

The Role of Peroxidase in Tolerance to Ozone in Bean (*Phaseolus vulgaris* L.)¹

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ABSTRACT

Seven accessions of bean (*Phaseolus vulgaris* L.) plant introductions, (PI's) 169-735, 171-790, 206-982, 370-569, 414-831, 209-493, and BBL-290 (cultivar Bush blue lake-290) were used to determine whether the peroxidase enzyme in plants is a contributing factor to a mechanism for ozone (O₃) resistance. These seven accessions were chosen based on variations in ozone tolerance in previous studies. Pre-treatment of plants with 0.2 ppm O₃/2 h significantly increased peroxidase activity in leaves and provided protection against a subsequent exposure to 0.4 ppm O₃/2 h. A positive correlation (r = 0.95) between increased peroxidase activity and enhanced O₃ tolerance in tolerant (T) and intermediate tolerant (IT) groups was found. The electrophoretic study showed two new bands of isoperoxidases that were induced only by O₃ exposure, and were predominant for the T, and IT group. These new bands may be involved in the O₃ resistance mechanism.

INTRODUCTION

A definitive role for peroxidase in plants has eluded plant scientists so far. Many researchers have reported the general involvement of peroxidases in lignin synthesis (Cattle and Kolatukuddy 1982; Egley et al., 1983; Harkin and Obst, 1973) and oxidation of endogenous indole acetic acid (Gramdow and Langen-Schwich, 1983) in beans (*Phaseolus vulgaris* L.). The varietal differences in ozone tolerance (Tingey et al., 1976; Beckerson et al., 1979; Butler and Tibitts et al., 1979a,b; Reinert et al., 1984) suggest that there is an inherent mechanism in plants that determines ozone susceptibility (Mebrahtu et al., 1990).

Peroxidase was cited as a screening parameter for different physiological stresses. An elevated peroxidase level is induced by cold, drought, hypoxia, and salt stress (Highkin, 1969; Rakova et al., 1969; Stutle and Todd, 1967; Siegl et al., 1966). Peroxidase activity was also used as a biochemical marker for different types of pollution (Curtis and Howell, 1971; Poddleskis et al., 1984). Ozone (O₃) was reported to have an effect on the level of peroxidase activity of several plant species (Dass and Weaver, 1968; Poddleskis et al., 1984; Tingey et al., 1975; Curtis et al., 1976; Egley et al., 1983; Cattle and Kolatukuddy, 1982). In very few cases, some

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TABLE 1. Selected Bean (*Phaseolus vulgaris* L.) genotypes and their tolerance to ozone.

Accession	O ₃ Tolerant (T)	Intermediate Tolerant (IT)	Sensitive (S)
PI169-735	+		
PI171-790	+		
PI206-962		+	
PI370-569		+	
PI414-831			+
PI209-493			+
BBL-290			+

effect on the isoperoxidase profile was reported (Shanon 1948; Curtis and Howell 1971; Curtis et al., 1976; Endress et al., 1980; Dass and Weaver 1972; Rier et al., 1983, 1987; Sood et al., 1985). Ozone was found to modify the patterns of peroxidase in ozone-sensitive plants more rapidly than in ozone-insensitive plants (Curtis et al., 1976).

The existence of multiple forms of peroxidase in plants was known for a number of years (Shanon, 1948), but the relationship of individual isoenzymes to specific biological functions is not clear. An increase in total peroxidase activity was often found when pathogens infect plants whose response can be classified as resistant (Andreev and Shaw, 1965; Farkas and Stahmann, 1966; Novackey and Hampton, 1968; Uritani and Stahmann, 1961). Such increases were caused by the activity of certain specific isoenzymes, as reflected by staining intensity in polyacrylamide gels (Seevers et al., 1971; Daly et al., 1970).

Toxic oxygen species, produced as by-products in many biological reactions and/or by air pollutants such as O₃, can lead to damage of almost all cell components:- DNA, lipid membrane, and protein (Tepperman and Dunsmuir, 1990; Storz, et al., 1990). Inducible defenses to counter oxidative damage in prokaryotic and eukaryotic cells were reported (Chan and Wiss, 1987; Keyse and Tyrrel, 1989; Christman et al., 1985), but the mechanisms by which the cell receives and responds to oxidative stress have not elucidated.

