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THE ROLE OF PLANT ENZYMES AND ETHYLENE DIUREA IN PROTECTION OF PINTO BEAN LEAVES FROM OZONE INJURY

A Thesis Presented

By

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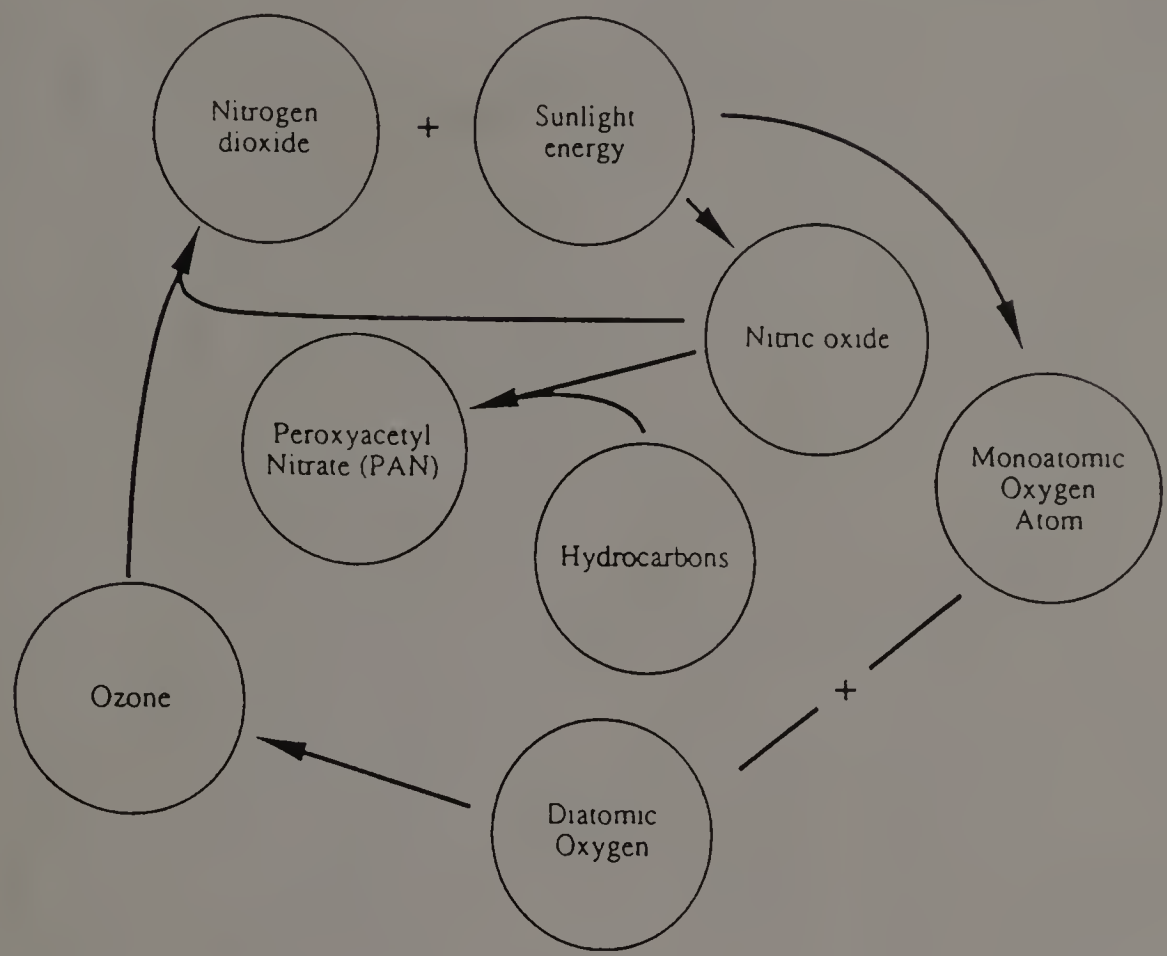
CHAPTER I

INTRODUCTION

Ozone, a triatomic form of oxygen, is both formed and eliminated naturally. Natural formation of this strong oxidant occurs in the troposphere when nitrogen dioxide in the presence of high levels of ultraviolet light (occurring on clear, sunny days) forms nitric oxide and a highly reactive monoatomic oxygen radical. This oxygen radical subsequently interacts with atmospheric oxygen to form ozone. Concentrations of natural ozone rarely exceed 0.05 ppm in the troposphere, as ozone subsequently decays by reacting with nitric oxide to reform oxygen and nitrogen dioxide. Ozone can also decay by absorbing light at 254 nm, an energy source that causes the ozone molecule to split into oxygen and a monoatomic oxygen radical (28). The sequence of these events (continual ozone formation/degradation) maintains the low level of ozone in the troposphere.

The natural ozone cycle in the troposphere becomes disrupted by the presence of reactive peroxy radicals and hydrocarbons in the atmosphere from automobile emissions and from the burning of fossil fuels (49). The hydrocarbons and peroxy radicals react with nitric oxide and form the photochemical oxidant peroxyacetyl nitrate (PAN), a reaction that essentially removes the nitric oxide from the ozone formation/degradation cycle (30), leaving little of the nitric oxide to react with monoatomic oxygen. Ozone formation thus continues unabated and elevated concentrations occur in the atmosphere. In addition, fossil fuel combustion releases nitrogen dioxide into the atmosphere, which produces more ozone as the increased nitrogen dioxide is split by ultraviolet light into nitric oxide and monatomic oxygen. Conditions

favorable to ozone formation are warm temperatures, intense sunlight and high nitrogen dioxide levels (49). The key ozone level determining reactions in the hydrocarbon polluted atmosphere are (20):



Currently, ozone is one of the most widespread air pollutants in the United States, and is particularly prevalent in the Northeast and Los Angeles basin. Ozone pollution levels within these area results from 2 sources. First, ozone formation occurs in the immediate vicinity from released hydrocarbons and second, from transport of hydrocarbons and ozone into the area. In Massachusetts, ozone pollution is aggravated by transport of photochemical air pollution formed from primary emissions in New York City. Pollution from New York is transported by prevailing winds through Connecticut and Massachusetts (19), resulting in elevated levels of ozone in the Hartford, Connecticut area in the afternoon and in the Boston, Massachusetts area in the evening (19). Connecticut and Massachusetts experience the highest ozone concentra-

tions in the Northeast, primarily due to long range transport. The amount of ambient ozone due to photochemical air pollution transport into Connecticut has been estimated at approximately 20 percent (20).

Chronic exposure to ozone levels as low as 0.05 ppm can cause damage to ozone sensitive vegetation (30). In the summer of 1984, the town of Ware and Fairhaven in central Massachusetts reached maximal peaks of 0.2 ppm ozone, four times the concentration necessary for damage to vegetation. Ware exceeded 0.12 ppm (The EPA limit for human exposure, 30) on 19 different days, and Fairhaven exceeded this level for 5 consecutive days. Other parts of Massachusetts were also subjected to high levels of ozone pollution. Sudbury in the eastern part of the state had a peak concentration of 0.17 ppm, and Agawam in the western part of the state reached an ozone peak of 0.15 ppm (30).

Extended periods of elevated ozone are known as an "ozone event" or "ozone episode". These ozone events occur when conditions for ozone formation are favorable for extended periods of time and result in toxic levels of this pollutant for many hours a day over several days (30). One of the results of elevated levels of ozone is extensive damage to vegetation. According to a 1974 estimate, annual crop loss to air pollutants (primarily ozone) in the United States was $500 million. Recent assessements have put losses to farmers from ozone and other air pollutants (sulfur dioxide) at $1 billion in agricultural crops (1). These estimates have not considered that ozone often works synergistically with other pollutants such as sulfur dioxide and results in secondary injury such as increased plant susceptibility to harmful pathogens (30, 51, 57, 72).

