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GENETICS AND THE MECHANISM OF OZONE TOLERANCE IN
SELECTED CULTIVARS OF PHASEOLUS VULGARIS L.

By

Asaf Zvi Guri

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ABSTRACT

GENETICS AND THE MECHANISM OF OZONE TOLERANCE IN SELECTED CULTIVARS OF PHASEOLUS VULGARIS L.

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The purposes of this thesis were: a) to establish the genetic background of differences in response to ozone in two tolerant and two sensitive cultivars of Phaseolus vulgaris L. and b) to suggest a physiological mechanism for the observed differences which would be consistent with the results from the genetic analysis.

Four varieties, two ozone-tolerant, Nep-2 and FH, and two ozone-sensitive, PHR and 0669, were selected from among 12 cultivars initially screened, to be used in this study.

The genetic study suggested that at least two major interacting dominant alleles at different loci control the expression of tolerance to ozone in these 4 varieties. In addition to the two major genes, there appeared to be an undetermined number of genes of minor effect involved in the overall genetic control system.

Measurements of stomatal conductivity (prior to and after 4 hours of ozone fumigation), and stomatal density, revealed that unlike some other studies in the past, the

two tolerant varieties exhibited a significantly higher capacity for gaseous conductivity (both abaxial and adaxial) than the two sensitive varieties. Similar results were obtained in regard to stomatal density. These findings imply that under the conditions of this study ozone uptake of the tolerant varieties might have been even higher than that of the sensitive varieties.

Production of ethane, due mainly to ozonation of linolenic acid, was similar in all four varieties, which suggests that the content of the most prevalent unsaturated fatty acid in bean plants (linolenic acid) is identical in the four varieties. Ethylene production, however, was higher in the ozone-injured leaves of the sensitive varieties. Since ethylene production increases after almost any kind of leaf injury, this difference in ethylene production between tolerant and sensitive varieties after ozone exposure cannot imply any particular mechanism. Production of ethylene due to ozone injury, however, can be used in the future for standardization of visual injury determinations.

The measurements of the two antioxidant substances; glutathione (GSH) and ascorbic acid (AA), which might be involved in repair of ozone injury, showed that leaves of the tolerant variety Nep-2 had a higher GSH concentration (on the basis of fresh weight) than the other three varieties immediately following ozone fumigation. No such difference was detected in AA concentration. Further research has revealed that the specific activity of the enzyme GSSG reductase, which catalyzed the conversion of oxidized

glutathione (GSSG) to its reduced form (GSH), was significantly higher in the two tolerant varieties, both before and after ozone fumigation. The inconsistency of high enzymatic activity and low GSH concentrations after fumigation in the tolerant variety FH is not understood and can be interpreted in different ways.

The fact that GSSG reductase in plants (similar to GSSG reductase in higher animals and yeast) is composed of two polypeptide subunits, could be related to the two major genes deduced from the genetic study.

In memory of my grandparents, Nehama and Yehoshua
Heshel Gurwitz for their caring and support. Their
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INTRODUCTION

For two decades air pollution in urban and many agricultural areas has remained one of the most serious man-made problems affecting crop production. Air pollutants may reduce growth and yield in many crops, depending on the pollutants, their concentration, time of exposure, sensitivity of the plant, and environmental factors. Pollutants may also mark and discolor foliage, reducing visual appeal and thereby the marketability of both ornamental and edible products. In the U.S. alone, it is estimated that annual crop losses due to pollution amount to well over one billion dollars. Damage to natural and horticultural vegetation cannot be estimated, but certainly is extensive (Ting and Heath, 1975).

Two serious types of air pollution exist in the world today. The first, commonly known as London smog, is composed of reducing components resulting largely from the combustion of fossil fuels of high sulfur content. The second type is comprised of oxidizing components in the air, primarily ozone (O_3), fluorine (F), nitrogen oxide (NO_x), and peroxyacetyl nitrate (PAN). Most photochemical smogs arise from the incomplete combustion of fuels by the internal combustion engine. High temperatures and insufficient combustion yield hydrocarbon fragments, which act as catalysts in oxidation

reactions. Also formed are oxides of nitrogen, largely NO_2 . The NO_2 is photochemically cleaved to NO and nascent oxygen. The resultant oxygen radical quickly reacts with molecular oxygen to form ozone. The ozone molecules are very reactive with a standard redox potential of approximately +2.1 V (Throp, 1954). The ozone active molecule is thought to be an ionic form (one resonance form is $\bar{\text{O}}-\overset{+}{\text{O}}=\text{O}$), in acidic media, in which ozone is more soluble in water than oxygen. In alkaline solutions, ozone rapidly decomposes, releasing molecular oxygen (Alder and Hill, 1950).

