Superoxide Dismutase

A POSSIBLE PROTECTIVE ENZYME AGAINST OZONE INJURY IN SNAP BEANS (PHASEOLUS VULGARIS L.)

Received for publication November 9, 1981 and in revised form February 3, 1982

EDWARD H. LEE AND JESSE H. BENNETT

Plant Stress Laboratory, Plant Physiology Institute, Science and Education Administration, Agricultural Research, United States Department of Agriculture, Beltsville, Maryland 20705

ABSTRACT

An experimental chemical N-[2-(2-oxo-1-imidazolidinyl)ethyl]-N'phenylurea (EDU), is an effective protectant against acute and chronic foliar injury due to ozone (O₃) when sprayed on intact leaves or supplied to the plants through soil application. An O₃-sensitive snap bean cultivar (Phaseolus vulgaris L. 'Bush Blue Lake 290') was systemically treated with EDU (0, 25, 50, and 100 milligrams per 15-centimeter diameter pot) to determine if EDU-induced or activated protective oxyradical and peroxyl scavenging enzymes. EDU-enhanced tolerance to O₃ injury always correlated with increases in superoxide dismutase (SOD) and catalase activities in the leaves. Peroxidase levels correlated more closely with foliar injury. Greater SOD levels in young leaves compared to older leaves were associated with lower ozone sensitivities in these tissues.

Polyacrylamide slab gel electrophoresis separations and specific determinations of SOD activity showed that EDU-treated plants possessed markedly greater SOD activity than non-treated plants. Tolerant plant tissues may have enhanced enzyme scavenging capabilities for the protection against toxix oxyradicals. Experimental confirmation for the oxyradical theory for O₃ phytotoxicity and SOD involvement in the detoxification process are presented.

Ozone (O₃) and certain oxy-free radicals are among the most damaging tissue toxins known (7-10, 15, 19). Nevertheless, products of these oxides have always been present in aerobic plant tissues subject to limited adaptation processes. At detrimentnal levels of O₃, visible and invisible injury to plants leads to leaf chlorosis or necrosis, decreased photosynthetic activity, altered metabolite pools, changes in enzyme activities, and effects on membrane permeability (9, 15, 22). Differential plant responses to O₃ have been related to environmental influences and genetic expression (22). Varieties of the same plant species can differ widely in their tolerances to oxidant stress, but the causes of such differences are still in question.

Previous studies (12, 13) have shown that O₃ injury can be prevented and senescence retarded in leaves of O₃-sensitive plants when treated with EDU. An adequate understanding of the mechanisms involved could provide important insights into both the basis for plant tolerance to O₃—the most important phytotoxic air pollutant—as well as to stress-induced aging of leaves. Biochemical and physiological processes in O₃-sensitive plants affected by EDU treatment have been investigated in our laboratory during the last 4 years (4, 12, 13). We report here results of experiments on EDU-induced tolerance which show higher tissue levels of SOD and catalase activities in leaves of a normally O₃-sensitive snap bean cultivar (Phaseolus vulgaris L. 'Bush Blue Lake 290') that had been transformed into a highly tolerant state by EDU treatment (12, 13).

MATERIALS AND METHODS

Plant Material and Chemical Treatment. Bush Blue Lake 290 (BBL-290) snap bean seeds were germinated and the plants were grown in 15-cm diameter clay pots containing sand-soil (3:1) media. The plants were cultured in a charcoal-filtered greenhouse (12). Ten days after sowing, the seedlings were treated with 200 ml 1% Peter's 20-20-20 fertilizer containing essential micronutrients. At 3 to 4 weeks of age, EDU doses of 0, 25, 50 or 100 mg/pot were applied as soil drenches in 100-ml aqueous applications. The plants were watered carefully to prevent loss of EDU. Twenty-four h after treatment, six replicate pots (one plant/pot) given each EDU treatment were subjected to O₃ fumigation to evaluate the tolerance induced. Unfumigated EDU-treated surrogate plants left in the greenhouse were assayed for total protein and enzyme contents on the day after O₃ fumigation, when O₃ tolerance of the test plants had been determined.