Because of the prevalence of ozone pollution, and the plant injury and crop loss resulting from exposure to this oxidant, scientists have studied possible means of protecting plants from ozone damage. Research has focused on why ozone injures plants, and the possibility of using plant breeding or antioxidants as measures of protecting plants from ozone. An antioxidant with potent capabilities is ethylene diurea (EDU). The limitations of EDU for ozone protection and how EDU provides protection are still not known.

Investigation of a possible ozone detoxifying system consisting of superoxide dismutase (SOD) and catalase (39) and determining the relationship of these enzymes with EDU would provide insight into the mode of action of EDU. While SOD has been studied considerably, little research has focused on catalase. Also of importance is quantifying the limitations of the protection EDU provides in relation to ozone exposure, time of EDU application, and concentration of EDU needed for protection.

Objectives

1. To observe the relationship between the activities of the enzymes superoxide dismutase (SOD), and catalase in relation to ozone exposure and ethylene diurea (EDU) application.

2. Establish the interaction between EDU and ozone in terms of visible injury and chlorophyll and carotenoid content.

CHAPTER II

LITERATURE REVIEW

Several environmental factors can influence whether a plant will be affected by ozone are known. High temperature (12, 44, 51, 55), humidity (52, 55), and light intensity (12, 51), and a sustained water supply (2, 17) are factors implicated in enhancing a plants susceptibility to ozone injury. Well watered plants are more likely to be injured by ozone than plants under drought or semi-drought conditions. Since ozone enters through the stomata, plants experiencing water stress would be less susceptible as ozone would not be able to enter the plant leaf and react with cellular membranes. Well fertilized plants appear to be more susceptible to ozone injury than plants suffering a nutrient deficiency (8, 50). Heavy metals in the soil, such as zinc, and cadmium can enhance ozone-induced phytotoxicity (26).

A plant's susceptibility to ozone is also often dependent upon stage of tissue development (18, 53), with leaves which have just matured more apt to exhibit foliar injury than immature leaves (18, 53). Immature leaves exposed to ozone are less likely to have elevated levels of peroxidase as older leaves, which are nearing senescence. Elevated levels of peroxidase activity has been associated with ozone injury (10).

Although a wide variety of plant species are susceptible to ozone, some species and cultivars are less sensitive than others. For example, the soybean cultivar 'Hark' is sensitive to ozone while the cultivar 'Hood' is tolerant and is able to withstand exposure to ozone episodes without visible signs of injury (41). Herbaceous plants with culitvars sensitive to ozone exposure include soybean (59), parsley (53), tomato

(42, 43, 45), pinto bean (73), navy bean (32), potato (18), tobacco (35), poinsettia (73), begonia (13), marigold (13), chrysanthemum (13) and snapdragon (13).

Several woody plants are also known to be sensitive to ozone, including White pine (4, 74), Ponderosa pine (71, 75), London plane (14), varnish tree (14), Honey locust (14), White birch (14), red maple (14), spruce (11), sycamore (55), green ash (55), silver maple (55) and poplar (54). Hardwood and evergreens suffer an additional problem in relation to most herbaceous species because hardwoods and evergreens are exposed to ambient ozone year after year, for decades and are more susceptible to chronic damage from ozone exposure (55). Ozone has been suggested to be a contributing factor in forest decline (55).

A common effect of ozone on plants is reduced growth (2, 17, 24, 55, 71). Research with soybeans (2) and Ponderosa pine (71) has demonstrated that both acute and chronic exposure of plant material to ozone results in a reduction of total plant biomass and a change in biomass allocation. In alfalfa exposed to ozone, root growth was reduced and plants had fewer leaves (24). In parsley exposed to ozone, more fixed carbon is transported to new leaves and less is transported to roots and mature leaves (53).

Another effect of ozone on plants is induction of increased ethylene production (21, 60). Within a few hours after exposure to ozone, tomato, tobacco, bean, onion, potato, spinach, squash, soybean, eucalyptus and English Ivy have a two to six-fold increase in ethylene emissions (22, 52, 60, 70, 72). Ethylene is known to disrupt flower development (22, 23), accelerate defoliation (52) and promote premature senescence (55, 70, 72).

Pollen growth is inhibited in plants exposed to ozone, a factor that may reduce or prevent seed production in plants. Working with an ozone sensitive cultivar (White Bountiful) and an ozone tolerant cultivar (Blue Lagoon) of petunia, Harrison and Feder (31) observed that the germination rate of the ozone sensitive cultivar was reduced by 80 percent and the tolerant by 15 percent. The germination rate may be reduced due to ultrastructural injury of pollen, as pollen organelles appeared to pull away from the cell membrane. This hypothesis is supported by more frequent changes in pollen organelles occuring in ozone sensitive species than in the ozone tolerant species. Ozone inhibits pollen tube growth in tobacco Bell-W3 at concentrations as low as 0.05 ppm (29). Pollen from tomato, corn and petunia have also been demonstrated to be sensitive to ozone (29).

Nutrient content of plants is altered by exposure to ozone. Plants exposed to ambient levels of ozone [7 h daylight mean (9 A.M. to 4 P.M.) of 0.06 ppm] have lowered concentrations of magnesium, calcium, potassium, and nitrogen (68). Plants exposed to 0.1 ppm ozone for 6 h/day for 5 days are observed to have increased concentrations of phosphorus, copper and iron (8). The exact mechanism for nutrient content alterations is unclear but could be related to changes in membrane permeability (35, 48). Clover nodulation has been observed to be inhibited by ozone exposure, resulting in a decrease in nitrogen fixation (36).

A common biochemical indication of plant exposure to ozone is an increase in membrane permeability. Solute leakage from *Phaseolus vulgaris* leaf discs upon acute ozone exposure was 2 to 4 fold greater than controls, resulting in significant losses of electrolytes, sugars, amino acids, and water from cells to intercellular spaces and causing a de-

crease in turgidity and a loss of plant cell integrity (35, 48). An increase in membrane permeability could result from oxidation of double and sulfhydryl bonds of fatty acids by ozone (55). Changes in fatty acids in the membrane following exposure of plant tissue to ozone may also alter membrane-bound photosynthesis systems as ozone in aqueous media can produce toxic photosynthetic imtermediates such as hydroxyl, perhydroxyl, and superoxide radicals (38).

Decreases in pigment content and the appearance of foliar injury such as necrosis and chlorosis are associated with ozone injury to plant tissue. Loss of chlorophyll has been observed in such plants as water cress, lettuce, and soybean (26, 39). Poplar leaves developed necrosis after exposure to 0.15 ppm ozone for 8 h/day, 5 days a week over 6 weeks (a chronic exposure), (54). Chlorosis, a symptom of chlorophyll degradation, has been observed in soybean and needles of Ponderosa pine trees after treatment with ozone (70, 72, 75).

Ozone decreases photosynthesis and causes a loss in plant chlorophyll. The loss of chlorophyll can result in a decrease of photosynthetic activity. Research with soybean indicates acute doses of ozone cause a rapid decrease in photosynthesis. With chronic exposure to this air pollutant, a period of several days or a few weeks is necessary for a drop in photosynthesis to occur process (59). Decreases in photosynthesis have been observed in four species of pine (4), and in poplar trees following exposure to ozone (58). A decrease in photosynthesis may be partially related to changes in nutrient content which can affect the manner in which photosynthetic complexes such as light harvesting complexes I and II bind to chloroplast membranes (68).

