1 with bacteria (*Azorhizobium caulinodans*) in the presence of 2 mM ASC and various concentrations of AP. Further details are described in the legend for Fig. 1.

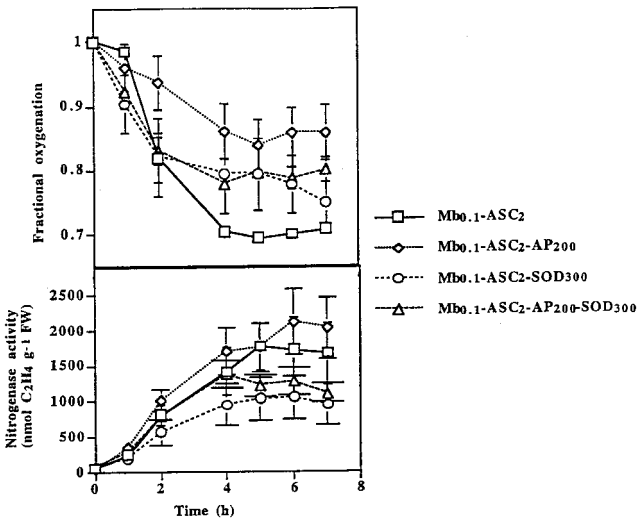


Fig. 5. Fractional oxygenation of Mb and nitrogenase (acetylene reduction) activity in model system 1 with bacteria (*Azorhizobium caulinodans*) in the presence of 2 mM ASC, AP (200 μg/ml) and SOD (300 units/ml). Further details are described in the legend for Fig. 1.

Each of the ASC treatments was statistically different from the $ASC_0$ treatment ($p < 0.04$ at 9 h).

Acetylene reduction (i.e. nitrogenase) activity was substantial and linear for the first 4 h (Fig. 3) in all treatments. No further accumulation of $C_2H_4$ was observed after 6 h. The highest nitrogenase activity was obtained with 2 mM ASC in which case the cumulative amount of acetylene reduction at 6 h was 42% higher than the amount observed with no ASC. Rates were substantially lower in vials containing K phosphate buffer alone (i.e. $Mb_0$–$ASC_0$–$AP_0$, data not shown).

Fig. 4 represents the results from the first complete (i.e. bacteria + ASC + AP) system. As in earlier cases, fractional oxygenation declined sharply in the Mb-alone ($Mb_{0.1}$) treatment (Fig. 4). Oxygenation was substantially improved with 2 mM ASC, but the best oxygenation was obtained when AP was also included along with ASC. The values for fractional oxygenation remained high (0.952 for $ASC_2$–$AP_{200}$) even after 9 h, a result that parallels the values obtained in the absence of bacteria. There were no significant differences between the responses to different concentrations of AP.

Nitrogenase activity was low in the presence of Mb alone and the inclusion of ASC resulted in only a small increase by 9 h (Fig. 4). However, much greater activity was observed when AP was included along with ASC. The greatest activity was observed with an AP concentration of 200 μg/ml. At 9 h, the cumulative production of $C_2H_4$ at this concentration of AP was 4.5-fold higher than the amount observed in the treatment containing Mb alone and 3.1-fold higher than the amount in the treatment containing Mb and ASC. Furthermore, the production of $C_2H_4$ continued in the $AP_{200}$ treatment even up to 9 h whereas $C_2H_4$ production generally had ceased at earlier times for other treatment, especially in the case of ASC alone treatments where little activity was observed after 6 h.