The objective of the present study was to investigate the possible role of peroxidase in tolerance to O₃ in selected bean accessions. The differences in peroxidase activity and in isoenzyme patterns were determined after ozone exposure and mechanical injury in sensitive and tolerant accessions.

MATERIALS AND METHODS

Plant Materials:

The seven bean accessions used in this study were selected based on a previous screening study for O₃ sensitivity (Reinert et al., 1984). These bean accessions and their degree of sensitivity to O₃ are listed in Table 1. Three seeds from each accession were planted in a 12 cm plastic pot with a 2:1 (v/v) mixture of gravel and buffered redi-earth, and seedlings were thinned to one plant per pot after emergence. Plants were grown in an environmentally controlled room with 28/22°C day/night temperature and illuminated by metal halide lamps providing 350

$\mu\text{Em}^{-2}\text{S}^{-2}$ photosynthetic photon flux density throughout the study, except under fumigation conditions.

Fumigation of plants with O_3 :

Plants were exposed to O_3 under environmentally controlled conditions at 16 and/or 18 days from planting for 2 h at $24 \pm 2^\circ\text{C}$, $65 \pm 3\%$ relative humidity, and a light intensity of $400 \mu\text{Em}^{-2}\text{S}^{-2}$ photosynthetic photon flux density provided by mixed incandescent and fluorescent lighting. Exposure to O_3 was initiated after plants were pre-equilibrated in the chamber for 2 h. Control plants were placed in a similar chamber, except that these plants were exposed to charcoal filtered air for 6 h. Ozone was generated by passing dry oxygen through an ozone generator (Griffin model GTC-A1 generator, Griffin Technics corp., Lodi, NJ). Ozone concentration in the growth chamber at plant height was monitored with a 8410 O_3 analyzer (Monitor Labs, Inc., San Diego, CA). The monitor was calibrated every 2 weeks with a Model 1003-pc ozone calibrator (Dasibi Environ Corp., Glendale, CA). After each fumigation with O_3 , plants were returned to the control environmental room.

In the first experiment, 18-day old plants were exposed to a single acute exposure of 0.6 ppm (v/v) O_3 for 2 h to determine the changes in peroxidase activity under screening conditions described by Reinert et al., (1984). The leaves of whole plants were extracted and assayed for peroxidase activity. In the second experiment, 18-day old plants were exposed to 0.0, 0.1, 0.2, 0.4 and 0.6 ppm (v/v) O_3 for 2 h to study plant responses to different O_3 concentrations. In this experiment, the first trifoliolate was collected, extracted and assayed for peroxidase activity. To study the effects of double exposure on the selected bean accessions, 16-day old plants were divided into four groups. The plants in the first and second group were pre-exposed to 0.1 and 0.2 ppm (v/v) O_3 for 2 h, followed by a second exposure of 0.6 and 0.4 ppm (v/v) O_3 for 2 h respectively, two days later. Plants in the third and fourth group were used as controls and were treated as described above. Trifoliate leaves were collected and peroxidase activity was estimated.

Ozone-treated plants were visually assessed for percentage foliar damage by estimating the percentage of visible injury on a 0% to 100 % scale in increments of 5 % (Reinert et al., 1984).

To distinguish between the effects of O_3 treatments and mechanical injury, plants from each accession were mechanically wounded as described in detail by Ryan (1974). Injured leaves were harvested 24 h following wounding and assayed for peroxidase activity.

Enzyme assay and electrophoresis of the isoenzymes:

The leaves from each plant were excised and deveined, individually weighed (3-5 g), and homogenized in a Waring Model 7011 blender (100 ml steel blender cup at high speed for 30 sec.) using 4 ml of cold 10 mM potassium phosphate buffer pH 8.0 containing 0.8 M KCl per 1 g leaf (Curtis and Howell, 1971). The homogenate was centrifuged for 10 min at $20,000 \times g$. The supernatant was collected and assayed for total peroxidase activity. All steps in the tissue processing were carried out at 4°C . Peroxidase activity was determined according to the modified method of Machly and Chance (1954) using O-dianisidine in a DU-8 spectrophotometer (Beckman Instruments, Inc., Columbia, MD). The change in absorbance at 470 nm was recorded at 20 sec. intervals, and the reaction was linear

for 5 min. Peroxidase activity (PA) was determined by measuring the change in $\Delta OD_{470nm} \text{ min}^{-1} \text{ g}^{-1}$ fresh weight as described by DunLeavy and Urs (1978).