Dark respiration increases in poplar leaves when plants are exposed to low levels of ozone (58). Dark respiration in Ponderosa pine needles almost doubled after exposure to 0.15 ppm ozone (4). Increased respiration and decreased photosynthesis would result in a dramatic loss in the amount of carbon fixed, decreasing plant growth.

Changes in carbon fixation and metabolism have been associated with ozone exposure. White pine trees display abberations in carbon fixation within ten minutes after exposure to 0.1 ppm ozone, with a decrease in soluble sugars and an increase in sugar phosphates and free amino acids, especially alanine (74). Changes within soybean tissue after exposure to 0.5 ppm ozone for 2 h lasted for 3 days and included a depression in the activity of glyceraldehyde 3-phosphate dehydrogenase and an activation of glucose 6-phosphate dehydrogenase (69).

Ozone can also cause alterations in other enzyme activities. Peroxidase is known to increase upon exposure of tissue to acute levels of ozone (0.4 ppm for 2 h), an increase suggested to be partially dependent upon a rise in calcium ions of the cytosol due to increased membrane permeability (10). Increases of peroxidase activity following ozone exposure have been observed in soybeans (66) spinach plants (67), and poplar trees (54). An increase in peroxidase activity could result in an increase in quinones and polymers that are harmful to the plant (54).

Besides peroxidase, several other enzymes are effected by ozone. Work with soybean cultivars has shown increases in the activities of glucose 6-phosphate dehydrogenase, phenylalanine ammonia lyase, and polyphenol oxidase (70). Increases in these enzyme activities occur earlier in ozone sensitive species than ozone tolerant species.

A decrease in ascorbic acid content of plant tissue has been associated with exposure to ozone. Ozone tolerant plants appear to have a greater ability to maintain their ascorbic acid (an antioxidant) content as compared with ozone sensitive plants (67). Maintainance of the cellular level of ascorbic acid is necessary for the continued functioning of the enzyme ascorbate peroxidase and the chloroplast's photosystem I hydrogen peroxide scavenging system (67). Ozone tolerant spinach species were able to maintain monodehydroascorbate (MDHA) reductase and ascorbate peroxidase activities in the presence of ozone (67). Other researchers have suggested that ozone tolerant strains are able to prevent peroxidase activities from becoming elevated (54).

Differences in tolerances to ozone have been associated with different catalase and SOD activities, ozone tolerant species appear to have a greater ability to maintain the activities of SOD and catalase than ozone sensitive species (39, 41). Data from research with Norway spruce trees (11) support this hypothesis, and suggest that SOD and catalase enzyme activties may be affected by the concentration of calcium and copper in the cytosol. Ozone tolerant species, which maintain their membrane structure under ozone stress, can prevent leakage of solutes such as calcium and copper into the cytosol, protecting the activities of these enzymes. Research has suggested membrane bound forms of SOD help maintain the integrity of the membrane (7). The ability of tolerant plants to sustain the SOD and catalase activity can be overcome by exposure to very high levels of ozone (0.4 ppm for several hours), (6).

The ozone detoxifying system of SOD and catalase catalyze the reactions (39):

(1)* $2H^+ + 2O_2^- \text{----->} H_2O_2 + O_2$ (SOD regulated)

(2)* $2H_2O_2 \text{----->} 2H_2O + O_2$ (catalase regulated)

Within the plant cells the SOD catalyzed reaction is rate limiting as catalase has a higher maximum velocity than SOD (56). SOD, a metal based enzyme, exists in higher plants as either a copper-zinc based or manganese based form (7). Copper-zinc based SOD is located mostly in the chloroplasts, but is also present in the cytosol. This form of SOD is characterized by sensitivity to cyanide and is inactivated by hydrogen peroxide. The manganese based SOD present in the mitochondrial matrix and in the cytosol, is insensitive to cyanide and is not inactivated by hydrogen peroxide (56).

Both tolerant and susceptible cultivars of *Phaseolus vulgaris* had increase levels of SOD after exposure to ozone, suggesting SOD alone is not responsible for ozone tolerance (47). Insensitivity of primary leaves to ozone was shown not to involve SOD (47). Other researchers have suggested SOD activity is not an important factor, citing the fact that SOD levels increased only after visible injury occurred (16). Visible injury continued to increase with time, even with elevated SOD activity (16).

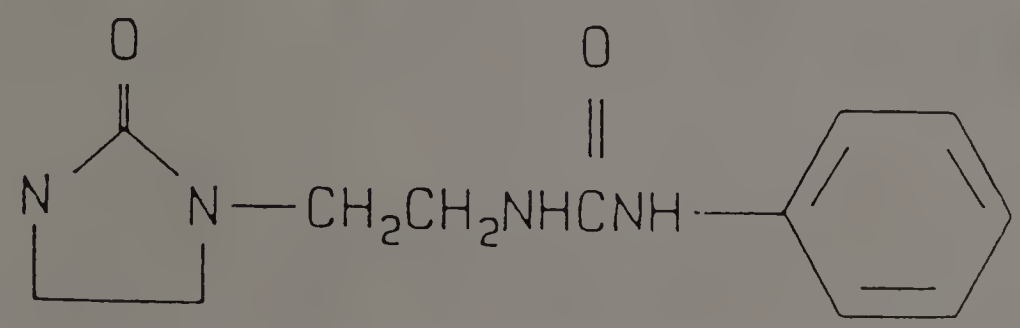
Whether or not a plant will be susceptible to ozone injury is often determined by a plant's genetic constitution. Tolerant cultivars may be able to maintain enzyme systems such as SOD and catalase that detoxify ozone (39, 41, 67).

While researchers have suggested screening various cultivars of a crop for sensitivity to ozone and utilizing tolerant species (34), as

ance, use of tolerant species may not be feasible. For example, an ozone sensitive species of petunia might have flowers more aesthetically pleasing than an ozone tolerant strain. Also, using tolerant hardwood species would not be a practical way of preventing the loss of forest stands as replanting millions of acres of forest would be time consuming and costly.

A more practical method of preventing ozone-induced injury to plants would be through the use of antioxidants. A variety of substance have been used as ozone-protectant chemicals, including ascorbic acid (41), and the fungicides benomyl (18, 32, 44, 45), triarimol, zineb, thiram and ferbam (51). Ascorbic acid is an effective antioxidant, but only at high concentrations (0.01 M or higher), (51). Benomyl is the most effective of the fungicides, however this compound and the other fungicides can alter plant-pathogen (harmful fungi) interactions in the field (32).

A more effective antioxidant appears to be an experimental chemical ethylene diurea (EDU, N-[2-(2-oxo-1-imidozolidinyl)ethyl]-N'-phenylurea), orginally synthesized by Carnahan et. al., for DuPont de Nemours E.I. Inc. in 1978 (9):



EDU has been used extensively in antioxidant research (6, 9, 12, 13, 14, 18, 32, 38, 39, 42, 46, 62, 63, 64, 73). Carnahan (9) applied EDU to Pinto beans (Pinto 111) and determined the ability of EDU both as a foliar spray and as a soil drench to protect plant tissue against ozone (28). A 6 ml foliar spray application of EDU at a concentration of 500 ug/ml (500 ppm) provided protection against visible injury induced by exposure to 0.8 ppm ozone for 150 minutes. Control plants not treated with EDU exhibited foliar injury on 100 percent of the leaf tissue, while EDU treated plants had injury on only 5 percent of the leaf tissue. Protection of plant tissue from ozone occurred within thirty minutes after foliar treatment (9). The prevention of the occurrence of visible injury EDU provided was greater than any other antioxidant.