Additional experiments in which SOD was included along with ASC and AP are shown in Fig. 5. SOD did

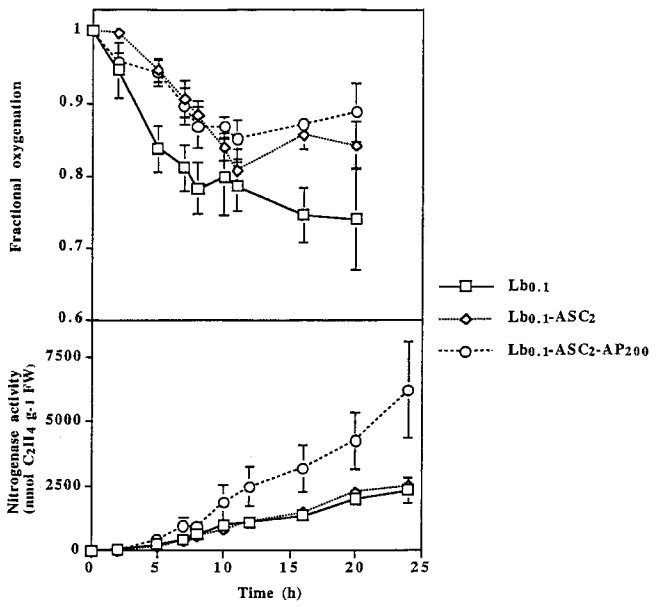


Fig. 6. Fractional oxygenation of Lb and nitrogenase (acetylene reduction) activity in model system 2 with isolated bacteroids of *Bradyrhizobium japonicum*, ASC (2 mM) and AP (200 μg/ml). Fractional oxygenation at 24 h is not shown because of anomalous readings probably due to degradation or microbial contamination. Further details are described in the legend for Fig. 1.

not provide protection against declines in oxygenation in any treatment and appeared to negate any advantage from AP in a fashion similar to that in Fig. 1. The highest oxygenation was obtained with ASC and AP ($Y = 0.858$ at 6 h), but if SOD was also included then $Y$ was only 0.786. Similarly, inclusion of SOD did not result in any increases in nitrogenase activity (Fig. 5) and the maximum protective effect was evident with ASC and AP.

### 2.3. System 2

The results with system 2 were consistent with those observed in system 1, however, bacteroids remained active for considerably longer (at least 24 h) and longer incubation times were required to see the maximum effects of each treatment. Fractional oxygenation of Lb declined from 1.0 to 0.741 after 20 hours in the absence of ASC (Fig. 6). The oxygenation decreased to only 0.843 in the presence of ASC. There was no additional protection from inclusion of AP in addition to ASC. These results are similar to those shown in Fig. 4 for system 1.

Nitrogenase activity was strongly promoted by inclusion of ASC and AP (Fig. 6). ASC did not result in higher activity when compared to treatments with Lb alone. The total accumulation of $C_2H_4$ was 2.6-fold higher in the ASC plus AP treatment when compared to the treatment with no ASC or AP.

## 3. Discussion

The results presented here illustrate the protective benefits of ASC and AP in two $N_2$-fixing model systems. Similar systems, as well as more elaborate flow-through systems, have been used to illustrate the effects of Lb, Mb, or various carbon sources on respiration and $N_2$ fixation by isolated bacteroids (Bergersen & Turner, 1975, 1990). These systems have proven useful in expanding understanding of the physiological role of key components in intact legume root nodules in which it is much more difficult to experimentally manipulate the levels of the compounds in question.

The observed changes in fractional oxygenation ($Y$) indicate a decrease in $Mb(Fe^{2+})O_2$ (or Lb) relative to the deoxygenated $Fe^{2+}$ form. This is not a direct indication of the presence of the $Fe^{3+}$ form, however it is likely that $Fe^{3+}$ form is continually produced in vivo by oxidation of $Lb^{2+}$ and $LbO_2$ (Becana & Klucas, 1992a). The $Lb^{3+}$ form has been detected with direct spectrophotometry in some nodules, especially nodules of moderately advanced age (54 days) or nodules exposed to high levels of $O_2$ (Lee et al., 1995).