Chemicals. EDU was obtained from E. I. duPont de Nemours and Company. Enzymes, cofactors, and substrates used for enzyme assays were obtained from '1 104 Sigma. Protein Sigma. Protein purity was verified by polyacrylamide gel electrophoresis and column chromatography using DEAE-cellulose. Enzyme solutions of freshly prepared buttermilk xanthine oxidase were diluted in 0.05 M K-phosphate (pH 7.8).

Environmental Conditions. Greenhouse environmental conditions during the growth period were: temperature, day (18-30°C)/night (15-20°C); RH, 50 to 98%; maximum daytime PAR intensities at plant height, <2000 μE m⁻² s⁻¹.

Ozone Fumigation. Ozone fumigations were conducted in a Controlled Environments, Inc., Model PGW 36 growth chamber. Chamber temperature, PAR, and RH conditions were: 24 to 27°C, 350 μE m⁻² s⁻¹, and 70 to 80% RH, respectively. CO₂ concentration was 360 ± 30 μL/L. The chamber light bank contained GE F96T12CW1500 cool-white fluorescent lamps supplemental with 100 W incandescent lamps. Test plants were preequilibrated in the chamber for 2 h before exposure to O₃.

Ozone was generated by passing pure O₃ through a high voltage

1 Abbreviations: EDU, N-[2-(2-oxo-1-imidazolidinyl)ethyl]-N'-phenylurea; SOD, superoxide dismutase; DEAE, diethylamino ethyl; PAGE, polyacrylamide gel electrophoresis.

2 Mention of a trademark or proprietary product does not constitute a guarantee or warranty of this product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products that also may be suitable.
electric discharge ozonizer. A REM Model 612B chemiluminescent O₃ analyzer and Mast Ozone Meter were used to monitor O₃ concentrations in the fumigation chamber. Four-h fumigations with 898 μg/m³ (0.45 μl/l) O₃ were conducted in the O₃ tolerance tests. After O₃ exposure, the plants were returned to the greenhouse. Ozone injury was assessed the following day.

Extraction of Unfumigated Plants. Unfumigated trifoliolate leaves from EDU-treated and untreated control plants corresponding to those that showed greatest sensitivity to O₃ in accompanying exposure trials were harvested for analyses 48 h after EDU treatment. Acetone powder extractions were based on the method of Nason with some modification (18). Leaf samples for different stages of development were excised, rapidly weighed, and immersed immediately in Dry Ice cooled acetone. The tissues were ground with a pestle in an ice-cold mortar. The slurries were suspended in 180 ml cold acetone and filtered through a Büchner funnel. The residues were washed once with cold ethyl ether. Dried, pigment-free acetone powders were stored in plastic bags desiccated at −20°C.

Preparation of Crude Extracts. Crude enzyme preparations were prepared by mixing 2.0 g acetone powder with 0.6 g insoluble PVP, and extracting for 10 min with 0.05 M K-phosphate (pH 7.3) with continuous stirring at 4°C. PVP additive was included as a phenol scavenger. The ratio of acetone powder to buffer was 1:10 (w/v). The suspensions were centrifuged at 13,000g for 15 min. Residues were reextracted two additional times by resuspension and centrifugation in 5-ml portions of phosphate buffer. Combined supernatants were brought to final sample volumes of 30 ml. Extracts at this stage were used for total soluble protein determinations, and for the estimation of peroxidase and catalase activities. Soluble protein in crude extracts was measured colorimetrically at 595 nm using the Bio-Rad protein assay (5). Peroxidase activity was measured by the O-dianisidine method (23). Catalase was assayed by the decrease of H₂O₂ absorbance at 240 nm according to Luck (14). One unit of peroxidase activity equaled that amount of enzyme decomposing 1 μM H₂O₂/min at 25°C. One unit of catalase activity equaled the amount of enzyme that liberated half the peroxide oxygen from a H₂O₂ solution in 100 seconds at 25°C (14).

