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Alteration of Extracellular Enzymes in Pinto Bean Leaves upon Exposure to Air Pollutants, Ozone and Sulfur Dioxide[1]

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ABSTRACT

Diamine oxidase and peroxidase, associated with the wall in pinto bean (*Phaseolus vulgaris* L. var Pinto) leaves, can be washed out by vacuum infiltration and assayed without grinding the leaf. The diamine oxidase activity is inhibited in vivo by exposure of the plants to ozone (dose of 0.6 microliters per liter × hour), whereas the peroxidase activity associated with the wall space is stimulated. This dose does not cause obvious necrosis or chlorosis of the leaf. These alterations are greater when the dose of ozone exposure is given as a triangular pulse (a slow rise to a peak of 0.24 microliters per liter followed by a slow fall) compared to that given as a constant square wave pulse of 0.15 microliters per liter for the same 4 hour period. Exposure of the plants to sulfur dioxide (at a concentration of 0.4 microliters per liter for 4 hours) does not result in any change in the diamine oxidase or peroxidase activities, yet the total sulfhydryl content of the leaf is increased, demonstrating the entry of sulfur dioxide. These two pollutants, with different chemical reactivities, affect the activities of the extracellular enzymes in different manners. In the case of ozone exposure, the inhibition of extracellular diamine oxidase could profoundly alter the movements of polyamines from cell to cell.

Exposure of plants to air pollutants induces many changes in physiological and biochemical processes (15, 25). Biochemical markers, such as enzyme activities and metabolite pools, can be used to detect early alterations in plants that occur before the appearance of visible injury or a decline in yield. However, the lack of specificity regarding the action of general plant stress upon a single marker creates a need for simultaneous screening of several biochemical markers or the use of more specific markers, currently undefined.

Peroxidases (donor: H_2O_2 oxidoreductase; EC 1.11.1.7) have frequently been used as biochemical markers of plant stress in general as well as air pollutant stress (5, 6, 16, 21). Stress usually increases the peroxidase activity, which is assayed as:

Reduced compound + H_2O_2

$$\rightarrow \text{Oxidized Compound} + H_2O$$

Certainly, air pollutants, such as ozone and sulfur dioxide, are known to increase the peroxidase activity (6, 21); however, the effect of ozone is found to be more pronounced on extracellular peroxidase activity than on the activity of the total cellular extract (5).

Increased amounts of phenolic compounds stimulate peroxidase activity and thus may regulate peroxidase activity *in vivo* (16). One phenolic compound, CA[4], was found to be present in *Phaseolus vulgaris* leaves, which had been exposed to ozone, at twice the concentration detected in unexposed leaves (13), thus implying a regulatory relationship.

Diamines and polyamines, which can stabilize membranes, retard senescence and buffer cells against changes in ionic composition (22), are thought to play a role in growth of rapidly expanding tissues. Furthermore, polyamines have been shown to prevent ozone-induced visible injury to plants (20). These polyamines are, in part, regulated by diamine oxidase. Diamine oxidase (diamine: oxygen oxidoreductase; EC 1.4.3.6), found notably in Leguminosae, primarily oxidizes putrescine, as shown in the equation below, to form hydrogen peroxide, ammonia, and a monoaldehyde, which spontaneously forms pyrroline (22).

$$H_2N\text{-}(CH_2)_4\text{-}NH_2 + H_2O + O_2 \rightarrow H_2O_2 + NH_3 + H_2N\text{-}(CH_2)_3\text{-}COOH$$

Although diamine oxidase was regarded as a soluble, cytoplasmic enzyme (16), investigators (9) have recently observed the presence of that enzyme in the cell wall region of lentil seedlings. They have also found a high level of diamine oxidase in the extracellular fluid obtained from pea epicotyls (8). Moreover, Kaur-Sawhney *et al.* (14) have reported that polyamine oxidase activity appears to be localized in the cell wall region of oat leaves. Extracellular amino oxidases could play a role in regulating the level of di- and poly amines in the apoplast and alter the movements of polyamines between cells (8, 22).

In this paper we report the effect of sulfur dioxide and different ozone exposures on the extracellular and total activity of diamine oxidase and guaiacol-dependent peroxidase in Pinto bean leaves. Further, the effects of CA on both guaiacol- and ascorbate-dependent peroxidase and the effect of H_2O_2

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[4] Abbreviations: CA, caffeic acid; CSTR, controlled stirred tank reactor; DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid).

on diamine oxidase were tested in both clean-air- and ozone-fumigated plants.