The beneficial effects of ASC are probably due at least in part to its ability to rapidly reduce the ferryl ($Fe^{4+}$) states of myoglobin and leghemoglobin to the ferric state (Giulivi & Cadenas, 1993; Moreau et al., 1995). A further benefit results from the slow conversion of the ferric state to the ferrous ($Fe^{2+}$) state at which $O_2$ binding may occur (Moreau et al., 1995). These reactions work to reverse the reactions in which the heme moiety is oxidized to the less desirable ferric and ferryl states by reactions involving autoxidation of $LbO_2$ with production of $O_2^{\cdot -}$ (reaction (2)), production of $H_2O_2$ by disproportionation via SOD (reaction (3)), and attack of the heme by $H_2O_2$ (reaction (4)) as represented below:

$$Lb^{2+} - O_2 \rightarrow Lb^{3+} + O_2^{\cdot -} \quad (2)$$

$$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (3)$$

$$Lb^{3+} + H_2O_2 \rightarrow Lb^{4+} + H_2O \quad (4)$$

When viewed in this manner, the reasons for the strong benefits of AP are also apparent since this enzyme has a high affinity for $H_2O_2$ and removes it before it has a chance to cause extensive oxidative damage.

The decline in fractional oxygenation of Mb in the presence of high concentrations of ASC (25 mM, Fig. 1A) illustrates the often-neglected ability of ASC to act as a prooxidant when present at high concentrations. Giulivi and Cadenas, (1993) have described in detail the prooxidant reactions of ASC with myoglobin and attribute this property to the reaction of ASC with $Mb^{2+}O_2$ to produce $Mb^{3+}$, $H_2O_2$ and monodehydroascorbate (the ascorbyl radical). However, Moreau et al. (1995) found no evidence for a similar reaction between ASC and $LbO_2$.

Free Fe is also a potential prooxidant in nodules. Free Fe is released from Lb as a consequence of attack by $H_2O_2$. Fe can react with ASC to produce $H_2O_2$ as well as causing the production of $^{\cdot}OH$ radicals via Haber–Weiss reactions (Puppo & Halliwell, 1988; Becana & Klucas, 1992b). Desferrioxamine prevents these harmful reactions by forming a complex with $Fe^{3+}$ that is extremely difficult to reduce (Halliwell & Gutteridge, 1986). Since desferrioxamine did not provide any benefits in the data presented here, we conclude that free Fe was not a major prooxidant in the model systems of this study.

Curiously, the addition of SOD consistently resulted in lower fractional oxygenation of Mb (Fig. 1C, 5) and lower nitrogenase activity (Fig. 5). SOD is generally regarded as an essential antioxidant defense as evidenced by its ubiquitous occurrence in aerobic cells, however, it is also a source of $H_2O_2$ (reaction (3)). Since heme proteins are particularly vulnerable to oxidation and degradation by $H_2O_2$ it may be that SOD

is having a deleterious effect in our model system by producing excess $H_2O_2$. This does not indicate that SOD is not an important antioxidant in vivo, but rather underscores the necessity of a balanced, multifaceted antioxidant defense.

The marginal benefits of catalase (Fig. 2) clearly illustrate the advantages of the ASC–AP system as opposed to catalase alone. Catalase has an extremely high apparent $K_m$ for $H_2O_2$ and is generally regarded as an ineffective scavenger of $H_2O_2$ (Dalton, 1995). Catalase is usually beneficial only in microbodies where its inefficiency is compensated for by an extremely high concentration of the enzyme. Nevertheless, the slight protection provided by catalase in this study supports the conclusion that the declines in fractional oxygenation of Mb were associated at least in part with $H_2O_2$.