Partially Purified Enzyme. Superoxide dismutase was partially purified by the modified method of Baker (1). Phosphate-buffered crude extracts were passed through DEAE-cellulose columns. This step was necessary because phenolics and polyphenol oxidase present in the crude extracts interfere with SOD activity measurements. DEAE-cellulose separation columns (2 × 25 cm, fine mesh) were equilibrated with 0.1 M K-phosphate (pH 7.8). After the crude extracts were loaded, the columns were washed and eluted with the same buffer. Clear effluents containing SOD activity were collected and checked both in solution and on negatively-stained polyacrylamide gel isolates.

Superoxide Dismutase Assay. The assay method for the determination of SOD activity, as described by McCord and Fridovich (16), was based on SOD inhibition of superoxide-mediated ferri-cytochrome c reduction. A standard assay mixture in a 3-ml cuvette consisted of 5 × 10⁻⁵ M K-phosphate (pH 7.8), 1.0 × 10⁻⁴ M Na-EDTA (pH 7.8), 1.0 × 10⁻⁵ M ferricytochrome c; type III,

Fig. 1. Bush Blue Lake 290 snap bean plants 2 d after exposure to O₃ at 0.45 μl/l for 4 h. Pots were treated with 100 ml of 500 μg/ml (50 mg/pot) EDU (right) or water (left) 24 h before exposure to ozone. Control pot (left): primary leaf (No. 4), fully expanded trifoliolate leaf (No. 3) and partially expanded trifoliolate (No. 2) were injured by O₃ treatment. The young trifoliates (No. 1) were not injured by O₃. No leaves on EDU-treated plants were visibly injured.
and $5 \times 10^{-5}$ M xanthine. The control rate was adjusted to 0.025 $A$ (at 550 nm) per min at room temperature by adding 0.033 unit xanthine oxidase solution to the reaction mixture. The rate of reaction was read at 15-s intervals for 1 to 2 min.

One unit of SOD activity was defined as that which inhibited 50% of the reaction rate under these conditions.

**Polyacrylamide Gel Electrophoresis.** Electrophoresis in polyacrylamide gels containing SDS was performed using a Bio-Rad vertical slab gel apparatus and the discontinuous system described by Laemmli (11). The proteins were completely dissociated by immersing the samples for 1.5 min in boiling water. Twenty-one $\mu$g protein from each sample were loaded in each well. Electrophoresis was carried out in 0.1 M Tris-glycine buffer (pH 8.3) at room temperature with constant voltage of 40 v until the bromophenol blue marker reached the bottom of the gel (about 16 h). Gels were stained for 1 h in a solution containing 0.1% Coomassie blue and 50% TCA and then destained in 7% acetic acid in a diffusion destainer. The gels were scanned on a Gilford 2530 Gel Scanner at 590 nm to obtain the optical density for each protein band. Mol wt of the proteins were estimated from the relative mobilities of marker proteins separated simultaneously with the samples by electrophoresis. The mol wt standards were phosphorylase B, 94,000; BSA, 68,000, ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 21,000; and lysozyme, 14,300.

**Locating SOD on Polyacrylamide Disc Gels.** Duplicate samples were prepared for the localization of both enzyme protein and enzyme activity. SOD was located by the negatively stained photochemical procedure described by Beauchamp and Fridovich (3). Subsequent to electrophoresis, the gels were removed from the glass tubes and immersed in ice-cold 0.05 M K-phosphate for 10 to 15 min prior to staining. The gels were rinsed with distilled H$_2$O then transferred to tubes containing 2.45 $\times 10^{-3}$ M nitroblue tetrazolium for 15 to 20 min at room temperature, followed by immersion for 15 min in a 0.05 M K-phosphate (pH 7.8) solution containing 2.8 $\times 10^{-3}$ M tetramethyltheline diamine, and 2.8 $\times 10^{-5}$ M riboflavin. After staining, the gels were washed and suspended in 0.05 M K-phosphate (pH 7.8) and $1 \times 10^{-4}$ M Na-EDTA prior to illumination in fresh buffered solution. The gels were stained blue except in the zones containing SOD. Photographs were then taken. In addition, the stained gels were scanned within

