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OXYGEN EFFECTS ON MAIZE LEAF SUPEROXIDE DISMUTASE AND GLUTATHIONE REDUCTASE

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Key Word Index—*Zea mays*; Gramineae; maize; hyperoxia; enzyme activity; superoxide dismutase; glutathione reductase.

Abstract—Exposure of maize seedlings to an atmosphere containing 75% O_2 and 350 ppm CO_2 resulted in a two- to three-fold increase in glutathione reductase activity in leaf tissue within 48 hr after initiation of the O_2 treatment. This elevated level of glutathione reductase was still evident in plants maintained in the hyperoxic environment for 7 days. Superoxide dismutase activity was not altered from its level in control tissue during the 7-day experimental period. These results suggest a key role for glutathione reductase in the protection of photosynthetic tissue against detrimental effects of intermediate reduction products of O_2.

INTRODUCTION

The free radical superoxide, O_2^-, results from the preferred, one-electron reduction sequence for O_2. At times when fixation of CO_2 by higher plants is low and electron transport is operating at normal rates, as during the induction phase of photosynthesis, limited quantities of $NADP^+$ are available to accept electrons, and O_2 functions as an alternative electron acceptor [1]. Utilization of this pseudocyclic pathway for electron transport causes an increase in stromal pH and provides additional molecules of ATP [1], but may impose upon leaf cells a high concentration of superoxide. This reactive chemical species and its subsequent reduction product H_2O_2 can have detrimental effects upon cellular components, necessitating endogenous mechanisms for protection.

Higher plants possess superoxide dismutases (EC 1.15.1.1) which catalyse the disproportionation of O_2 to H_2O_2 and ground state molecular oxygen [2]. Involvement of this enzyme in the protection of aerobic organisms against oxygen toxicity has been inferred from observations of elevated levels of SOD activity in animals [4], bacteria [5, 6], fungi [7], blue-green algae [8], and green algae [9] exposed to atmospheres enriched in O_2. We failed to observe a similar effect in leaf tissue of the C_3 cotton plant, *Gossypium herbaceum* [10].

In leaves of C_4 NADP-malic enzyme species such as maize, bundle sheath chloroplasts are thought to function under high CO_2 concentrations and a deficiency of Photosystem II [11], conditions which may minimize the need for superoxide dismutase in these cells. In the present study, we evaluated the effect of 75% atmospheric O_2 upon levels of superoxide dismutase activity and examined responses of other oxidoreductases, including catalase (EC 1.11.1.6), peroxidase (EC 1.11.1.7), and glutathione reductase (NADPH) (NADPH: oxidized glutathione reductase, (EC 1.6.4.2), which may function in the protection of photosynthetic tissue against oxidative reactions involving intermediate reduction products of O_2.

RESULTS

Preliminary investigations of O_2 effects on superoxide dismutase activity in maize leaf tissue showed the level of this enzyme to be unaltered by exposure of seedlings to 45% O_2 for 7 days. Similarly, etiolated tissue which contained all forms of the enzyme but lower levels of activity than photosynthetically active tissue of a comparable age, also failed to exhibit any change in superoxide dismutase activity in response to a doubling of the atmospheric O_2 concentration (data not shown).

Levels of soluble protein and specific activities of several oxidoreductases in extracts from seedling maize leaf tissue exposed for up to 7 days to an atmosphere containing 350 ppm CO_2, but enriched to 75% O_2, are given in Table 1. Superoxide dismutase (SOD)‡ activity remained constant throughout the treatment period. Levels of cyanide-insensitive superoxide dismutase activity in this tissue were relatively low and changes in this level may not be apparent in quantitative determinations of total activity reported in Table 1. Spectrophotometric assays conducted in the presence of 1 mM cyanide,

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‡Abbreviation: SOD, superoxide dismutase.

Table 1. Levels of soluble protein and oxidative enzyme activities in seedling maize leaf tissue exposed to 75% O_2