The use of the term ozone injury is common and it includes several symptoms, like "water-logging", "bronzing", "flecking", and "necrosis". These symptoms refer to a generalized dullness of the leaf surface, although each may represent slightly different processes occurring within the leaf. Under most conditions, exposure to ozone is likely to be expressed as a combination of the above symptoms. Visible injury is associated with a decrease in total leaf photosynthetic activity, and hence reduced leaf productivity (Todd, 1958), and ultimately cell death. In addition, the following cryptic symptoms occur within the leaf after exposure to ozone: 1) a reduction of the plant's photosynthetic activity, 2) a build-up of the pollutant's by-products within the leaf, 3) overall unhealthy appearance without necrotic lesions, 4) reduced growth or yield over a considerable length of time, and 5) increased susceptibility to disease, parasite or insect invasion (McCune et al., 1967). It is quite obvious that visible injury is the result of a series of events beginning

at the primary site of damage within the leaf and leading to the final collapse of whole cellular regions.

It is generally accepted that ozone, like other pollutant gases, enters the plant through the stomates. Stomatal closure results in little or no injury. Ozone then reacts with water in the substomatal cavity and can be modified to other active species like hydrogen peroxide and hydroxyl ions, both of which can be detrimental to leaf cells (Heath, 1980). Next, ozone may react with the charged groups, such as cellulose, amino acids, galacturonic acid residues, lignic acid and bound Ca^{2+} , in the cell wall or with the few enzymes present (Somers, 1973). Reactions of ozone at the plasmalemma are likely to be with the: (a) unsaturated fatty acid residues, (b) aromatic residues and primary amines, and (c) exposed sulfhydryl groups (Ting and Heath, 1975). Reaction at the membrane may alter binding sites of various plant pathogens and alter membrane permeability. Changes in permeability would, in turn, affect the movement of ions through membranes, affecting the net osmotic potential across the membrane. Chloroplasts and mitochondria may be particularly sensitive to osmotic changes (Heath and Frederick, 1979). Consequently, regulatory mechanisms of normal cellular metabolism begin to fail and eventually this leads to tissue collapse and death.

The objectives of this thesis are:

- 1) To continue attempts to explain the heritable basis of ozone tolerance in selected cultivars of Phaseolus vulgaris.
- 2) To try to identify a physiological mechanism

which might be compatible with data obtained in the genetic study:

- a) To determine whether ozone-tolerant plants absorb less ozone into their leaves (due to lower stomatal conductivities) than ozone-sensitive varieties.
- b) To determine whether leaf tissues of tolerant varieties have higher concentrations than sensitive varieties of antioxidant compounds, such as ascorbic acid and glutathione.
- c) To determine whether ethane and ethylene production in the leaves may be related to ozone injury.

LITERATURE REVIEW

The role of stomata in ozone injury has been recognized since the beginning of air pollution study with plants, due to the fact that ozone must enter the leaf before it can elicit the physiological responses of cells and bring about the characteristic necrotic spotting. Treatments which cause the stomata to close or partially close, such as withholding irrigation, reduce damage from weather fleck (Dean, 1972). Application of the phytohormone abscisic acid (ABA) which closes stomata also reduces ozone injury in the treated plants (Flecher et al., 1972). The stomata of resistant onion varieties were reported to close upon exposure to low concentrations of ozone (Engel and Gabelman, 1966). Ozone at low concentrations caused a loss of differential permeability in the guard cells, resulting in stomatal closure. The guard cells recovered soon after the ozone level was lowered. In addition, Dean (1972) in tobacco, and Butler and Tibbitts (1979) in beans, showed that ozone-resistant cultivars have lower stomatal densities than ozone-sensitive cultivars.

Evans and Ting (1973) and Perchorowicz and Ting (1974), reported that ozone had a considerable effect on cell permeability. Following ozone fumigation of bean leaves, membrane

permeability to titrated water decreased, but increased for internal solutes and labeled rubidium and glucose. The authors believed that cellular membranes might be a primary target for ozone.

Tomlinson and Rich (1968) believe that sulfhydryl compounds are critically involved in ozone injury, based on evidence that an ozone-resistant tobacco variety had fewer sulfhydryl groups than an ozone-susceptible variety. They found a slight drop in total sulfhydryl content after ozone exposure of beans and spinach plants. Furthermore, tobacco varieties treated with sulfhydryl-binding agents, such as iodoacetate and iodoacetamide, developed symptoms similar to those produced by ozone. Since sulfhydryl groups are essential for fatty acid synthesis, it was suggested that ozone affected membrane permeability by inhibiting fatty acid synthesis (Tomlinson and Rich, 1969).