Gel electrophoresis (Davis, 1964) was performed on leaf extracts to determine the changes in isoenzyme profile caused by different treatments. Sodium ascorbate was added to the leaf homogenate to a final concentration of 50 mM and a portion of this solution was dialyzed against tris-glycine buffer (pH 8.3) in a 18 mm spectrapore membrane tubing (MW cut-off 3500 Dalton). Protein was determined in the dialyzed extracts using folin-phenol reagent (Lowery et al., 1951) and no significant differences in protein concentration were found among the three groups and between treatments. Details of gel electrophoresis procedures and staining are given elsewhere (Curtis and Howell, 1971; Habeck and Curtis, 1974). Immediately after the run was completed, the gels were removed and incubated for 15 min in a mixture of 0.1 % O-dianisidine reagent and 0.3 % H_2O_2 in a Na acetate buffer, pH 5.1 (Curtis and Howell, 1971). The gels were transferred to 7 % acetic acid for 1-3 min, then washed with distilled water, and photographed. Because of the large number of gels generated and the unavailability of a gel scanning densitometer, the results were summarized in a computer generated graph (Figure 4).

Experiments had four replications and were repeated twice. The collected data were subjected to analysis of variance as a randomized complete block design and least significant difference ($P = 0.05$) was used to compare means. Correlations between observed variables were examined using linear regression analyses. Statistical analyses were performed using the general linear model (GLM), SAS program (Barr et al., 1976). A probability of < 0.05 was required for significance in all statistical analyses.

RESULTS AND DISCUSSION

The induction of peroxidase activity (PA) by a single acute O_3 -exposure was investigated and the results are presented in Figure 1. The data showed a significant increase in PA in O_3 -treated plants, especially in the sensitive group (S, 370%). This increase was much higher than that of plants in the tolerant (T, 53%) and in the intermediate tolerant (IT, 86%) group. This significant increase in PA in sensitive plants may be due to leaf damage that was higher in the sensitive group throughout the study (Figure 3). Moreover, the data in Figure 1 also indicated that control plants in the T and IT groups had a significantly higher PA than that of the S group.

A significant increase in PA was observed in all mechanically injured plants (Figure 1). However, the T and IT group showed higher increases in the enzymatic activity than the S group. The data in Figure 1 also showed that the increase in PA caused by O_3 -treatment was significantly higher than that of mechanically injured ones. The increase in PA following O_3 -exposure has been reported earlier (Curtis et al., 1976; Tingey, 1975; Endress et al., 1980; Dass and Weaver, 1972; Curtis and Howell, 1971; Runeckles and Rosen, 1977a,b). The increase in PA by mechanical injury is in agreement with other research conducted on sunflower, *Helianthus annuus* L. (Lipez, 1970); tobacco, *Nicotiana tabacum* L. (Birecka et al., 1975); and sweet potato, *Ipomoea batatas* pair (Birecka et al., 1976).

To study the plant response to different O_3 concentrations, plants were exposed to 0.1, 0.2, 0.4 and 0.6 ppm $\text{O}_3/2$ h. Peroxidase activity increased as O_3 level