	2 days*		7 days*	
	21% O_2	75% O_2	21% O_2	75% O_2
Enzyme activity	units/mg protein†			
Superoxide dismutase	12.0 ± 3.75	12.0 ± 1.71	10.0 ± 1.08	10.0 ± 1.74
CN^--sensitive‡	10.0 ± 2.73	9.0 ± 2.10	7.8 ± 1.5	7.8 ± 1.86
CN^--insensitive	2.0 ± 1.95	2.7 ± 1.20	2.7 ± 1.20	2.4 ± 0.87
Glutathione reductase	0.05 ± 0.023	0.12 ± 0.019§	0.06 ± 0.018	0.14 ± 0.014§
Peroxidase	13 ± 1.5	13 ± 1.2	15 ± 1.2	13 ± 1.7
Catalase	930 ± 103	1594 ± 255	880 ± 73	780 ± 77
Glycolate oxidase	6.4 ± 0.69	4.2 ± 1.03	9.8 ± 0.72	9.6 1.6
Malate dehydrogenase	63 ± 5.5	72 ± 5.7	50 ± 4.3	60 ± 3.8
Acid phosphatase	10 ± 1.5	10 ± 1.5	10 ± 1.0	8 ± 2.3
	mg/g tissue			
Soluble protein	6.3 ± 1.09	5.8 ± 0.54	6.4 ± 0.46	5.6 ± 0.32

*All plants were acclimatized in air (21% O_2) in vinyl chambers for 48 hr; O_2 in one chamber was then elevated to 75 ± 5%. CO_2 was maintained at 350 ± 50 ppm.

†A unit of dismutase activity is defined as that amount which inhibits by 50% a standardized rate of 0.025 $A_{550\,nm}$/min for ferricytochrome *c* reduction. A unit of glycolate oxidase activity is defined as 0.01 $A_{352\,nm}$. Activities of all other enzymes are expressed in units of μkat. Data for each enzyme are expressed as the average ±1 s.d. for eight samples, each prepared from 3–4-week-old leaf tissue harvested at the midpoint of the photoperiod.

‡Cyanide-sensitive SOD activity was calculated by subtracting SOD activity observed in the presence of 1 mM cyanide from total SOD activity observed in the absence of cyanide.

§Enzyme activities in O_2-treated tissue which are significantly different from control levels at $P < 0.005$.

however, revealed the levels of the cyanide-insensitive form, and consequently the cyanide-sensitive SOD, to be unaltered by the hyperoxic treatment of intact plants. Specific activities of glycolate oxidase (EC 1.1.3.1.), catalase, peroxidase, NAD^+-malate dehydrogenase (EC 1.1.1.37), and acid phosphatase (EC 3.1.3.2), as well as the level of soluble protein, in O_2-treated tissue were comparable to their corresponding levels in extracts from control tissue maintained in air throughout the experimental period. In contrast, glutathione reductase (NADPH) activity had increased two- to three-fold after 48 hr of O_2 treatment. This elevated level of reductase activity was still evident at the conclusion of the experiment 5 days later.

Comparison of data for O_2-treated and control leaf tissue, expressed as a function of fresh tissue weight, are presented in Fig. 1. The dramatic increase in glutathione reductase and relatively unchanged level of SOD activity in tissue exposed to 75% O_2 (Fig. 1a and b) are consistent with trends observed when enzymatic activities were expressed on the basis of soluble protein (Table 1). With the exception of catalase, levels of all other enzymes examined in O_2-treated tissue were similar to or less than levels observed in control tissue after experimental periods of both 2 and 7 days. Catalase activity initially increased in response to the hyperoxic environment (Fig. 1b), but decreased by day 7 to a level below air controls (Fig. 1a).

Compared to companion plants maintained outside vinyl chambers, maize seedlings inside vinyl chambers became progressively chlorotic. Chlorophyll content examined in 80% acetone extracts of leaf tissue (Table 2) was consistent with the physical appearance of leaves. Chlorophyll was dramatically reduced in tissue exposed to 75% O_2; although the chlorophyll *a*/*b* ratio in O_2-treated tissue was comparable to that in control tissue in vinyl chambers containing normal concentrations of O_2 (21%). Chlorophyll was quantified only at the conclusion of the week-long study, but tissue appearance indicated a continual loss of chlorophyll during the exposure to elevated O_2 concentration. Interveinal regions were most affected, and the development of this chlorosis was exacerbated by the supra-atmospheric concentration of O_2 used in these studies.

Despite the chlorosis, plants maintained in vinyl chambers in air under controlled conditions of light, temperature, humidity, and CO_2 exhibited constant levels of soluble protein and enzymatic activities in leaf tissue throughout the experimental period (Table 1), providing evidence that changes in enzyme levels in O_2-treated tissue occurred in response to the increase in atmospheric O_2, and not simply to transfer to the vinyl chambers. Chlorotic tissue from both control and O_2-treated plants showed gradual signs of recovery upon removal of plants from vinyl chambers, and new leaves exhibited no symptoms of stress.