Research with EDU applied both as an aqueous soil drench and as a foliar spray, has indicated EDU provides protection against ozone injury. Research with seedlings of red maple, white birch, White dogwood and White pine have indicated a 500 ppm EDU soil drench will prevent injury to these tree seedlings when exposed to 0.4 ppm ozone for 3 h (13). A comparison of soil drenches and foliar sprays suggests the soil drench method is more effective and lasts longer in protecting plants from ozone injury. An elapsed time of three to seven days between application of EDU and ozone exposure increased EDU's effectiveness as a soil drench. This research was supported by studies done with begonias, snapdragon, tomato, pepper, aster, lettuce and English Ivy (13).

Stem injection (61, 62) has also been suggested as an effective means of EDU application. Data gathered from treatment of yellow-poplar seedlings indicated that injection of 5 ml of a 500 ppm EDU solution

can maintain photosynthesis and prevent foliar injury when plants are exposed to 0.35 ppm ozone for 3 h (62). The protection afforded by EDU was overcome when seedlings were exposed to 0.95 ppm ozone for 3 h. EDU treated plants still suffered much less damage than control plants. Further research with seedlings of red maple, honey-locust, sweetgum, and Pin oak indicates much less EDU (5ml vs. 250 ml) is needed for protection when applied by stem injection instead of an aqueous soil drench (61). Both methods appear to be most effective when EDU is applied seven days before ozone exposure. The advantages of stem injection of EDU are avoidance of drift, precise dosage control, and improved applicator safety. The disadvantages with stem injection of EDU is this method is time consuming, requires greater training and inflicts a minor injury to the plant (61).

The effectiveness of EDU appears unrelated to light, temperature, humidity, and nutrient content of the plant (12). When ozone stress is the dominant factor affecting a plant, EDU can prevent significant ozone injury. However, an ozone sensitive plant suffering from severe water stress and exposed to ozone will not be helped by EDU application as osmotic stress and not ozone is the dominant stress. Research has suggested that application of EDU in the absence of ozone stress may have a slight detrimental effect on plants (33). EDU doesn't appear to affect stomatal resistance (63), suggesting EDU protection of plants is not from the prevention of ozone absorbance, yet EDU is still able to reduce damage from ozone (18, 32).

Past results have suggested that a wide variety of plants can be protected from ozone injury by EDU application. Some species which have been protected from ozone injury by EDU application include snap

beans (39), pinto beans (9), tomato, pepper, lettuce, English ivy (13), soybean (64), Navy bean (32), potato (18), petunia (12), begonia (13) red clover (38) red maple, white birch, white dogwood, white pine (14), sugar maple, white ash, dogwood, crabapple (63), and yellow poplar (62). All research with EDU has involved plants that were ozone sensitive, and to be effective, EDU levels should be adjusted for a particular species. EDU is most effective when applied three to seven days before ozone exposure (13, 61, 63).

Recent research has focused on EDU's mode of action. Some data has suggested EDU either maintains or enhances the activities of the catalytic oxidoreductase enzymes SOD and catalase (6, 39, 63) while other research suggests SOD is not responsible for the ozone protection EDU affords (15). These enzymes have been demonstrated to be related to photoxidative injury symptoms such as senescence (27), altered membrane permeability (27), lipid peroxidation (27) and ethylene production (3). One proposed hypothesis is SOD and catalase detoxify ozone and ozone-induced radicals before they can alter the biochemistry of a plant, which in turn prevents physical injury. Once the activities of these enzymes have decreased to a critical point (unknown), injury occurs. Therefore EDU may provide a plant with protection from ozone damage by sustaining the activities of SOD and catalase (6, 39).

Other research has suggested that only the imidazole component (also part of Benomyl) of EDU affords ozone resistance while the phenylurea part of EDU has a cell proliferating effect similar to the effect of the synthetic cytokinin, kinetin (40). EDU may protect plants from ozone injury by increasing the amount of soluble carbohydrates in leave tissue as treatment of *Phaseolus vulgaris* with EDU has increased the

levels of erythritol, fructose, glucose, sucrose, ribose and arbitol (37). Carbohydrates have been indicated as a mechanism of detoxifying hydroxyl radicals produced by ozone exposure (7).

EDU has also been known to increase peroxidase activity (up to two to three fold), and toxicity from high levels of EDU (greater than 1,000 ppm) maybe from the enhancement of this enzyme (39).

CHAPTER III

MATERIALS AND METHODS

Plant Material

Bean plants, *Phaseolus vulgaris* L. cv. Pinto 111 Idaho, were used in these studies. The plants were grown in a peat- sand-soil mix (1:1:1 by volume) contained in square black plastic pots (13 cm x 13 cm x 15 cm deep) from seed (2 plants per pot) in a controlled environment room (27 ± 2°C, SHO fluorescent lights, 16 h photoperiod, irridiance 20 W/m^2). Water was supplied as required to maintain moist soil conditions and fertilizer (20-20-20, N-P_2O_5-K_2O) was applied weekly in 150 ml aliquots from a solution containing 11.3 g dissolved in 2 liters, a concentration that promoted vigorous growth. Four plants per pot were thinned to two plants being as similar to each other as possible.

Ethylene Diurea Treatment

A wettable powder of ethylene diurea (EDU) (50 % active ingredient) at a concentration of 400 ppm active ingredient (unless specified otherwise) was dissolved and thoroughly mixed in distilled water. Except for temporal studies where EDU was applied at different times, a 150 ml aliquot of the EDU solution was applied as a soil drench to each pot 3 days before exposure of plants to ozone, an application procedure previously described (T. Craig Weidensaul, 73). For temporal experiments, EDU was applied at various stages of plant development, from seed to three days before treating the plants with ozone. Plants were exposed to ozone when the central leaflet of the fourth trifoliate was 0.5-1.5 cm long.

Ozone Treatment

Plants were treated with ozone for 4 h in a previously described ozone treatment chamber (1.03 m x 0.36 m x 0.83 m high), (21). Exposure to ozone was started 4 h into the 16 h photoperiod, a time period chosen to reflect the usual time when ozone episodes usually occur in the field. The ozone was generated by passing charcoal-filtered air across an ultraviolet bulb and the levels of ozone were monitored by a Dasibi Model 1008-RS ozone meter with the sampling tube at the surface of the central leaflet on the second trifoliate leaf of the experimental plant.

To assure open stomates during treatment with ozone, plants were thoroughly watered before exposure and relative humidity was maintained at 95 percent during the treatment period. Temperature in the treatment chamber was 27 ± 2°C during the day, 15 ± 2°C at night, corresponding to an expected summertime diurnal cycle. During the treatment period (both during and following ozone exposure), plants were under light from SHO fluorescent tubes at an irradiance of 20 W/m^2.

Enzyme Analysis

Leaf tissue was analyzed for catalase and SOD activity 1 day (47) after the termination of ozone exposure, using the procedure of Tanaka (66, 67). A 0.25 g portion of leaflet was selected from the second trifoliate (60-80% mature, a stage of development considered moderately/highly ozone sensitive), was macerated in 5 ml potassium phosphate buffer pH 7.8, containing 600 ppm polyvinylpyrrolidone as a phenol scavenger, using a chilled mortar and pestle. A 2 ml aliquot of the slurry was centrifuged at 13,600 G for 15 min and the supernatant was used for enzyme analyses. Samples were chilled throughout the isolation procedure.