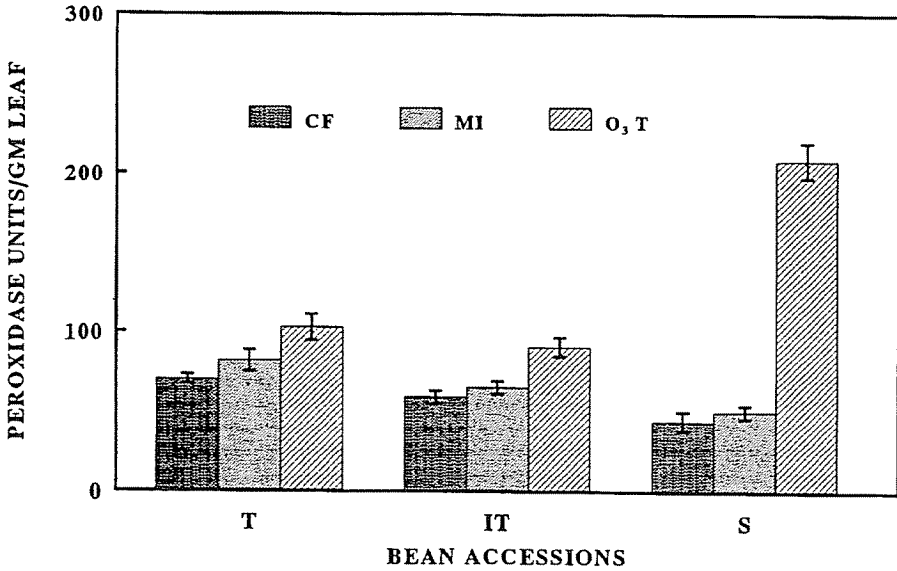


FIGURE 1. Effect of O₃ and mechanical injury on the peroxidase activity in beans (*Phaseolus vulgaris* L.). CF; Charcoal Filter (control), O₃ treated (0.6 ppm O₃/ 2 h), MI; Mechanical Injury. T; Tolerant, IT; Intermediate Tolerant, and S; Sensitive accessions. Means of control and O₃ treatment and means of control and mechanically injured plants are significantly different according to paired analysis at p > 0.05, LSD = 12 and 4.99 for O₃ and mechanical injury, respectively.

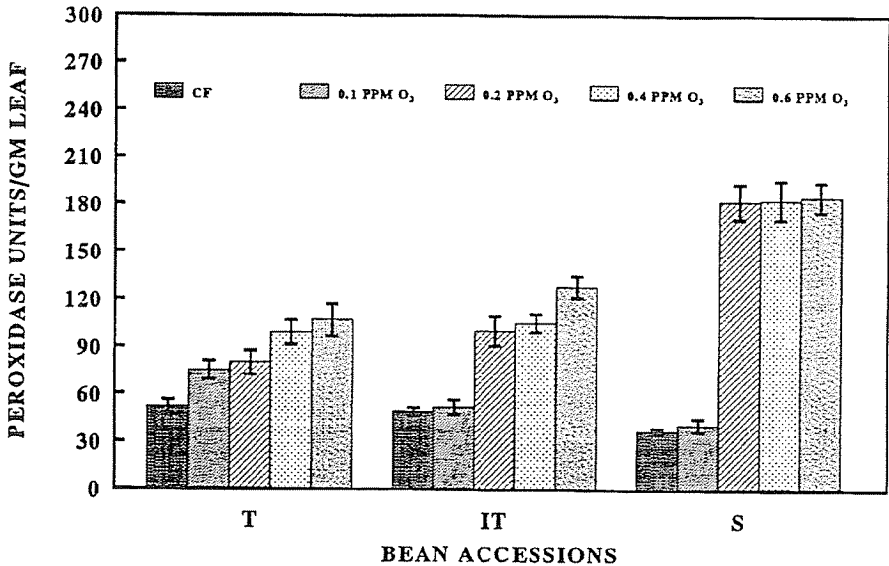


FIGURE 2. Effect of different O₃ concentrations on the peroxidase activity in beans (*Phaseolus vulgaris* L.). CF; Charcoal Filter, 0.1, 0.2, 0.4, and 0.6 ppm O₃/2 h. T; Tolerant, IT; Intermediate Tolerant, and S; Sensitive accessions.

TABLE 2. Induced peroxidase activity (PA) and percentage of injured leaf area (LD) of first trifoliolate of bean exposed to 0.6 ppm O₃ or pre-exposed to 0.1 ppm/2 h at day 16 and followed 0.6 ppm O₃/2 h, two days later.