MATERIALS AND METHODS

Plant Material

Seeds of *Phaseolus vulgaris L.* var Pinto (W. Atler Burpee Co., Warminster, PA 18974) were germinated in the dark in a growth chamber at 18°C in pots containing vermiculite. After 48 h, the pots were transferred to a lighted growth chamber (24°C/20°C day/night temperature, about 50% RH day, 12 h light regime at a PAR intensity of 400 μE m^{-2} s^{-1}). The plants were watered every other day with deionized water. Half-strength Hoagland solution was supplied to the plants once a week. Unifoliate and first trifoliate leaves were used in the experiments.

Fumigation

The plants were exposed to atmospheres of ozone and sulfur dioxide in 1.22 m diameter, Teflon-covered, CSTR chambers in the greenhouse of the Statewide Air Pollution Research Center at Riverside, CA. Fumigations were accomplished by adding the pollutant to the filtered air entering the chambers. To generate ozone, tank oxygen was passed through a Griffin Ozonizer, and the ozone in the CSTR chamber was monitored with a Dasibi Ultraviolet Ozone Analyzer (19). Sulfur dioxide was from a commercial tank (Matheson Gas), and its concentration within the CSTR was monitored by a TECO model 43A pulse fluorescence SO_2 Monitor. The analyzers were calibrated quarterly with a transfer standard obtained from the California Air Resources Board office in El Monte, CA.

Twenty-four plants, of an age of 18 to 20 d, were randomly assigned to three treatments (eight plants per treatment). The treatments were: (a) control, i.e. filtered air alone; (b) square wave of ozone, generated with an immediate rise of ozone from the control level to a level of 0.15 μL/L (v/v) of ozone for 4 h, followed by an immediate decline back to the control level; (c) triangular wave of ozone, in which the level of ozone would slowly rise over 1.5 h up to the final peak of 0.24 μL/L which was held for 1 h, followed by a slow decline over the next 1.5 h back to the control level. The total dose of the ozone to each group of the ozone-exposed plants was the same (0.60 μL/L × h). Under most circumstances, these exposure levels resulted in very little visible injury to the plants, but they did result in a decline in total wet weight accumulation of the plant grown over 4 weeks (with two exposures per week, 15% decline; D Olszyk, B Takemoto, RL Heath, unpublished data). In rare instances, when visible injury was noted in the margin (only with the triangular pulse), those leaves were not used.

Plants were selected and exposed to sulfur dioxide in a similar manner, except only a single exposure of 0.4 μL/L SO_2 was given in a square pulse over 4 h which resulted in no visible injury. Exposure of slightly higher levels (0.5 μL/L) gave slight injury patterns in the margins (about 10% of total leaf area), but the visible injury varied slightly from day to day and was dependent upon the environmental conditions.

Preparation of Extracellular Fluid

The leaves were harvested 24 h after exposure to ozone and vacuum infiltrated (at −65 kPa; four periods of 30 s each, followed by a release of the pressure) with 100 mM KCl, 66 mM K-phosphate buffer at pH 7.0, according to the method of Castillo *et al.* (4). After infiltration, the leaves were blotted with absorbent tissue and centrifuged (1000*g* for 10 min at 4°C) in nested tubes. The upper tube, containing the leaves, had a perforated bottom through which the extracellular fluid passed and was collected in the lower tube. The fluid was maintained in an ice bath and analyzed immediately for enzymic activities and protein content.

Preparation of Total Cell Material Extract

Leaf material (0.5 g) was ground in liquid nitrogen with insoluble PVP (1 g/g fresh weight tissue) and 3 mL of 100 mM KCl, 66 mM K-phosphate buffer (pH 7.0) in a mortar. The extract was filtered and centrifuged at 10,000*g* for 10 min. The resulting supernatant fluid constituted the soluble fraction of total cell material and was analyzed immediately for enzyme activities and protein content.

Peroxidase Assay

Peroxidase activity was assayed with two different electron donors (5). Guaiacol peroxidase was determined in a reaction medium containing 66 mM K-phosphate buffer (pH 6.1), with 30 mM guaiacol and 6 mM H_2O_2 and an aliquot of the plant extract. The increase in absorbance was recorded at 470 nm with a Beckman DU-7 spectrophotometer. Guaiacol-peroxidase activity is expressed as ΔA_{470} min^{-1} mg^{-1} protein.