To determine catalase activity a 60 ul sample of the isolated supernanant was added to 2 ml of a phosphate buffer (pH 7.8). The solution was thoroughly mixed and 60 ul of 3 percent hydrogen peroxide was added. The resulting mixture was homogenized and decrease in absorbance measured at 240 nm for 2 min. Extract prepared from a 20 replicate sample of control plants (no ozone and no EDU) was used to establish a measurement standard defined as 10 units of enzyme activity (absorbance decrease of 0.208/min). The data for samples assayed at the beginning of the measurement procedure and assayed again two hours later (the time required for analysis of all treatments) was the same.

Superoxide dismutase (SOD) activity was measured using the method of Beauchamp and Fridovich (5) and is based on the inhibition of a color change in nitro-blue tetrazolium (NBT) by SOD activity. A 35 ul sample of the supernatant from the enzyme isolation process (containing the SOD enzyme) was added to 2 ml of the SOD reaction mixture (Appendix A) and illuminated with a 60 W bulb in an aluminum foil lined box (12 cm x 12 cm x 10 cm high) for 6 min. Changes in absorbance were measured at 560 nm and compared to changes that occurred in a 20 replicate sample of control solutions having no supernatant added (absorbance increase was 0.22 units over six min). As with catalase, samples assayed at the beginning of the measurement procdure and assayed again at the end yielded nearly identical data, indicating time of measurement wasn't a significant variable.

Visible Injury

Visual injury to plants was monitored 1 day after ozone exposure. All recognizable forms of ozone damage to leaf tissue such as

flecking, stippling, chlorosis, and necrosis, were recorded and expressed as a percentage of leaf area injured.

Chlorophyll and Carotenoid Content

The chlorophyll and carotenoid content of leaf tissue were measured 48 h after ozone treatment, to allow for the development of injury (16). A 0.5 g sample of leaf tissue was selected from the third trifiolate (50-60% mature). The tissue was macerated with mortar and pestle in 10 ml of 80 percent acetone and 0.05 g of magnesium carbonate. The resultant slurry was allowed to set for 2 h in the dark at 5°C and subsequently filtered through 4 layers of cheesecloth. This solution was diluted 25 fold and absorbance measured at 645 and 663 nm for chlorophyll content and 475 nm for carotenoid content (65). The concentration of pigments were calculated as:

$$\text{total chlorophyll (ug/ml)} = 8.02 \times \text{Abs.}_{663} + 20.2 \times \text{Abs.}_{645}$$

$$\text{carotenoid content (ug/ml)} = \text{Abs.}_{475} \times 4.0$$

Experimental Design

All experiments were of completely random design and were replicated a minimum of three times unless specified otherwise. For each experiment there were four treatments; ozone/EDU, ozone/No EDU, No ozone/EDU, No ozone/No EDU (control), with four plants per treatment. Analysis of variance (F test) was used to determine differences between treatments at $p \leq 0.05$ or $p \leq 0.01$ (Appendix B).

CHAPTER IV

RESULTS

The dosage required to ensure extensive damage to leave tissue was determined by exposing Pinto bean plants to various hourly levels of ozone. An hourly level of 0.3 ppm ozone 4h/day over 3 days resulted in severe visible injury (Table 1). Visible injury included flecking, stippling, chlorosis and necrosis on the margins of the second and third trifoliate leaves. Plants treated with a lower concentration of ozone (0.12 ppm ozone, the EPA limit for human exposure) had injury on 5-10 percent of the leaf tissue (Table 9).

The total amount of visible injury remained constant as ozone exposure increased in duration (4 days) or concentration (0.4 ppm ozone) (Tables 5 and 6), although most of the visble injury at the higher levels of ozone was due to necrosis rather than chlorosis. Plants exposed to the longer duration of ozone (4 days) exhibited injury on 75 percent of the leaf tissue.

Besides visible injury, plants exposed to ozone had losses in chlorophyll and carotenoid pigments. Chlorophyll and carotenoid levels in leaf tissue were reduced when plants were exposed to 0.12 ppm ozone 4 h/day for three days. Although a slight decrease in chorophyll concentration of 5 percent was observed as compared with controls not treated with ozone, these differences were not significant (Appendix B). Increasing the ozone level to 0.3 ppm increased the loss of chlorophyll to 24 percent, a highly significant loss (Appendix B). Plants treated with 0.3 ppm ozone also had a 14 percent loss of carotenoids (Appendix B). Pinto bean leaves treated with 0.3 ppm ozone 4 h/day for 4 days had a 25 percent loss in chlorophyll content in the leaf tissue,

a highly significant loss (Table 5). In all experiments, decreases in chlorophyll were strongly correlated with decreases in carotenoids (r = 0.99).

The decreases in pigment content and the appearance of visible injury, were accompanied by a decrease in catalase activity in leaf tissue. In plants exposed to a low level of ozone (0.12 ppm, 4 h/day for 3 days), catalase activity in leaves was reduced by 22 percent as compared with controls (Table 9). Increasing the level of ozone treatment (0.3 ppm, 4 h/day for 3 days) caused catalase activity to decrease 61 percent (Table 5), a highly significant decrease. Exposing plants to an acute level of ozone (0.45 ppm for 4 h) resulted in a 32 percent loss of catalase activity in leaf tissue (Table 8), a significant loss in catalase activity as compared with plants not exposed to ozone.

Treatment of plants with ozone did not affect superoxide dismutase (SOD) activity. Plants exposed to 0.12 ppm ozone 4 h/day for 3 days had SOD activity similar to that of control plants (Table 9), with no significant differences among any treatments. Plants exposed to 0.3 ppm ozone 4 h/day for 3 days had maintained 93 percent of the SOD activity of the control plants (Table 3). Ozone exposure sometimes exhibited an unexplained increase in SOD activity (Tables 4, 8).

Injury from ozone treatments were altered by the application of the antioxidant EDU as a soil drench. A concentration of 400 ppm EDU reduced visible injury from 70-80 percent to 5-10 percent (Table 2). EDU also reduced visible injury when plants were exposed to 0.3 ppm ozone 4 h/day for 3 days. Plants treated with EDU had five to ten percent of the leaf tissue injured, while plants not treated with EDU, but exposed to the same ozone concentration had damage to 75 percent of the leaf

tissue (Table 3). Pinto beans treated with a longer duration of ozone (0.3 ppm ozone 4 h/day for 4 days versus 3 days) had 25 percent visible leaf injury when treated with EDU, but plants not treated with EDU had 75 percent visible leaf injury (Table 5).

Application of EDU reduced the amount of pigment lost in leaves following exposure to ozone. In most experiments, application of EDU prevented a significant loss of chlorophyll content. Only when plants were treated with 0.4 ppm ozone 4 h/day for 3 days did EDU treated plants suffer a significant loss of chorophyll compared to controls (Table 6). Increasing the level of ozone reduced the protection against chlorophyll damage that EDU could provide. Plants exposed to ozone and treated with EDU also had a smaller loss of carotenoids than plants exposed to ozone and not treated with EDU.