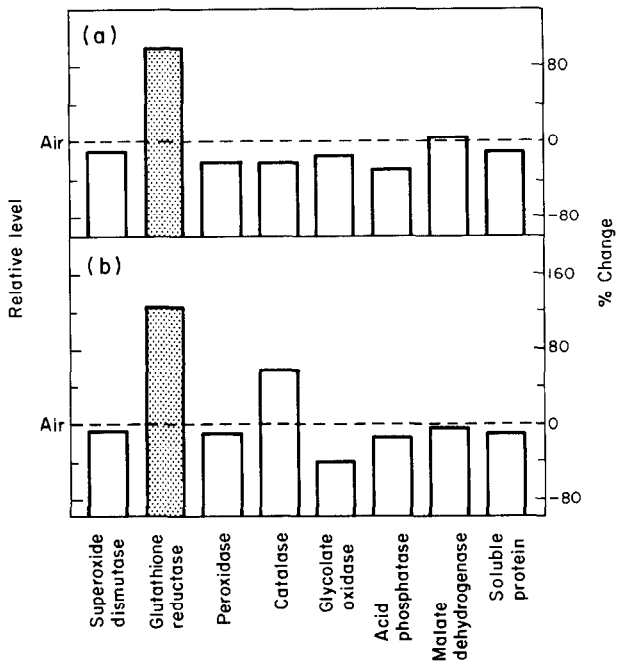

Fig. 1. Changes in levels of oxidative enzymes in *Zea mays* leaf tissue exposed to 75% O_2. Deviations of data for O_2-treated tissue from data for control tissue were calculated using averages of protein levels and enzyme activities, expressed as a function of tissue weight, in eight extracts each from 3–4-week-old tissue obtained from seedlings exposed to air or 75% O_2, 350 ppm CO_2 during the experimental period. Hatched bars represent those enzyme activities in O_2-treated tissue which were significantly different from corresponding activities in control tissue at $P < 0.005$. Units of enzyme activities are defined as in Table 1. (a) Tissue harvested 7 days after initiation of 75% O_2 treatment. Enzyme activities in air controls (units/g tissue) are comparable to those of day 2: superoxide dismutase, 66 ± 6.9; NADP-glutathione reductase, 0.38 ± 0.112. (b) Tissue harvested 2 days after initiation of 75% O_2 treatment. Enzyme activities in air controls (units/g tissue): superoxide dismutase, 72 ± 17.0; NADP-glutathione reductase, 0.32 ± 0.091; catalase, 5860 ± 650; peroxidase, 82 ± 8.3; NAD-malate dehydrogenase, 400 ± 47; acid phosphatase, 63 ± 1.09; glycolate oxidase, 40 ± 6.6. Soluble protein (mg/g tissue): 6.3 ± 1.09.

Table 2. Chlorophyll content of maize leaf tissue grown in controlled environmental chambers and in vinyl growth chambers

	Atmosphere		
	Inside vinyl chamber		Outside vinyl chamber
Chlorophyll*	Air	75% O_2†	Air‡
Total (mg/g fr. wt)	1.12 ± 0.070	0.34 ± 0.064	1.48 ± 0.244
a (mg/g fr. wt)	0.82 ± 0.054	0.25 ± 0.050	1.14 ± 0.160
b (mg/g fr. wt)	0.30 ± 0.028	0.09 ± 0.014	0.34 ± 0.089
a/*b*	2.78	2.63	3.41

*Data are averages ± 1 s.d. for eight samples harvested from plants maintained in vinyl chambers for 9 days.

†Plants were acclimatized in air for 2 days, then O_2 was increased to 75%. CO_2 was maintained at 350 ppm.

‡These plants were of identical age to the other plants but were not placed within the vinyl chamber. Other growth conditions were identical to the plants in the vinyl chambers.

DISCUSSION

In seedling maize leaf tissue exposed to 75% O_2 for 48 hr, glutathione reductase (NADPH) activity was elevated two- to three-fold over its level in control tissue and was present at this higher level after 7 days exposure of plants to the hyperoxic environment. Only constitutive levels of superoxide dismutase activity, however, were present, even after seedlings were treated with 75% O_2 for a week. These results are consistent with our previous observations with cotton, a C_3 plant [10]. Although low Photosystem II activity and associated deficiency of noncyclic electron transport occur in bundle sheath cells [11] apparently adequate constitutive levels of superoxide dismutase activity exist in maize leaf tissue so that no increase results on exposure to an O_2-enriched atmosphere. Foster and Edwards [3] recently reported a uniform distribution of superoxide dismutase activity between mesophyll and bundle sheath cells.