Tolerance Group ^a	Accession	O ₃ Concentration							
		Control		0.6 ppm		0.1 and 0.6ppm		LSD (0.05)	
		PA	LD	PA	LD	PA	LD	PA	LD
T	169-735	45	0	104	70	125	60	19	18
	171-790	50	0	210	85	220	78	10	5
IT	206-982	45	0	129	90	156	90	25	2
	370-569	48	0	126	85	139	80	9	4
	414-831	53	0	136	65	146	80	9	13
S	209-493	40	0	183	100	191	100	8	5
	BBL-290	34	0	177	98	71	80	5	15

^aT: tolerance to O₃; IT: Intermediate tolerance to O₃; S: sensitive to O₃

increased (Figure 2). At 0.1 ppm O₃/2 h, the T group showed significantly higher increase in PA than the IT and S groups and no leaf injury was observed in all treated plants (Figure 3). Both the T and IT group showed a gradual increase in PA when O₃ concentration was gradually increased from 0.2 to 0.6 ppm/2 h (Figure 2). In contrast, sharp increases in PA were observed in the S group at 0.2 ppm O₃/2 h. This sharp increase was associated with significantly higher leaf injury (72% at 0.2 ppm O₃/2 h) compared to the T (9%) and IT (19%) groups, respectively (Figure 3). In addition, a significant positive correlation ($r=0.95$) was found between leaf injury and the increase in PA at different O₃ concentrations.

Pre-exposing treatment (0.1 ppm O₃/2 h) for the IT and S groups caused a minor increase in PA that was insufficient to provide protection against the subsequent O₃-exposure. As indicated in Table 2, the 0.6 ppm and 0.1 and 0.6 ppm O₃/2 h treatments showed high foliar injury (83 - 99 %) in the S and IT groups. In contrast, a small but, significant reduction in leaf injury (11.5 %, $p < 0.05$) was observed for the T group.

A second exposure experiment was conducted with concentration of 0.2 ppm O₃/2 h 16 days from planting, followed by exposure to 0.4 ppm O₃/2 h two days later. The pre-induction of peroxidase by 0.2 ppm O₃/2 h allowed for significantly higher PA in the T and IT groups (Table 3). As a result, there was a significant decrease in foliar damage following the 0.4 ppm/2 h treatment in the T and IT groups which were pre-exposed to 0.2 ppm O₃. The data from these two groups confirm Runeckles and Rosen's (1977a,b) conclusion that pre-treatment with low doses of O₃ induced resistance to more concentrated acute-O₃-exposure.

The 0.1 ppm O₃ pre-exposure study showed that the pre-induction of PA was not significant and therefore did not help to protect plants in the IT and S groups against O₃ injury in the 0.1 and 0.6 ppm O₃/2 h treatment. In contrast, a significant reduction in leaf injury was found in plants in the T group. During pre-exposure

TABLE 3. Induced peroxidase activity (PA) and percentage of injured leaf area (LD) of first trifoliolate of bean exposed to 0.4 ppm O₃ or pre-exposed to 0.2 ppm/2 hr at day 16 and followed 0.4 ppm O₃/2 hr, two days later.

Tolerance Group ^a	Accession	O ₃ Concentration							
		Control		0.4 ppm		0.2 and 0.4 ppm		LSD(0.05)	
		PA	LD	PA	LD	PA	LD	PA	LD
T	169-735	45	0	60	20	85	13	19	5
	171-790	58	0	138	50	216	38	50	5
IT	206-982	45	0	125	45	229	31	69	6
	370-569	48	0	98	75	136	51	23	12
	414-831	53	0	94	21	198	15	47	4
S	209-493	40	0	108	98	128	90	19	7
	BBL-290	34	0	207	85	249	83	43	3