Treatment with EDU also reduced the loss of catalase activity in plants exposed to ozone. Plants exposed to an acute level of ozone (0.45 ppm ozone for 4 h), and treated with EDU had 80 percent of the catalase activity of the controls (no ozone, EDU), while plants without EDU application and treated with ozone had 68 percent of the catalase activity of matching control plants (no ozone, no EDU) (Table 8). Plants treated with a chronic ozone exposure (0.3 ppm ozone 4 h/day over 3 days) had a 61 percent loss of catalase activity while those treated with EDU had an 8 percent loss (Table 5). Increasing the ozone dosage reduced the ratio of catalase activity between plants treated with ozone (EDU/No EDU). When the ozone exposure increased in duration the ratio fell from 2.21 to 1.40 (Table 5). An increase in ozone intensity resulted in the ratio falling from 2.21 to 1.57 (Table 6).

Application of EDU had no discernible effects on the level of SOD activity within the leaf tissue of a plant. Plants exposed to 0.3 ppm ozone 4 h/day for 3 days and treated with a 400 ppm solution of EDU had 103 percent of the SOD activity of controls, while plants exposed to the same level of ozone and treated with a 300 ppm solution of EDU had 105 percent SOD activity of the controls (Tables 3 and 4). Plants treated with 0.3 ppm ozone 4 h/day for 3 days and not treated with EDU had 93 percent SOD activity of the controls (Table 3). None of these changes were significant.

Varying time of application of EDU to plants before ozone treatment did not result in any significant changes in any of the observed parameters (Table 7). When exposed to 0.3 ppm ozone 4 h/day for 3 days, and treated with 400 ppm EDU 3, 12 and 19 days before ozone treatment, there were no significant losses of chlorophyll. All plants not treated with EDU had highly significant losses of chlorophyll (Appendix B). Catalase activity of plants receiving EDU applications 3, 12, and 19 days before ozone treatment decreased 14, 13 and 15 percent, respectively. Plants not treated with EDU had highly significant (3 and 12 days) or significant (19 days) decreases (40 to 83%) in catalase activity (Table 7).

When the level of EDU was reduced by 25 percent (from 400 ppm to 300 ppm) and the ozone level remained at 0.3 ppm 4 h/day for 3 days, visible injury increased from 5-10 percent to 10-20 percent. The decrease in catalase activity rose from 8 percent to 17 percent (Table 4).

Increasing the duration or intensity of ozone treatment while maintaining the level of EDU at 400 ppm resulted in a rise in the amount of visible injury, a decrease in chlorophyll content and catalase activity when compared to plants treated with a lower amount of ozone (Tables 5 and 6).

Table 1
Relationship Between Various Ozone Concentrations and Chlorophyll Loss

Ozone Concentration* (ppm)	Chlorophyll Loss (%)
0.15	1.5
0.20	5.0
0.25	12.2
0.30	24.1

*Ozone concentration for 4 h/day for 3 days.

Table 2
Relationship Between Various EDU Concentrations and Visible Injury

EDU (ppm)*	Visible Injury (%)
0	70-80
100	50-60
200	20-30
300	15-20
400	5-10
600	10-20

*Ozone concentration of 0.3 ppm 4 h/day for 3 days.

Table 3
The Effect of EDU on the Development of Ozone Injury in Beans

Treatment[a] Ozone	EDU (ppm)	Visible Injury (%)	SOD (units)	Catalase (units)
No	0	0	3.0	9.0
	400	0	2.9	7.8
Yes	0	70-80	2.8	3.5**
	400	5-10	3.1	7.2

[a]Plants exposed to 0.3 ppm ozone 4 h/day for 3 days. One unit of catalase activity is defined as a 0.0208 decrease in absorbance at 240 nm. One unit of SOD activity is defined as a 10 percent inhibition of the absorbance change of the dye NBT at 560 nm. Each number is an average of 12 data points.

** Significantly different from other treatments at $p \leq 0.01$.

Table 4
The Effect of a Reduced EDU Concentration on the Development of Ozone Injury

Treatment[a] Ozone	EDU (ppm)	Visible Injury (%)	SOD (units)	Catalase (units)
No	0	0	7.2	5.4
	300	0	6.8	7.1
Yes	0	70-80	7.4**	2.3**
	300	10-20	7.6**	5.9

[a]Plants exposed to 0.3 ppm ozone 4 h/day for 3 days. One unit of catalase activity is defined as a 0.0208 decrease in absorbance at 240 nm. One unit of SOD activity is defined as a 10 percent inhibition of the absorbance change of the dye NBT at 560 nm. Each number is an average of 12 data points.

** Significantly different from other treatments at $p \leq 0.01$.

Table 5
The Effect of Extended Ozone Treatment on EDU

Treatment[a] Ozone (days)	EDU (ppm)	Visible Injury (%)	Catalase Activity (% control)	Relative Catalase Activity (EDU/No EDU)
3	0	70-80	39	
				2.21
	400	5-10	86**	
4	0	70-80	57	
				1.40
	400	20-30	80	

[a]Plants exposed to 0.3 ppm ozone 4h/day for 3 or 4 days. Data for catalase expressed as percent of control, where control plants were those not exposed to ozone or EDU and are considered to be 100 percent. Each number is an average of 12 data points.

** Plants treated with a 3-day ozone exposure and treated with EDU were different at $p \leq 0.01$ as compared with plants not treated with EDU.

Table 6
The Effect of Ozone Concentration on EDU Efficacy

Treatment[a] Ozone (ppm)	EDU (ppm)	Visible Injury (%)	Catalase Activity (% control)	Relative Catalase Activity (EDU/ No EDU)
0.3	0	70-80	39	
				2.21
	400	5-10	86**	
0.4	0	60-70	44	
				1.57
	400	20-30	69*	

[a]Plants exposed to either 0.3 ppm or 0.4 ppm ozone 4 h/day for 3 days. Data for catalase expressed as percent of control, where control plants were those not exposed to ozone or EDU and are considered to be 100 percent. Each number is an average of 12 data points.

**/* Plants treated with ozone and with EDU were significantly different at $p \leq 0.01$ (**) or $p \leq 0.05$ (*) as compared with plants treated with ozone but not with EDU.

Table 7
Variation of Time of EDU Application and the Resultant Effect on the Development of Ozone Injury.

Treatment[a] Application (days)	EDU (ppm)	Visible Injury (%)	Catalase Activity (% control)
3	0	70-80	39
	400	5-10	86**
12	0	60-70	17
	400	5-10	87**
19	0	50-60	60
	400	5-10	85*

[a]Plants exposed to 0.3 ppm ozone, 4 h/day for 3 days. EDU applied either 3, 12, or 19 days before exposure to ozone. Data for catalase and expressed as percent of control, where control plants were those not exposed to ozone or EDU and are considered to be 100 percent. Each number is an average of 12 data points.

**/* Plants treated with EDU were significantly different at $p \leq 0.01$ (**) or $p \leq 0.05$ (*) from plants not treated with EDU.

Table 8
Effects of an Acute Ozone Exposure
and EDU on Pinto Beans

Treatment[a]		Visible		
Ozone	EDU (ppm)	Injury (%)	SOD (units)	Catalase (units)
No	0	0	3.3	10.5
	400	0	4.1	12.4
Yes	0	10-15	5.3*	7.1*
	400	0-5	4.4*	9.9*

[a]Plants exposed to 0.45 ppm ozone for 4 hours. One unit of catalase activity is defined as a 0.0208 decrease in absorbance at 240 nm. One unit of SOD activity is defined as a ten percent inhibition of the absorbance change of the dye NBT at 560 nm. Each number is an average of 4 data points.

* Significantly different at $p \leq 0.05$ (*) from all other treatments.