![Image](image.png)

**RESULTS**

**Leaf Injury and Enzyme Activities.** Results comparing O$_3$ fumigation of intact EDU-treated and control snap beans are shown in Figures 1 and 2. Symptoms of O$_3$ injury on the trifoliate were expressed as stippling, bifacial necrosis, or marginal and tipburn injury. Plants pretreated with 100 ml 500 $\mu$g/ml EDU (50 mg/pot) showed no O$_3$ injury. Plants given 100 ml 250 $\mu$g/ml (25 mg/pot) EDU also showed no injury at the end of O$_3$ fumigation, but after 24 h, some injury could be observed. The lowest concentration of EDU (12.5 mg/pot) provided little ozone protection to the plants. Control plants showed severe injury after 2 to 3 h O$_3$ exposure. Soil applications of EDU given 24 h before fumigation consistently reduced O$_3$ injury to the trifoliate in relation to the increasing EDU concentrations applied. Leaves ranging from about 70 to 95% of their full size were most sensitive to ozone (Fig. 3). Expanding leaves (50-70%) exhibited less injury; very young trifoliate on both treated and nontreated plants were not injured by ozonation.

Superoxide dismutase, catalase, and peroxidase activities in extracts from EDU-treated BBL-290 trifoliate leaves are shown in Table I. Three experiments were conducted on each stage of development and the results were analyzed statistically for significance. Repeated tests gave similar patterns. At low EDU doses, 25 mg/pot, only slight stimulation of SOD and catalase activities (107 and 117% of control, respectively) were observed 2 d after treatment. The effects of 25 mg/pot EDU were, however, not statistically significant in any of the three experiments. Fifty mg/pot EDU greatly increased SOD activities in all three experiments. Catalase activity was also significantly increased by this treatment. EDU application at this level, which markedly reduced O$_3$ damage, was always associated with increasing SOD and catalase activity. EDU applied at twice this dose (100 mg/pot) caused some foliar damage. This was accompanied by lower SOD and
Younger leaves at different stages of development. Table I. Effect of Various Applied EDU Concentrations on SOD, Catalase, and Peroxidase Activity of BBL-290 trifoliate leaves.

<table>
<thead>
<tr>
<th>Conc. of EDU (mg/pot)</th>
<th>SOD* (units/g dry wt)</th>
<th>Catalase* (units/mg protein)</th>
<th>Peroxidase* (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>282</td>
<td>129.0 ± 7.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.8 ± 2.4</td>
</tr>
<tr>
<td>25</td>
<td>340</td>
<td>138.7 ± 16.8</td>
<td>20.9 ± 2.6</td>
</tr>
<tr>
<td>50</td>
<td>868</td>
<td>298.3 ± 19.9</td>
<td>36.4 ± 3.5</td>
</tr>
<tr>
<td>100</td>
<td>430</td>
<td>170.3 ± 17.5</td>
<td>29.3 ± 6.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> One unit of SOD activity is the amount of enzyme which inhibited 50% of the Cyt c reduction reaction at 25°C.

<sup>b</sup> One unit of catalase activity is the amount of enzyme which liberated half of the peroxide oxygen from a H₂O₂ solution in 100 s at 25°C.

<sup>c</sup> One unit of peroxidase activity equaled that amount of enzyme decomposing 1 µM of H₂O₂/min at 25°C.

<sup>d</sup> Means ± SD, n = 3.

Table II. SOD Activity Determined for Leaves at Different Stages of Development Taken from EDU-Treated (50 mg/pot) and Control Plants. Plants were treated 48 h before sampling.

<table>
<thead>
<tr>
<th>Stage of Development</th>
<th>SOD Activity* (units/mg protein)</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Younger leaves (less than 40% expanded)</td>
<td>305 a</td>
<td>307 a</td>
</tr>
<tr>
<td>Expanded leaves (50–70% expanded)</td>
<td>142 b</td>
<td>270 a</td>
</tr>
<tr>
<td>Mature leaves (fully expanded)</td>
<td>125 b</td>
<td>297 a</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values in each column followed by the same letter are statistically different at the 5% level of probability.