Ascorbate peroxidase was determined in a reaction mixture of 100 mM Hepes-KOH buffer (pH 7.0) with 0.1 mM EDTA, 100 μM Na-ascorbate, and 100 μM H_2O_2, to which was added an aliquot of the plant extract. The enzymic activity was measured by following the oxidation of ascorbate at 265 nm. Ascorbate peroxidase activity is expressed as μmol min^{-1} mg^{-1} protein, calculated from the ascorbate extinction coefficient of 13.4 mM^{-1} cm^{-1}.

Diamine Oxidase Assay

The diamine oxidase activity was determined according to the method of Federico *et al.* (9). Reactions were carried out in 110 mM K-phosphate buffer at pH 7.0 with 10 mM putrescine as the substrate. After incubation at 37°C for 10 min, the reaction was stopped by adding 200 μL 10% (w/v) TCA to the 1.5 mL reaction sample, followed by 20 μL of *o*-amino-benzaldehyde (10 mg/mL) in ethanol to detect pyrroline. The absorbance of *o*-amino-benzaldehyde was measured at 430 nm on a Beckman DU-7 spectrophotometer 10 min later. Diamine oxidase activity is expressed as nmol pyrroline min^{-1} mg^{-1} protein, using an extinction coefficient of 1.86 mM^{-1} cm^{-1}.

Sulfhydryl Assay

The total SH-content of the extracellular fluid was assayed as described by DeKok *et al.* (7). Sulfhydryl was determined

in 0.2 M Tris-HCl, 1 mM K-phosphate buffer (pH 8.0), 2% SDS, and 0.5 mM 5,5′-dithio-bis (2-nitrobenzoic Acid) (DTNB). After 15 min the developed color was measured at 412 nm with a Beckman DU-7 spectrophotometer. Correction was made for the absorbance of DTNB-reactive compounds in the incubation mixture in the absence of the extracellular fluid. The total SH-content is expressed as nmol SH mg^{-1} protein, using an extinction coefficient of 13.6 mM^{-1} cm^{-1}.

Other Methods

Protein was determined by the Bio-Rad protein assay with bovine serum albumin as the standard (2). All data were analyzed by a two-way analysis of variance (ANOVA) and a Duncan's multiple range test of mean difference.

RESULTS

The variations of extracellular and total guaiacol-dependent peroxidase activities within primary leaves of Pinto bean after ozone exposure are shown in Table I. Exposure of the plant to ozone induced an apparent increase of leaf extracellular activity (defined as that activity which can be washed out of the intact leaves by vacuum infiltration) and an increase in the total activity within the leaf extract. The increase of the extracellular activity (51%) was statistically significant only in plants exposed to the triangular pulse of ozone. The activity per unit of protein was higher in the extracellular fluid than that of the total cell extract. However, the total activity within the cell was much greater because the amount of protein contained in the extracellular fluid is only 0.11 ± 0.02% of cellular total.

Extracellular peroxidase activity assayed with ascorbate as the electron donor was also increased after exposure to a triangular wave of ozone (that with a peak distribution, Table II). Caffeic acid at 10 μM in the assay mixture stimulated this activity in both the control and exposed plants. Yet this stimulation was more pronounced in control plants (4-fold) than in ozone-treated plants (2.5-fold). Thus, in the presence of caffeic acid, peroxidase activity was only slightly inhibited by ozone exposure. The stimulation of peroxidase activity by caffeic acid was specific for ascorbate-dependent peroxidase activity; guaiacol-dependent peroxidase activity was only slightly (8%) increased in the presence of caffeic acid (data not shown).

The response of diamine oxidase to ozone fumigation was similar, although in the opposite direction, to the response of peroxidase. As shown in Table III, ozone fumigation, under both exposure regimes, caused a significant decline in diamine oxidase activity within the extracellular fluid, while the activity of the total leaf extract indicated no net change in any case. Again the triangular ozone wave (with the higher peak) caused the greatest decline in activity (nearly 50%).