Efforts were made throughout these experiments to insure that assay conditions remained similar in comparable experiments, however, some minor variations in responses were observed. For instance, there was a lag time of several hours for nitrogenase activity in Fig. 3 but not in Fig. 4. These differences probably reflect variations in the physiological condition of *Azorhizobium* which is particularly sensitive to minor differences in timing of the growth phase and to manipulations required to induce $N_2$ fixation. The data presented here are representative of more than 30 experiments with at least five replicates of each treatment. These experiments all indicated positive responses consistent with the interpretations previously discussed and vary generally only with regards to minor differences in kinetics. The leveling off of nitrogenase activity towards the end of several experiments could reflect either an acetylene-induced nitrogen starvation or a depletion of carbon reserves. No attempts were made to add additional nutrients during the course of the experiments.

The concentrations required for optimum protection in system 1 were 2 mM for ASC and 200 μg/ml for AP. These concentrations closely match those present in intact soybean root nodules. Specifically, the ASC concentration in soybean nodules has been reported to be 2.1 mM (Dalton et al., 1986). AP is one of the most abundant proteins in nodule cytosol and comprises about 0.9% of total soluble protein (Dalton et al., 1987). Assuming uniform distribution throughout the nodule, we estimate that this corresponds to an approximate concentration of 150 μg/ml within intact nodules. The substantial resources required to maintain these high levels of antioxidants in nodules is further evidence of their criticality.

These observations indicate that ASC and AP are capable of minimizing decreases in oxygenation of Mb and Lb in $N_2$-fixing model systems. ASC and AP also result in higher rates of $N_2$ fixation (acetylene reduction) in these systems. This supports the hypothesis that these antioxidants play critical roles in $N_2$-fixing nodules where they are particularly abundant and is consistent with other studies noting a stimulation by ASC of respiration and nitrogenase in isolated bacteroids of Siratro (*Macroptilium*) (Udvardi et al., 1986), strong correlations of soybean nodule effectiveness with levels of antioxidants (Dalton et al., 1993) and enhanced levels of $N_2$ fixation following application of ASC by either endogenous feeding of roots or intravascular infusion of stems of soybean (Bashor & Dalton, 1999).

## 4. Experimental

### 4.1. General

Both systems were constructed in sealed serum vials (24.3 ml capacity) with an initial gas head space consisting of 97% $N_2$ and 3% $O_2$ and a reaction volume of 9.0 ml. System 1 consisted of *Azorhizobium caulinodans* ORS 571 and $MbO_2$. This system had the advantage of ease of preparation as $N_2$-fixing *Azorhizobium* can be grown in culture and Mb may be purchased commercially. Mb and Lb have shown to be useful as sources of low concentrations of free dissolved $O_2$ in experiments with suspensions of bacteria (Bergersen & Turner, 1975, 1990). System 2 consisted of bacteroids of *Bradyrhizobium japonicum* and purified $LbO_2$, both obtained from soybean root nodules. This system was considerably more difficult to prepare but had the advantage of more closely resembling a nodule. Throughout this report, the concentrations for the reaction components are indicated as subscripts following the abbreviation for that component. For example, $Mb_{0.1}$–$ASC_1$–$AP_{200}$–$SOD_{300}$–$Cat_{100}$–$Des_{0.2}$ indicates 0.1 mM $MbO_2$, 1 mM ASC, 200 μg/ml AP, 300 units/ml SOD, 100 units/ml catalase, and 0.2 M desferrioxamine. Buffered ASC was added to the reaction vials from a concentrated stock of 250 mM K ascorbate (pH 6.8 for system 1, 7.2 for system 2). Affinity-purified recombinant soybean AP was prepared from a heterologous expression system (Dalton et al., 1996). Concentrated stocks (typically 1–2 mg/ml) of pure AP in 1 mM K phosphate, pH 6.8 were stored at −20°C with no loss of activity. SOD and catalase from bovine liver and desferrioxamine mesylate (Sigma) were also prepared in concentrated stocks in 1 mM K phosphate, pH 6.8. For SOD, one activity unit was defined to inhibit the rate of reduction of cytochrome c by 50% in a coupled system with xanthine and xanthine oxidase. For catalase, one activity unit was defined to decompose 1 μmol of $H_2O_2$ per min at pH 7.0 at 25°C.