Fig. 4. Retention of SOD activity induced in BBL-290 trifoliate by EDU treatment 2 and 5 wk after soil application.

Catalase activities than those obtained with 50 mg EDU/pot.

To assess the possible adverse effects of excessive EDU applications, the second trifoliate leaves of plants treated with 0, 25, 50, and 100 mg/pot EDU were analyzed for peroxidase activity. As shown in Table I, no statistical differences were observed between the controls and those receiving the lower applied concentrations (25 and 50 mg/pot), but activities were significantly increased for the 100 mg/pot treatment. Plants given the highest EDU application showed nearly a 6-fold increase in peroxidase activity which appeared to be linked primarily to tissue injury.

The relationship between SOD activity and leaf damage caused by O₃, given as a function of the stage of leaf development, is shown in Figure 3. The SOD activity decreased from high values in young tertiary trifoliate (stage 1) to proportionately lower levels in 40 to 50% expanded secondary trifoliate (stage 2), 60 to 70% expanded trifoliate (stage 3), fully expanded leaves (stage 4), and primary leaves (stage 5). Maximum injury in the most sensitive leaves corresponded to SOD levels below 150 units/mg protein. SOD activity in extracts from young leaves with less than 40% expansion, 50 to 70% expanded leaves, and mature leaves are given in Table II. Visible injury increased in the order: expanded leaves > 50 to 70% expanded leaves > young tertiary leaves or EDU-treated plants. The data also indicate that all leaves on EDU-treated (tolerant) plants exhibited SOD activities equivalent to those of the control plant.
bands in EDU-treated plants (Fig. 5, gel column b), compared to those of untreated controls (Fig. 5, gel column a). Scanned gels showed the largest peak present was a protein with mol wt of approximately 32,000 followed by a 16,000 peak. The close proximity of these denatured protein bands with those of denatured commercial SOD protein is shown in Figure 5 gel column c. Commercial SOD showed two bands after SOD-PAGE: the undissociated dimer of mol wt 32,000 and a dissociated subunit at 16,000.

In another experiment, we measured SOD activities of unde-natured commercial enzyme and partially purified enzyme extracts from BBL-290 leaves by disc gel electrophoresis followed by negative staining techniques (Fig. 6). SOD activity from crude extracts was found in several distinct bands. Four major active bands (b, c, d, and e-band) were detected from both EDU-treated and untreated BBL-290 snap bean leaves. Scans of gels with commercially purified SOD exhibited three distinguishable bands (a, b, and c-band). A close correspondence existed between these three bands present in BBL 290 leaves and those of commercial SOD. Dialysis and ammonium sulfate fractionation of the crude extracts did not alter the SOD banding patterns.

**DISCUSSION**

Protein extraction from lyophilized acetone powders and *in vitro* detection of SOD activity was complicated by interaction among proteins and phenolic compounds. Phenols form complexes with proteins and are readily oxidized to quinones. Quinones in turn oxidize essential protein functional groups or form covalent bonds with the proteins. PVP, included as a phenol scavenger, overcame this difficulty. Buffered extraction solutions with a pH of 7.8 were originally tested, but the pH was altered to 7.3 to improve the effectiveness of PVP.

Highest SOD activity, accompanied by excellent O₃ protection, occurred for BBL-290 snap bean plants treated with 50 mg/pot EDU. Differences in leaf age and stage of development caused variations in O₃ response of control plant trifoliates. The relative susceptibility or tolerance of these leaves, however, was found to correlate well with the tissue SOD content. Superoxide dismutase catalyzes the reaction: O₃₂⁻ + O₂⁻ + 2H⁺ → H₂O₂ + O₂. It plays an essential role in scavenging superoxide radicals, protecting cells against O₃ or oxy-radical reaction products. The phytotoxic action of O₃ may be largely mediated through the formation of active oxygen intermediates, such as superoxide anion, hydroxyl- and perhydroxyl-radicals, or H₂O₂ and lipid peroxides generated in plant and animal tissues during ozone exposure (7, 8). Superoxide dismutase in conjunction with catalase may act as an enzymic oxidant detoxification system (7, 8, 17). In recent years, increasing evidence has been presented in support of such a hypothesis in higher plants (2, 4, 12, 20). SOD activities have also been associated with the protection of plant tissues from Sₐ₂₂₉ injury in plants (21).