^aT: tolerance to O₃; IT: Intermediate tolerance to O₃; S: sensitive to O₃

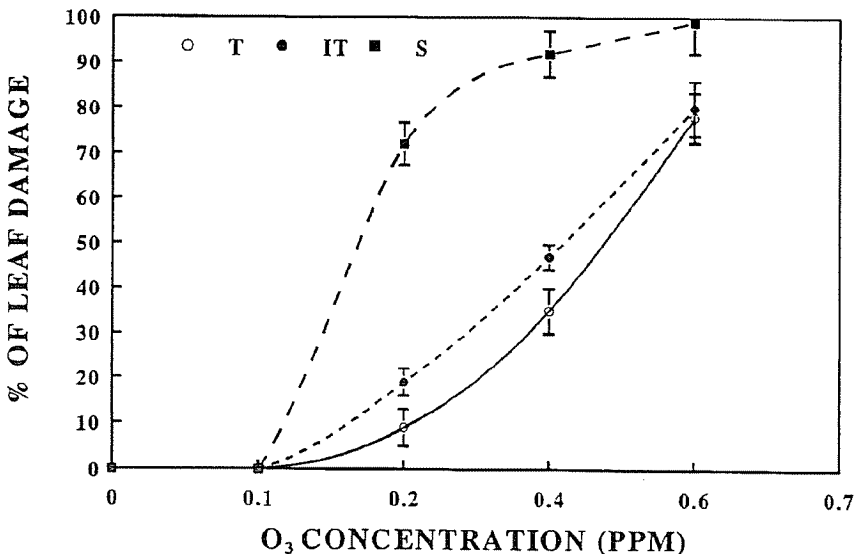


FIGURE 3. Percentage of leaf damage at different O₃ concentration in beans (*Phaseolus vulgaris* L.). T; Tolerant, IT; Intermediate Tolerant, and S; Sensitive accessions.

with 0.2 ppm, our study showed a possible relationship between increased levels of PA and enhanced tolerance to O₃ in the plants of the T and IT groups. The T and IT group showed moderate increases in PA by pre-exposure treatment and an average 28% reduction in foliar injury was observed. The sharp increase in PA and high foliar damage in sensitive plants suggests an alternative explanation of the association between peroxidase and foliar injury by O₃. The extent of injury to the

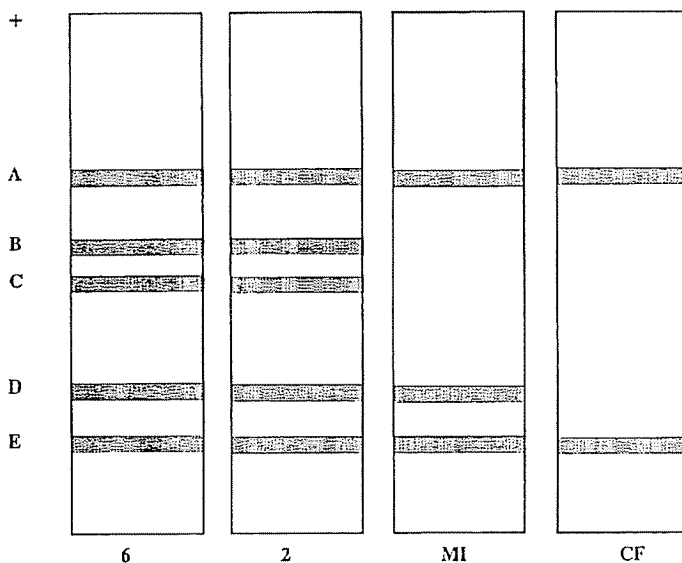


FIGURE 4. Gel electrophoresis representative of *Phaseolus vulgaris* isoperoxidase pattern following O_3 exposure. A, B, C, D, and E represent specific isoperoxidase bands. The four different treatments are designated as follows: CF; Charcoal Filter (control), 2, O_3 -treated (0.2 ppm O_3 / 2 h); 6, O_3 -treated (0.6 ppm O_3 / 2 h); MI; Mechanical Injury and the origin located on the top.

S group (65.5%) from low dose exposure showed a significant protection would be unlikely and leads us to speculate that the increase in PA in these plants may be a part of an injury response to O_3 rather than a protective mechanism (Nadolny and Sequeira 1980; Seevers et al., 1971). Since PA was increased by O_3 pre-treatment in tolerant and intermediate tolerant accessions, it suggests the presence of a factor(s) which control(s) the dose-dependent increase in PA with increasing O_3 concentration (Figure 2), and this factor(s) could be absent in sensitive accessions.