Table 9
Effects of EDU and a Low Level
Ozone Exposure on Pinto Beans

Treatment[a]		Visible		
Ozone	EDU (ppm)	Injury (%)	SOD (units)	Catalase (units)
No	0	0	5.0	6.3
	400	0	5.3	7.5*
Yes	0	5-10	6.6	4.9
	400	0	5.8	8.4*

[a]Plants exposed to 0.12 ppm ozone 4 h/day for 3 days. One unit of catalase activity is defined as a 0.0208 decrease in absorbance at 240 nm. One unit of SOD activity is defined as a ten percent inhibition of the absorbance change of the dye NBT at 560 nm. Each number is an average of 4 data points.

* Significantly different at $p \leq 0.05$ from all other treatments.

CHAPTER V

DISCUSSION AND CONCLUSION

Discussion

Exposing Pinto bean plants to an ozone level of 0.12 ppm for 4 h/day over three days resulted in visible injury to the leaf tissue. Damage to foliage occurred on the second and third trifoliate leaflets in the form of flecking, stippling, chlorosis and necrosis. As ozone treatments increased either in intensity or duration, visible injury also increased. After exposure to 0.3 ppm ozone 4 h/day over 3 days, plants exhibited visible injury over 70-80 percent of the canopy.

Coinciding with the occurrence of visible injury was a decrease in the content of the pigments chlorophyll and carotenoids. EDU application helped prevent the degradation of these pigments and in turn inhibited the occurence of visble injury. How carotenoids are affected by ozone exposure and EDU treatment has not been studied. Carotenoids are necessary accessory pigments for chlorophyll. Carotenoid data followed the same patterns as chlorophyll data for all treatments and was strongly correlated with chlorophyll data ($r = 0.99$). Plants exposed to ozone suffered a significant loss of chlorophyll and usually a significant loss of carotenoids.

There was no relationship between the activity of superoxide dismutase (SOD) and treatment with EDU and/or exposure to ozone. Some research (7, 27, 39) has indicated SOD to be an important enzyme in detoxifying ozone, while other research (15, 16, 47) has suggested SOD plays no significant role in ozone tolerance. A reason for the discrepancy could be different isolation procedures, inconsistencies in assay procedures and a variation in species of plants (7, 15). For example,

in some instances SOD activity was measured using only crude extracts (16), while other researchers isolate SOD by dialysis (47) or by DEAE-cellulose columns (39). All assays were based on the superoxide scavenging ability of SOD, preventing the reduction of a chemical or complex. Caution must be taken to remove peroxidase from the solution to be assayed, or a false low reading could result. This is more likely to occur in ozone damaged tissue which will have higher peroxidase levels (66). There was variation in the compound used to accept the electron from the superoxide molecule; some researchers used ferricytochrome c (39, 47), while others used NBT (11, 15, 16). There was no discernible pattern between isolation techniques, assay procedures or SOD data. In other studies, activity in other studies was measured using a change in absorbance on a per weight basis (39), versus using only change in absorbance as used in this research. Measuring on a per weight bases adds another variable (weight) to the measurement of SOD activity.

Another important factor pertaining to variability in SOD activity is the fact that the copper-zinc based form of SOD is inhibited by a build up of hydrogen peroxide (56), and accounts for 60-75 percent of the SOD activity in a cell (manganese based SOD accounts for the rest, 16). If catalase activity decreases, levels of hydrogen peroxide would increase in the leaf, inhibiting the activity of copper zinc based SOD, potentially lowering activity by 75 percent. The procedures of isolation and measurement of SOD removes hydrogen peroxide, leading to a reading of SOD activity that may not accurately reflect SOD activity whithin the leaf. The procedure used for measuring SOD activity in these studies was orientated towards the measure of the activity of the hydrogen peroxide resistant form of SOD (manganese based) not the

hydrogen peroxide sensitive form of SOD. Lee and Bennett (39) used isolation (purified extract) and assay procedures (ferricytochrome c) different from the isolation (crude extracts) and assay techniques (NBT) used in this study. There isolation procedure howver, still removes SOD from the presence of hydrogen peroxide. There is no practical procedure to date that can accurately measure SOD activity within the leaf or to measure how much hydrogen peroxide is necessary for the inhibition of SOD activity.

The data supports the concept of EDU protection of plant tissue from visible injury by ozone was due to sustained catalase activity. In our experiments, plants exhibiting ozone induced foliar injury had catalase activities lower than controls. EDU application either directly or indirectly results in catalase activity being maintained and secondary injury symptoms such as visble injury and pigment loss being prevented. When the capacity of EDU to protect plants is overcome by increased hourly ozone levels or longer duration of ozone exposure, catalase activity subsequently decreases. A drop in catalase activity occurred approximately one day before the onset of visible injury and pigment loss. While catalase activity is sustained, a build up of hydrogen peroxide is prevented and SOD activity is not inhibited.

Experiments varying EDU concentration against a constant ozone level (0.3 ppm ozone 4 h/day over 3 days) showed a 400 ppm solution to be effective in preventing visible injury on Pinto 111 bean leaves. Concentrations below 300 ppm failed to provide adequate protection, resulting in a rapid increase in visible injury of leave tissue along with decreases in catalase activity and chlorophyll and carotenoid content. A common problem in EDU research is determining an effective

concentration of EDU (especially for field studies) and EDU application levels are either too high resulting in toxicity, or too low preventing EDU from being effective.

The protection a plant receives from EDU can be overcome by increasing the hourly ozone levels or duration of exposure. When hourly ozone levels were increased by 33 percent (from 0.3 ppm/h to 0.4 ppm/h), plants treated with EDU had an increase in visible injury and a decrease in catalase activity in comparison with plants treated with EDU and exposed to the lower concentration of ozone. Increasing the duration of exposure from three days to four days (0.3 ppm 4 h/day) had the same effect on EDU treated plant as increasing the hourly ozone levels; catalase activity decreased, pigment loss and visible injury increased.

Another important issue addressed in this study was the temporal aspect of EDU application. Our data suggest EDU could be applied the same day as seeds were sown and the resultant plants exposed to ozone nineteen days later and EDU still was able to shield plants from ozone injury. Protection from oxidant injury was just as effective as if plants were treated three days before ozone exposure, an indicated time for optimal EDU efficacy (literature review). EDU may not be able to afford protection for nineteen days in the field however, due to possible higher leaching rates and microbial activity. Also, soil conditions could affect the availability of EDU for uptake. Our prolonged protection could have been due to the fact that EDU may have been bound to the organic material in the soil media (peat moss) which allowed fo continual uptake by the plant root system.

Our data indicates that EDU at a concentration of 400 ppm could provide ample protection for potted Pinto bean 111 plants from ambient

ozone pollution occurring within nineteen days after application of EDU if the rooting media is the same as used in this study. The highest ambient levels of ozone occurring in western Massachusetts are $\leq$0.2 ppm for 1-4 h. A 150 ml aliquot of a 400 ppm EDU solution protected plants from on ozone level as high as 0.45 ppm for four hours. This information could provide a base for field studies with similar media as used here, and using the same cultivar of Pinto beans. In both cases (ambient and acute ozone exposure) protection was correlated with sustained catalase activity which prevented secondary injury symptoms from occurring such as loss of chlorophyll and carotenoids.

Conclusion

Ozone is a prevalent air pollutant in the United States causing a wide variety of detrimental effects on plants (30). Prevention of oxidant injury and understanding the mechanism(s) of protection could aid in preventing further decreases in crop yields and damage to ozone sensitive plants.

One possible mechanism plants posses for detoxifying ozone is the utilzation of the enzymes superoxide dismutase (SOD) and catalase. SOD catalayzes the reduction of an oxygen radical to hydrogen peroxide and oxygen. Catalase enzyme action converts hydrogen peroxide to water and oxygen.