Hydrogen peroxide, one of the products of diamine oxidase, can inhibit the enzyme itself (18, 22). Since ozone decomposition in water can induce the formation of hydrogen peroxide (12), the effect of hydrogen peroxide upon diamine oxidase after ozone fumigation was tested (Fig. 1). The activity of the extracellular enzyme from the control plants was inhibited when hydrogen peroxide was added; a 50% inhibition occurred at a concentration of about 2.2 mM H_2O_2 (open circles, Fig. 1). The enzymatic activity of diamine oxidase in the extracellular fluid from ozone-exposed leaf tissue was reduced by about 50% even without added hydrogen peroxide, but adding hydrogen peroxide induced a further decline in activity (down to 25% by 2.2 mM; open triangles, Fig. 1). Thus, the H_2O_2-inhibition curve for postfumigation activity from ozone-exposed plants paralleled the control inhibition curve

Table I. *Extracellular and Total Guaiacol-Dependent Peroxidase Activities from Pinto Bean Primary Leaves after Ozone Fumigation*

Assays of the peroxidase activity, with guaiacol as the substrate, were carried out after preparation of the leaf material according to "Materials and Methods." The plants were exposed to ozone as described in "Materials and Methods" for control-filtered air, square wave, or triangular wave exposure. All ozone fumigations had an average dose of 0.6 μL/L × h. Values are the average of two different experiments with three replicates in each experiment. Values followed by the same letter in the same column are not significantly different (above the 5% level).

Treatments	Extracellular Fluid	Total Extract
	ΔA_{470} min^{-1} mg^{-1} *protein*	
Control	32.0 (a)	15.1 (a)
Ozone		
Square wave	37.4 (ab)	16.8 (a)
Triangular wave	48.2 (b)	17.7 (a)

Table II. *Effect of Caffeic Acid on the Extracellular Ascorbate-Dependent Peroxidase Activity from Pinto Bean Primary Leaves*

The assay of peroxidase activity, with ascorbic acid as the electron donor, in extracts from exposed plants was carried out according to "Materials and Methods." Plants were exposed to either control-filtered air, or to the triangular wave of ozone (at a dose of 0.6 μL/L × h) according to "Materials and Methods." Caffeic acid (CA, 10 μM) was either withheld from (−) or added (+) to the reaction before beginning the assay. Values are averages ± SD for 8 replicates.

Treatment	−CA	+CA
	μmol min^{-1} mg^{-1} *protein*	
Control	0.43 ± 0.10	1.75 ± 0.19
+Ozone	0.61 ± 0.06	1.55 ± 0.32

Table III. *Extracellular and Total Diamine Oxidase Activities from Pinto Bean First Trifoiate Leaves after Ozone Exposure*

Enzyme assays were carried out as described in "Materials & Methods" and Table I. Values are an average of two different experiments with three replicates in each experiment. Values followed by the same letter in the same column are not significantly different (at a 5% level).

Treatments	Extracellular	Total
	nmol pyrroline min^{-1} mg^{-1} *protein*	
Control	1076.2 (a)	31.9 (a)
Ozone		
Square wave	774.2 (b)	34.1 (a)
Triangular wave	527.8 (c)	32.3 (a)