Comparing one plant species or plant tissues from one variety with another in regard to SOD content and O₃ tolerance involves many inherent physiological and environment factors that cannot be easily controlled. Therefore, a more straightforward approach was sought in this investigation—*i.e.*, studying tissues from a normally O₃-sensitive plant variety which can be made highly tolerant to ozone in a short period of time by EDU treatment. This technique overcomes serious problems relating to plant age and stage of development, nutritional differences in mineral 'efficient' and 'inefficient' plant varieties and environmentally induced enzyme activities.

Certain plant growth regulators or environmental factors that cause stomatal closure will reduce plant sensitivity to O₃ exposures. In previous studies, we showed that EDU-induced O₃ tolerance did not result from stomatal closure or reduced leaf O₃ absorption rates (4). All evidence to date indicates that the protection occurs...
at the cellular level of organization and is biochemical rather than biophysical in its basic nature.

At the O<sub>3</sub> test concentration used (0.45 μl/l O<sub>3</sub>), non-EDU-treated (control) plants showed severe injury after 2 to 3 h O<sub>3</sub> fumigation. EDU soil applications in every case reduced O<sub>3</sub> injury to expanded leaves depending upon the concentration applied. EDU at the optimal dose that promoted maximum O<sub>3</sub> tolerance was also the most effective in inducing SOD and catachalase activities. We do not know to what extent EDU treatment affects the activities of other possible protective enzymes, such as glutathione peroxidase, which have been associated with enzymatic detoxification mechanisms and are found to respond to oxidant exposure in animal tissues (6, 17). We do know that EDU treatment enhances and sustains RNA and protein levels generally in leaves (12). EDU-treated leaf tissues also show increased sugar levels which seem to be caused by improved efficiency in cellular carbohydrate utilization. These metabolic effects are associated with the retardation of senescence in leaf discs held in the dark (under starvation conditions) or exposed to low PAR levels near the CO<sub>2</sub> compensation point (13). Retardation of senescence could be aided by more efficient carbohydrate utilization and the preservation of cell structure and integrity by protective oxidant scavenging systems.

Evidence to date suggests that EDU treatment enhances the basic aerobic nature of cells through the induction and regulation of oxidant-scavenging enzymes and cell sustaining mechanisms. These enzymes protect aerobic cells against tissue oxyrads and peroxides formed during photoxynthesis and sugar-dependent respiratory oxidase activity (13) as well as from active oxidants arising from exogenous sources such as environmental oxidants (8). EDU-potentiated tolerance brought about by the induction of oxidant scavenging enzymes could both mitigate injury and delay senescence. EDU-induced protection against oxidant stress or aging may depend in part upon maintaining the structural integrity of cells under stress conditions and improving the efficiency of cellular metabolic processes.

Acknowledgments—We thank C. Y. Wang of Horticulture Crops Quality Laboratory, United States Department of Agriculture for performing some of SDS-PAGE separation and L. C. Fraizer for technical assistance. The advice of J. E. Baker, Plant Hormone Laboratory, United States Department of Agriculture, Beltsville, MD, during this work is also gratefully acknowledged.

LITERATURE CITED


2. BAUM J, JG SCANDALIOS 1979 Developmental expression and intracellular localization of superoxide dismutases in maize. Differentiation 13: 133-140

3. BEAUCHAMP C, I FRIDOVICH 1971 Superoxide dismutase: Improved assays and an assay applicable to acrylamide gel. Anal Biochem 44: 276-287


6. Deleted in proof.


10. HECK WW 1968 Factors influencing expression of oxidant damage to plant. Annu Rev Phytopathol 6: 165-188