Our data suggests that EDU protects plants from ozone injury by maintaining catalase activity. The detoxification of hydrogen peroxide by catalase allows SOD activity to continue, decreasing the concentration of oxygen radicals. A build up of hydrogen peroxide has been

associated with inhibition of SOD (56). With the continued functioning of these 2 enzymes, harmful oxy radicals from ozone are readily converted to hydrogen peroxide and in turn water and oxygen. This prevents a loss of structural integrity resulting in pigment degradation and visible injury.

A 400 ppm solution of EDU prevented plants from suffering ozone induced foliar injury when exposed to a high level of ozone (0.3 ppm 4 h/day over 3 days). The protection EDU afforded was overwhelmed by increasing the hourly ozone concentration or the duration of ozone exposure. A decrease in catalase activity always occurred as the protection by EDU receded. EDU applied as a soil drench nineteen days before plants were exposed to ozone was still able to maintain catalase activity during ozone treatment.

By completing EDU/ozone response studies, a determination of the amount of EDU necessary for protection against a particular oxidant level can be interpolated. Soil application of EDU can maintain catalase activity in plant leaf tissue preventing a loss of structural integrity and disruption of physiological activity from ozone exposure.

APPENDIX A

SUPEROXIDE DISMUTASE REACTION MIXTURE

Superoxide dismutase reaction mixture contained 1.17 x 10^{-6} M riboflavin, 0.01 M methionine, 2 x 10^{-5} M sodium cyanide, 5.61 x 10^{-5} M p-nitroblue tetrazolium (NBT), and 0.05 M potassium phosphate (KH_2PO_4) at pH 7.8 (5).

APPENDIX B

ANALYSIS OF VARIANCE TABLES

ANOVA Table for Catalase (Table 3)

Source	DF	MS	F Value
Experiment	2	14.919	2.977
Ozone	1	111.021	22.154**
EDU	1	18.750	3.741
Ozone/EDU	1	69.120	13.793**
Within	42	210.480	5.011
Total	47		

ANOVA Table for SOD (Table 3)

Source	DF	MS	F Value
Experiment	2	8.607	9.892**
Ozone	1	0.010	0.011
EDU	1	0.075	0.086
Ozone/EDU	1	0.351	0.403
Within	42	0.870	
Total	47		

ANOVA Table for Chlorophyll (Table 3)

Source	DF	MS	F Value
Experiment	2	4053.377	6.166**
Ozone	1	7313.672	11.126**
EDU	1	1986.380	2.900
Ozone/EDU	1	5082.061	7.731**
Within	42	657.359	
Total	47		

ANOVA Table for Carotenoids (Table 3)

Source	DF	MS	F Value
Experiment	2	85.084	2.977
Ozone	1	126.750	4.435*
EDU	1	50.021	1.750
Ozone/EDU	1	63.023	2.205
Within	42	28.576	
Total	47		

ANOVA Table for Catalase (Table 4)

Source	DF	MS	F Value
Experiment	2	7.126	2.517
Ozone	1	38.342	13.539**
EDU	1	62.792	22.172**
Ozone/EDU	1	3.049	1.077
Within	42	18.718	
Total	47		

ANOVA Table for SOD (Table 4)

Source	DF	MS	F Value
Experiment	2	7.948	18.366**
Ozone	1	3.521	8.136**
EDU	1	0.041	0.100
Ozone/EDU	1	0.907	2.097
Within	42	0.433	
Total	47		

ANOVA Table for Chlorophyll (Table 4)

Source	DF	MS	F Value
Experiment	2	2521.850	8.441**
Ozone	1	17995.508	60.236**
EDU	1	593.613	1.987
Ozone/EDU	1	2311.500	7.737**
Within	42	298.752	
Total	47		

ANOVA Table for Catalase (Table 5)

Source	DF	MS	F Value
Experiment	2	3.813	0.626
Ozone	1	99.763	16.387**
EDU	1	10.268	1.687
Ozone/EDU	1	22.688	3.727
Within	42	6.088	
Total	47		

ANOVA Table for Chlorophyll (Table 5)

Source	DF	MS	F Value
Experiment	2	664.351	4.639*
Ozone	1	14466.435	101.025**
EDU	1	2094.842	14.629**
Ozone/EDU	1	297.533	2.078
Within	42	143.196	
Total	47		

ANOVA Table for Catalase (Table 6)

Source	DF	MS	F Value
Experiment	2	6.794	1.681
Ozone	1	120.968	29.934**
EDU	1	21.068	5.213*
Ozone/EDU	1	2.253	0.558
Within	42	4.041	
Total	47		

ANOVA Table for Chlorophyll (Table 6)

Source	DF	MS	F Value
Experiment	2	2039.200	29.546**
Ozone	1	5731.257	83.042**
EDU	1	1338.799	19.398**
Ozone/EDU	1	11.894	0.172
Within	42	69.016	
Total	47		

ANOVA Table for Catalase (Table 7)

Source	DF	MS	F Value
Experiment	2	14.425	2.892
Ozone	1	49.613	9.947**
EDU	1	11.077	2.221
Ozone/EDU	1	30.237	6.062*
Within	42	4.988	
Total	47		

ANOVA Table for Chlorophyll (Table 7)

Source	DF	MS	F Value
Experiment	2	460.754	4.916*
Ozone	1	4820.021	51.427**
EDU	1	3407.070	36.352**
Ozone/EDU	1	1350.448	14.409**
Within	42	93.725	
Total	47		

ANOVA Table for Catalase (Table 8)

Source	DF	MS	F Value
Ozone	1	35.403	5.999*
EDU	1	22.090	3.743
Ozone/EDU	1	0.809	0.137
Within	12	5.901	
Total	15		

ANOVA Table for SOD (Table 8)

Source	DF	MS	F Value
Ozone	1	4.625	7.323*
EDU	1	0.010	0.015
Ozone/EDU	1	2.890	4.578
Within	12	0.631	
Total	15		

ANOVA Table for Chlorophyll (Table 8)

Source	DF	MS	F Value
Ozone	1	222.756	2.594
EDU	1	130.531	1.520
Ozone/EDU	1	1736.803	20.229**
Within	12	85.859	
Total	15		

ANOVA Table for Catalase (Table 9)

Source	DF	MS	F Value
Ozone	1	0.181	0.042
EDU	1	21.856	5.067*
Ozone/EDU	1	5.176	1.200
Within	12	4.314	
Total	15		

ANOVA Table for SOD (Table 9)

Source	DF	MS	F Value
Ozone	1	4.515	3.618
EDU	1	0.330	0.264
Ozone/EDU	1	1.052	0.843
Within	12	1.248	
Total	15		

ANOVA Table for Chlorophyll (Table 9)

Source	DF	MS	F Value
Ozone	1	0.276	0.002
EDU	1	446.266	3.015
Ozone/EDU	1	205.208	1.386
Within	12	148.015	
Total	15		

APPENDIX C

THE EFFECT OF EDU AND OZONE ON PIGMENTS

Treatment[a]		Chlorophyll	Carotenoids
Ozone	EDU	(ug/ml)	(ug/ml)
No	0	186.7	40.7
	400	178.7	40.4
Yes	0	141.7**	35.2**
	400	174.7	39.5**

[a] Plants exposed to 0.3 ppm ozone 4 h/day for 3 days. EDU concentration in ppm. Each number is an average of 12 data points.

** Significantly different from other treatments at $p \leq 0.01$.

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