### 4.2. System 1

Pure horse heart $MbO_2$ (Sigma) was prepared in 1 mM K phosphate (pH 6.8) as described by Appleby and Bergersen, (1980). After reduction with excess sodium dithionite, the solution was desalted on a 23.5 cm × 2.5 cm Sephadex G-25 column. Heme concentration of the eluent was calculated using the pyridine hemochromogen assay (Appleby & Bergersen, 1980). Stock solutions of $MbO_2$ thus prepared were stored at −20°C until needed for the vial reactions. The desired concentration (typically 0.1 mM) of $MbO_2$ in each vial was obtained by adding the appropriate volume of concentrated $MbO_2$ stock and diluting with 1 mM K phosphate (pH 6.8). *Azorhizobium caulinodans* ORS 571 was grown on YLS medium (Elmerich et al., 1982) with the addition of 2X vitamins. Cultures were grown overnight in air at 37°C while shaking at 200 rpm. The cultures were then centrifuged for 5 min at 12,000 × *g* and resuspended in sufficient LSO medium (YLS minus N) to obtain an $A_{600}$ of ~0.7. No additional nutrients were added. $N_2$ fixation was induced by incubating this resuspension in an atmosphere of 97% $N_2$ and 3% $O_2$ at 37°C with shaking for 12 hours at which time the bacteria were placed in the vials along with the other components of system 1. Sufficient bacteria were added to obtain an $A_{600}$ of 0.35 in each vial thus insuring that the initial amount of nutrients in each vial was identical. After all components had been added, the reaction vials were incubated at 37°C with constant shaking at 150 rpm.

### 4.3. System 2

Purified leghemoglobin was prepared from frozen nodules (−80°C) of soybean (*Glycine max* (L.) Merr. cv. Williams 82) inoculated with *Bradyrhizobium japonicum* 122DES as described by Appleby and Bergersen, (1980). Protocols for preparing and storing $LbO_2$ solutions were as described above for $MbO_2$ except that the buffer used throughout system 2 was 50 mM K phosphate (pH 7.2). Bacteroids of *B. japonicum* were prepared anaerobically from freshly harvested 54-day old nodules of soybean plants that had been grown as described by Dalton et al. (1986). The extraction was as described by Koch et al., (1967) and was performed in a large polyvinyl glove bag filled with $N_2$ that had passed through an $O_2$ scrubber (Advanced Gas Specialty Equipment model SG6160). All buffers were sparged with $O_2$-free $N_2$ for 30 min. Fresh bacteroids were prepared for each experiment. The bacteroids were suspended in 50 mM K phosphate (pH 7.2) containing 10 mM Na succinate and 1 mM $MgCl_2$. Each vial contained sufficient bacteroids to obtain an $A_{600}$ of 2.0. After all components had been added, the reaction vials were incubated at 25°C with constant shaking at 150 rpm.

### 4.4. Acetylene reduction

Sufficient acetylene was added to the gas head of each vial such that the concentration of acetylene was 10% (v/v). Gas samples (0.5 ml) were removed at selected time intervals and analyzed for ethylene on a Varian model 330 gas chromatograph equipped with a 0.318 cm × 76 cm stainless steel column of Poropak N and a flame ionization detector. All activity was expressed on the basis of fresh weight of bacteria as determined by weighing pellets after centrifuging at 11,000 × *g* for 15 min and decanting the supernatant. Activity expressed on a dry weight basis was determined to be approximately 10-fold higher.