Suggestions that superoxide dismutase is induced in aerobic organisms are based upon failures to observe increases in enzyme activity under 'inducing' hyperoxic conditions when protein synthesis inhibitors were also present [6, 8]. Such studies do not eliminate the possibility that changes in levels of enzyme activity may be due to regulation by some other protein factor. That inactive molecules of superoxide dismutase may be present in aerobic organisms is demonstrated by data of Reiss and Gershon [12] which show, with aging, there is significantly reduced enzyme activity per unit of enzyme in rat liver. We submit that in photosynthetically active leaves of higher plants, high active constitutive levels of superoxide dismutase are present in order to protect the particularly sensitive photosynthetic apparatus against actual or potential detrimental effects of O_2 metabolism. Obviously the process by which levels of superoxide dismutase activity are elevated during the greening process needs to be evaluated.

Although glutathione reductase activity in maize leaf tissue is low (Table 1), its level in control tissue is comparable to that observed in leaves from numerous other plants [13], and the two- or three-fold higher level of activity consistently observed in leaf tissue exposed to 75% O_2 is more than can be attributed to variations in tissue samples (Table 1, Fig. 1). The increase in glutathione reductase activity which occurred early in the experimental period and the specificity of this response suggest a prominent role for this enzyme in protection of leaf tissue against damage by chemically reactive intermediate reduction products of O_2. Further, these data complement reported seasonal fluctuations in glutathione reductase activity in response to temperature-dependent changes in oxidative phenomena in leaves of winter-hardy evergreens [14] and our observation of O_2-dependent increases in glutathione reductase activity in cotton leaf tissue [10].

Definitive mass spectral data verify the *in vivo* occurrence of an oxygen cycle [16] in green plants which becomes quantitatively greater under conditions of limiting CO_2 [17]. Glutathione reductase (NADPH) which is present in the chloroplast [15] may have multiple functions within that organelle which relate to the oxygen cycle. By utilizing reducing equivalents from NADPH to regenerate the pool of reduced glutathione within the chloroplast, glutathione reductase ensures the availability of $NADP^+$ to accept electrons derived from photosynthetic oxidation of H_2O. Glutathione reductase thereby directs electrons away from O_2 and minimizes production of O_2^-. Secondly, reduced glutathione generated by glutathione reductase can function indirectly via an ascorbate–dehydroascorbate cycle [15, 18] to maintain low endogenous levels of H_2O_2, which must be accomplished within the chloroplast[19].

The dramatic response of glutathione reductase to the increase in environmental O_2 concentration was unparalleled by any other oxidoreductase examined. Catalase activity in O_2-treated tissue was 50% higher than in control tissue after 48 hr of treatment (Fig. 1b), but the change in specific activity was small (Table 1). Unlike the elevated level of glutathione reductase activity, the increase in catalase activity was not sustained over the entire experimental period, and in fact, a decrease in activity, relative to controls, was apparent after plants were exposed to 75% O_2 for 7 days. At this time catalase must contribute less toward protection of leaf tissue against oxidation by H_2O_2 and may reflect the development of senescence caused by environmental stress. It should be noted that Pritchard and Hudson [20] found changes in levels of catalase in plants treated with hyperbaric O_2 to be a more important parameter than the absolute level of activity.

The chlorosis of the plants placed in the vinyl chambers remains unexplained. The vinyl transmitted virtually no light below 300 nm which could contribute to abnormal leaf tissue [21]. Furthermore, certain volatile components from the vinyl chamber might have also occurred in sufficient concentration to account for this effect [22]. The oxygen stress did result in greater loss of chlorophyll (Table 2). These same chambers had been used for studies with cotton [10] and these plants did not reflect decreased chlorophyll content as did the corn plants used in this study. Clearly the leaf chlorophyll content of the C_3 plant was much less affected by the elevated O_2 concentration in contrast to the C_4 corn plants. Although this result may seem surprising, it is recognized that the C_4 mesophyll cells have reduced capacity for photorespiration so that photobleaching of these cells may be more easily accomplished. However, CO_2 levels were monitored at 350 μl/l. so that chlorosis was not due to an absence of this essential electron acceptor.