As seen in Figure 1, the increase in PA (19%) as a result of mechanical injury was lower than the increase in PA by O_3 exposure treatment (Figure 1). Thus, it was unlikely for mechanical injury to provide significant protection against O_3 . However, leaves which had been mechanically injured showed O_3 damage only on the proximal end of the leaf. Leaf tissue on the distal side of the mechanical injury site showed little or no damage by O_3 , while leaf tissue of the proximal side of the injury showed typical symptoms of O_3 phytotoxicity. However, results were extremely variable and statistically insignificant.

The effect of O_3 exposure and mechanical injury on the peroxidase isoenzymes are presented in Figure 4. Inspection of the stained gels revealed visual differences in band intensity between O_3 -treated and control plants. All of the bands showed an increase in the stain intensity as results of O_3 -exposure. Plants exposed to 0.6 ppm O_3 /2 h showed the greatest amount of staining. Two new bands (B and C) were identified in the T and IT plants after O_3 exposure, and were similar in stain intensity in those two groups of plants. These two bands were absent in control and mechanically injured plants in the T, IT, and S groups, as well as in S accessions that was exposed to O_3 (Figure 4). Bands A and E were common to all accessions

with increasing stain intensity following both O₃ and mechanical injury treatments. The band D tended to be a variable among accessions and was induced by O₃ and mechanical injury treatments. Similar alterations in isoperoxidase profiles were reported earlier (Farkas and Stahman, 1966; Dass and Weaver, 1972; Endress et al., 1980; Curtis and Howell, 1971; and Curtis et al., 1976). However, no correlation between tolerance to O₃ and specific isozyme in beans was documented. Alterations in isoperoxidase profile were also observed in O₃-exposed callus (Sood et al. 1985, Rier et al. 1983, 1987).

Isoenzyme profiles are generally believed to be genetically controlled. Changes in the isoenzyme patterns due to changed environmental conditions or infections were explained by altered combinations of protein subunits to yield different molecular species or by activation or derepression of latent synthetic potentialities (Schwartz, 1966; Scandalios 1964).

It is suggested that some of the isoperoxidase induced by O₃ exposure may be a part of the mechanism for tolerance and missing in the O₃-sensitive plants. Consequently, the presence of these two bands may be responsible for the protection against O₃ injury rather than the increase in the total peroxidase activity.

The reason why plant peroxidases are more active under pollution is not known. Recently, the Oxy-R-controlled regulon of hydrogen peroxidase-inducible genes in *E. coli* was used as a model to study the cellular response to oxidative stress. When bacterial cells were treated with low doses of hydrogen peroxide, the synthesis of at least 30 proteins was induced, and the cell became resistant to subsequent doses of hydrogen peroxide, that would otherwise be lethal (Storz et al., 1990). Christman et al. (1985) reported that the expression of nine of these proteins induced by hydrogen peroxide treatment was under the control of the Oxy-R gene. Strains carrying deletions of Oxy-R were unable to induce the nine proteins and were hypersensitive to hydrogen peroxide and other oxidants (Storz et al., 1990). Some of the nine proteins that are regulated by the Oxy-R gene were identified and included catalase and alkyl hydroperoxide reductase (Morgan et al., 1986). The presence of similar systems in plants is possible. More research is needed in this area.

Several comparisons may be made between the reaction of peroxidases to air pollutants such as O₃ and to infection. Curtis et al. (1976) found that upon O₃ exposure, the PA of an O₃-tolerant cultivar of soybean was less affected than the activity of an O₃-sensitive cultivar, and this finding is in agreement with our finding. This also may be compared to the differences observed between plants resistant and susceptible to specific infection. Horsman and Wellburn (1977) reported that plants already exposed to a pollutant appear to be less responsive to new exposure as compared to plants coming from non-polluted environments, and this resembles the acquired resistance following an infection. Our investigation established similar correlations in beans for both O₃-tolerant and intermediate tolerant plants, and not for O₃-sensitive ones. Finally, the results showed a positive relationship between higher peroxidase levels, isoenzymes, and enhanced O₃-tolerance. This research is preliminary in nature but shows possible trends that may lead to an understanding of the mechanism of O₃-tolerance in plants. Further research is needed to determine the exact significance of pre-induced PA and its relevance to

O₃-tolerance. The inheritance of specific isoperoxidases that are induced by O₃-treatment (band B and C) is under investigation.

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