### 4.5. Fractional oxygenation of Mb and Lb

At each time point approximately 0.1 ml of reaction mixture was removed from each vial and immediately placed in a microcuvette for analysis on a Beckman model DU-640 UV/Vis spectrophotometer. The fractional oxygenation ($Y$) was determined using the following equation (Appleby & Bergersen, 1980):

$$Y = \frac{\Delta A_t - \Delta A_{red}}{\Delta A_{oxy} - \Delta A_{red}}$$

where $\Delta A_t = A_{581} - A_{562}$ for $MbO_2$ or $A_{575} - A_{562}$ for $LbO_2$ at a particular time point, $\Delta A_{oxy}$ is equal to the above values at time zero with $Y$ assumed to be 1.0, and $\Delta A_{red}$ is equal to the above values as determined on an aliquot that had been reduced by addition of excess dithionite.

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## References

Appleby, C. A. (1984). *Ann. Rev. Plant Physiol*, *35*, 443.
Appleby, C. A., & Bergersen, F. J. (1980). In F. J. Bergersen, *Methods for evaluating biological nitrogen fixation* (pp. 315–335). Chichester: Wiley.
Asada, K. (1992). *Physiol. Plant*, *85*, 235.
Bashor, C. J., & Dalton, D. A. (1999). *New Phytol*, *142*, 19.
Becana, M., & Klucas, R. V. (1992a). *Plant Physiol*, *98*, 1217.
Becana, M., & Klucas, R. V. (1992b). *Proc. Natl. Acad. Sci. USA*, *89*, 8958.

Bergersen, F. J. (1982). In *Root nodules of legumes: Structure and function* (pp. 23–50). Chichester: Research Studies Press.
Bergersen, F. J., & Turner, G. L. (1975). *J. Gen. Microbiol*, *89*, 31.
Bergersen, F. J., & Turner, G. L. (1990). *Proc. R. Soc. Lond. B*, *238*, 295.
Dalton, D. A. (1995). In S. Ahmad, *Oxidative stress and antioxidant defenses in biology* (pp. 298–355). New York: Chapman and Hall.
Dalton, D. A., Diaz del Castillo, L., Kahn, M. L., Joyner, S. L., & Chatfield, J. M. (1996). *Arch. Biochem. Biophy*, *328*, 1.
Dalton, D. A., Hanus, F. J., Russell, S. A., & Evans, H. J. (1987). *Plant Physiol*, *83*, 789.
Dalton, D. A., Langberg, L., & Treneman, N. (1993). *Physiol. Plant*, *87*, 365.
Dalton, D. A., Russell, S. A., Hanus, F. J., Pascoe, G. A., & Evans, H. J. (1986). *Proc. Natl. Acad. Sci. USA*, *83*, 3811.
Elmerich, C., Dreyfus, B. L., Reysset, G., & Aubert, J. P. (1982). *EMBO J*, *1*, 499.
Everse, J., & Hsia, N. (1997). *Free Rad. Biol. Med*, *22*, 1075.
Gibson, Q. H., Wittenberg, J. B., Wittenberg, B. A., Bogusz, D., & Appleby, C. A. (1989). *J. Biol. Chem*, *264*, 100.
Giulivi, C., & Cadenas, E. (1993). *FEBS Lett*, *332*, 287.
Halliwell, B., & Gutteridge, J. M. (1986). *Arch. Biochem. Biophys*, *246*, 501.
Ji, L., Wood, S., Becana, M., & Klucas, R. V. (1991). *Plant Physiol*, *96*, 32.
Koch, B., Evans, H. J., & Russell, S. R. (1967). *Plant Physiol*, *42*, 466.
Layzell, D. B., & Hunt, S. (1990). *Physiol. Plant*, *80*, 322.
Lee, K., Shearman, L. L., Erickson, B. K., & Klucas, R. V. (1995). *Plant Physiol*, *109*, 261.
Moreau, S., Puppo, A., & Davies, M. J. (1995). *Phytochem*, *39*, 1281.
Puppo, A., & Halliwell, B. (1988). *Planta*, *173*, 405.
Udvardi, M. K., Day, D. A., & Gresshof, P. M. (1986). *Plant Cell Reports*, *5*, 207.