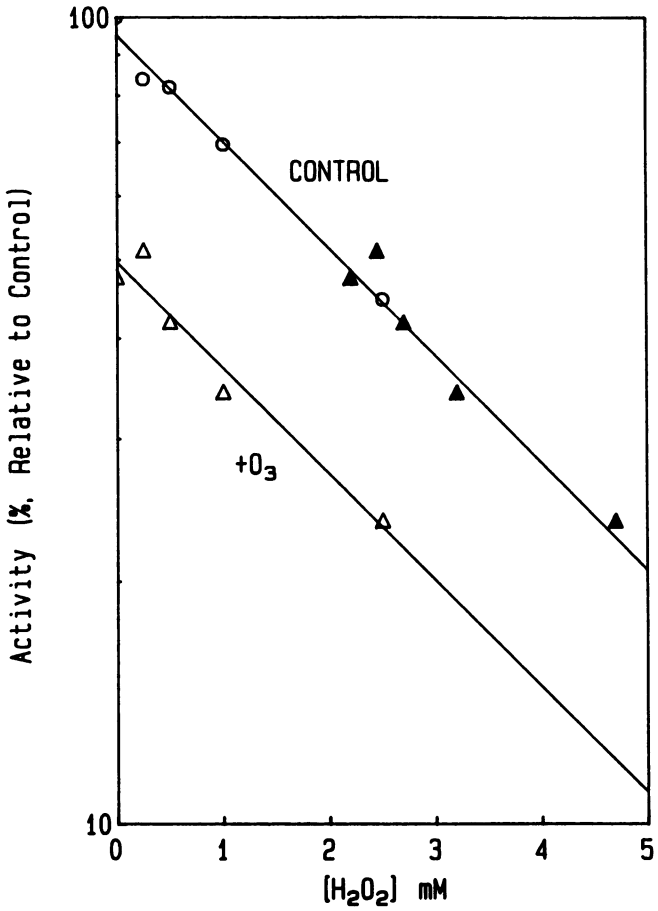

Figure 1. Effect of hydrogen peroxide on the extracellular diamine oxidase activities of pinto bean first trifoliate leaves from control and ozone-treated plants. The assay and preparation of plant material were carried out as described in "Materials and Methods." H_2O_2 was added after extraction of the extracellular fluid. Full diamine oxidase activity (100%) of the control plants in the absence of H_2O_2 was 55 nmol pyrroline min^{-1} g^{-1} fresh weight. Symbols: Open circles, activity of extract from control plants; open triangles, activity of extract from fumigated plants; closed triangles, data from as the open triangles, but the activities were shifted to the control line as if 2.2 mM H_2O_2 were added to the actual concentrations of H_2O_2 within the solution.

but was shifted toward lower activity at all levels of H_2O_2 concentration (closed triangles, Fig. 1).

Sulfur dioxide fumigation alters sulfur biochemistry and increases internal pools of osmotically active material in leaves and so can affect the leaf biochemistry differently than ozone (25). Table IV shows evidence of a differential effect of sulfur dioxide upon the enzyme chemistry within the cell wall. The plants were fumigated with a level (0.4 μL/L for 4 h) of sulfur dioxide which caused no visible injury. Under these conditions, the extracellular peroxidase and diamine oxidase activities did not change significantly, but the level of reduced sulfur in the extracellular fluid from exposed leaves was nearly doubled compared with that of the nonexposed leaves. When visible injury was observed at higher levels of sulfur dioxide (0.5 μL/L × 4 h), the activity of peroxidase increased somewhat (20%), but no change in diamine oxidase was observed (data not shown).

Table IV. *Effect of Sulfur Dioxide Fumigation on the Extracellular Activities of Several Enzymes and Sulfhydryl Groups within the First Trifoliate Leaves of Pinto Bean*

The enzyme activities and level of sulfhydryl were assayed as described in "Materials and Methods," from the extracellular fluid extracted from the wall space of the leaves. The fumigation was carried out as with ozone fumigation with 0.4 μL/L SO_2 given for 4 h as a square pulse. The assays were done 24 h after the fumigation. Values are the average of two different experiments with three replicates in each experiment.

Enzyme	Units*	Activity	
		Control	Fumigated
		units mg^{-1}-protein*	
Diamine oxidase	(nmol/min)	1190 ± 160	1070 ± 60
Peroxidase	(ΔA_{470}/min)	136 ± 18	151 ± 46
Sulfhydryl	(nmol)	860 ± 160	1560 ± 70

DISCUSSION

The observation that peroxidase activity increases under air pollutant fumigation is not new (6, 20). Castillo and his coworkers (4, 5) showed that the activity of extracellular peroxidases, especially the basic form whose activity was supported by ascorbate oxidation, increased markedly and rapidly after fumigation. Most of their work was done with ozone, but sulfur dioxide fumigation likewise caused an increase in general peroxidase activity. In this paper, we report two new observations: (a) extracellular peroxidase activity rises even upon ozone exposure at a level where there is no visible injury and (b) a triangular pulse of ozone stimulates a higher peroxidase activity than does a square wave, even though the total dose is the same.

Musselman *et al.* (19) were the first to report that triangular pulses of ozone cause a greater visible injury to Red Kidney bean. Under the conditions reported here, the triangular pulse caused a greater yield reduction in total wet and dry weight of spinach compared with the square pulse, even though both ozone exposures were given over the same time period (4 h) and at the same total dose (concentration × time). The transpiration rate, which is a measure of the aperture of the stomates, did not change during either fumigation (data not shown). Thus, there may be a mechanism which allows a detoxification of the ozone entering the leaf under these low doses of ozone. At high peak levels, the detoxification mechanism may be overwhelmed and other biochemical alterations occur.