Levels of protein or enzymes which catalyse reactions involving intermediate reaction products of O_2 metabolism were not affected by the vinyl chamber. Likewise the marker enzymes, acid phosphatase and NAD^+-malate dehydrogenase, appeared insensitive to the effect of the vinyl chambers on O_2 concentration (Table 1). We therefore conclude that the large increase in glutathione reductase activity but constant level of superoxide dismutase activity in tissue exposed to 75% O_2 reflects an adequate capacity for metabolizing even high levels of O_2 but an increased

requirement for reduced glutathione for protection of chloroplast components against oxidative damage and probably H_2O_2 reduction. The increased acid phosphatase activity observed in cotton leaf tissue under elevated O_2 [10] was not observed in these experiments with corn tissue.

EXPERIMENTAL

Plant culture. Zea mays cv Pioneer 3369A seeds were obtained from the Virginia Tech Agronomy Department. Seeds were imbibed overnight and planted (25–50/25-cm plastic pot) in a Perlite–Vermiculite (1:1) potting medium in controlled environment chambers at 30° and 70° ± 10% r.h. A 16 hr day/8 hr night cycle was provided with irradiances of 200–300 $\mu E/m^2$ per sec supplied by a mixture of cool-white fluorescent and incandescent bulbs. Plants were watered with full-strength Hoagland's nutrient every fourth day.

Elevated O_2 *studies.* For studies of effects of altered concentrations of atmospheric O_2, two $60 \times 60 \times 120$ cm vinyl chambers were erected inside a controlled environment growth cabinet adjusted to maintain light and temp. at constant levels during plant culture. Filtered air from a compressed air-line, alone or supplemented with specified quantities of CO_2 and O_2 from compressed gas cylinders, was introduced at a rate of 10 l/min to provide about 1.5 vol. changes/hr. CO_2 was maintained at 350 ± 50 ppm and monitored by IR gas analysis during the experiment. Atmospheric O_2 concn was monitored using a Beckman Model 1008 Fieldlab oxygen analyser. Humidity was maintained at 70 ± 10% by bubbling gases through distilled H_2O as necessary.

Four pots of seedlings, 3–4 weeks of age were introduced into vinyl chambers and acclimatized in air for 48 hr. O_2 concn in one chamber was then elevated to 75 ± 5% for the duration of the experiment.

Protein preparation. Tissue was harvested from two pots in each chamber 2 and 7 days after initiation of the O_2 treatment, and all harvests were made at the midpoint of the light period to prevent variations in data arising from possible diurnal rhythms. Samples (3 g) of leaf tissue were crushed in liquid N_2, then homogenized in 15 ml 0.1 M Tris–HCl, pH 6.9, containing 0.01 M iso-ascorbate, 2% PVP-10 and 1.5 g Polyclar AT. After centrifugration of the homogenate at 24 000 g for 10 min, gel filtration chromatography of the supernatant on Sephadex G-50 coarse routinely yielded a soluble protein (free of small MW compounds) suitable for reliable enzyme assays.

Analytical procedures. Protein yields were determined using the Coomassie G-250 dye-binding assay [23] with defatted crystalline BSA as the standard reference protein.

Spectrophotometric assays of enzymatic activities were conducted using 10–200 μl aliquots of soluble protein extracts so that reaction rates were proportional to the amount of protein added. No loss of activity occurred between times of extraction and assay. Superoxide dismutase was monitored according to ref. [24]. A unit of superoxide dismutase activity is defined as that amount which inhibited by 50% a standardized rate of 0.025 $A_{550\,nm}$/min for ferricytochrome *c* reduction. Cyanide-insensitive superoxide dismutase was quantified by the inclusion of 1 nM cyanide in the assay mixture; cyanide-sensitive activity was calculated by difference. Peroxidase was assayed by monitoring H_2O_2-dependent oxidation of leuco-2,3′,6-trichloroindophenol at 675 nm [25]; a mM extinction coefficient of 273 was used to determine activity. Glycolate oxidase activity was detected by a modification of a general protocol proposed by Cohen [26]; H_2O_2, formed as a product of glycolate oxidation, was coupled to diaminobenzidine oxidation. One unit was defined as a change of 0.01 $A_{351\,nm}$. Relatively insoluble diaminobenzidine was prepared as a stock reagent by suspending 0.5 mg/ml with a glass homogenizer; the 3-ml reaction mixture contained 0.1 ml diaminobenzidine stock, 60 μg horseradish peroxidase, 10 mM glycolate and 300 mM KPi, pH 8.3. Other enzymes with reference to their assays are: glutathione reductase (NADPH) [27], catalase [28], NAD^+-malate dehydrogenase [29] and acid phosphatase [30].

Chlorophyll content of leaf tissue was determined in 80% acetone according to ref. [31].

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