It may be that the stimulation of ascorbate peroxidase *in vivo* is induced by higher levels of internal caffeic acid within ozone-treated plants (13). Mehlhorn and Kunert (16) found that caffeic acid increased the reaction rates of horseradish peroxidase when ascorbic acid was the electron donor. This enzyme from fumigated plants reacts *in vitro* upon the addition of caffeic acid to the same extent as it does from the control plants. Thus, if ozone fumigation induced an increase in the levels of caffeic acid, the activity of the ascorbate-dependent peroxidase within the cell wall would be likewise increased, perhaps to facilitate the scavenging of H_2O_2. In this manner, phenolic compounds, by regulating ascorbate-de-

pendent peroxidase, may limit oxidative events in the wall region external to the membrane.

Possible mechanisms for peroxidase activation have been previously discussed (11) and, in part, involve injury to the membrane which induces a shift in Ca^{2+} levels within the wall and ultimately within the cell. Another possibility involves the production of H_2O_2 within the wall space by ozone (12). The inactivation of diamine oxidase by H_2O_2 (Fig. 1) supports this concept; the activity of diamine oxidase from the exposed plants was the same as that to which 2.2 mM H_2O_2 had been added *in vitro*. One can calculate that the amount of ozone within the wall might be as large as 4×10^{-10} mol cm^{-2} of cell surface, over the exposure of about 4 h or, if all were converted to H_2O_2, the H_2O_2 concentration would be about 2 mM for a 2 μ thick cell wall (12). Thus, the production of H_2O_2 within the wall by ozone breakdown may have inactivated diamine oxidase *in vivo*.

The biochemical mechanism of inactivation of the diamine oxidase is problematical. Recently, Floris *et al.* (10) showed that diamine oxidase from *Euphorbia* has a sulfhydryl which is implicated in the enzymic activity. If the diamine oxidase associated with the wall space of bean is similar, both H_2O_2 and ozone could easily inactivate it by oxidation. It has been previously shown that the K^+-stimulated ATPase, located within the plasma membrane, is inhibited by ozone both *in vivo* and *in vitro* by an oxidation of its critical sulfhydryl (12). Similar events may occur with diamine oxidase.

One consequence of a decrease of the diamine oxidase activity might be an increase in its substrate. Indeed, the ozone-exposed plants show an increase in putrescine and polyamines (3, 24). Perhaps plants regulate their diamine and polyamine levels in order to stabilize the plasma membrane (22) and thus to protect the cells against oxidant stress.

Polyamines can activate the membrane-bound ATPases, partially inhibit the peroxidases located in the membrane-wall region, and slow the release of stress ethylene upon the wounding of the tissue (22). These polyamine-induced processes are in opposition to the processes induced by ozone (14). The amounts of chlorotic and necrotic tissue of tomatoes after exposure to ozone are reduced when polyamines are added to the shoots before fumigation (the transpiration stream carries the polyamines at a concentration of 0.01 mM from the petiole into the leaves, Ref. 25). Putrescine and spermine reduced the damage to the tissue by more than 50%.

The possible interaction of polyamines with membrane stability highlights another metabolic relationship. Both polyamines, spermidine and spermine, and ethylene arise from methionine (26). Tingey and his coworkers (1, 23) have observed such a release of stress ethylene upon ozone fumigation of plants. The connection between ethylene and ozone injury was strengthened recently by Mehlhorn and Wellburn (17), who found that the amount of visible injury could be reduced by spraying with aminoethoxyvinylglycine, an inhibitor of 1-aminocyclopropane-1-carboxylic acid synthase, the enzyme in the pathway of ethylene and polyamine biosynthesis. Blocking ethylene production could have stimulated polyamine production.

In conclusion, peroxidase and diamine oxidase activities within the extracellular fluid provide a sensitive assay of actual biochemical alterations induced by ozone even at low levels of ozone fumigation, where tissue damage is not visible. The increase in peroxidase and decline in diamine oxidase may provide a method for standardization of the initial events of ozone-induced biochemical alterations at the cell wall and membrane sites. Further, polyamines may be ultimately associated with the localization of the injury patterns within the leaf and with stress protective mechanisms.

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