Many workers believe that the critical sites for ozone injury are the unsaturated fatty acid residues of the membrane lipids and that damage occurs by a process similar to lipid peroxidation. Lipid peroxides are formed by a cyclic reaction involving:

- a) extraction of a hydrogen atom from a methylene carbon between the double bonds,
- b) attack of the free radical by molecular oxygen, and
- c) a further extraction of hydrogen from another fatty acid, in a cyclic reaction (Lundberg, 1962).

The first reaction of ozone with unsaturated fatty acids is believed to involve the production of an ozonide (ozone addition across the double bond) with one possible breakdown product being melanodialdehyde upon multiple ozonide formation (Ting and Heath, 1975).

The loss of fatty acid material from ozonated tissue would be evidence of ozone attack of fatty acid residues. Actually, such losses are reported to be very low. Swanson et al. (1973) showed that while relative concentrations of C16:2 (fatty acid with 16 carbon atoms and 2 double bonds) and C16:3 declined slightly (5-10%), the concentration of C16:0, C16:1, C18:0, and C18:1 increased in leaves of ozone-treated plants as compared with control. Tomlinson and Rich (1969) reported a decline in all fatty acids in ozonated tobacco leaves, with the largest decline in C16:0 and C18:3.

There appears to be some relationship between levels of some nitrogenous compounds and ozone injury. MacDowell (1965) reported that tobacco leaves were most sensitive to ozone just after full leaf expansion and that sensitivity was associated with a decline in total protein. Ting and Mukerji (1971) suggested that free amino acids play a role in ozone sensitivity, since their concentration declines at about the same time as maximum ozone sensitivity. In addition, many amino acids increased after ozone exposure while protein content decreased. Therefore, ozone may affect protein metabolism either by enhancing protein hydrolysis, resulting in an increase of free amino acids, or by interfering with protein synthesis without affecting amino acid accumulation

(Ting and Heath, 1975). The decline in protein synthesis could occur if the endoplasmic reticulum was disrupted, or if the internal ionic medium (K^+ or Mg^{++}) was altered (Pestka, 1971).

Dugger et al. (1962) first noted that concentrations of reducing and soluble sugars in beans were lowest in leaves of greatest ozone sensitivity. External application of a simple hexose solution reduced ozone sensitivity of leaves, although it was not clear how much entered the leaf. In addition, the ozone sensitive developmental stage in cotton leaves was correlated with a depletion of soluble sugars (Ting and Mukerji, 1971).

Both Tomlinson and Rich (1968) and Pell and Brennan (1973) have observed a small decline in ATP levels in bean plants exposed to ozone. This decline was observed within one hour of exposure and was interpreted as an initial response. In addition, Mudd et al. (1974) have shown that the nicotine-amide ring of NADH is cleaved when ozone is bubbled through an aqueous system containing this compound. Since the ratio of NADH : NAD^+ regulates cellular metabolism, it is likely that metabolism would be affected by the reduced nucleotide loss. Cracker and Starbuck (1972) found, in beans, that ozone fumigation decreased RNA content in primary leaves due to a corresponding increase in the level of RNAase. On the other hand, Tingey et al. (1975) could find no increase in the activity of RNAase in soybean plants after ozone fumigation.

Ozone has a deleterious affect on photosynthesis. Nobel

(1974) reported that ozone fumigation can reduce the chlorophyll content of pea chloroplasts. Nobel and Wang (1973) found that ozone fumigation could inhibit photophosphorylation in pea chloroplasts. This was apparently related to an increase in chloroplast membrane permeability. In contrast, Coulson and Heath (1974) found that ozone fumigation inhibited the electron transport of photosystem I and photosystem II without uncoupling photophosphorylation. The authors postulated that ozone, unlike detergents, disrupted the normal pathway of energy flow from light-excited chlorophyll into the electron transfer compounds by "loosening" but not completely disrupting the membrane.

The importance of the age at which tissue is exposed to ozone has become evident. Early studies showed that older, more mature leaves were injured more readily than young leaves (Dugger et al., 1962). Bobrov (1955) observed in oats that neither young nor mature leaves were ozone susceptible, and that only leaves which had just completed expansion were injured.

Engel and Gabelman (1966) observed that an ozone sensitive inbred onion line maintained open stomata after ozone exposure, whereas stomata of the resistant inbred line closed. They hypothesized that the guard cells of the resistant plants became leaky after ozone exposure, resulting in subsequent closure; later, stomata functioned normally. Genetic crosses in this case suggested that resistance was controlled by a dominant genetic system, but it was not ascertained that resistance was due to a single gene difference. Butler et

al. (1979) in beans, Huang et al. (1975), and Povilaitis (1967) in tobacco, reported that ozone resistance is recessive and cytoplasmic factors are not involved. Other studies by Hanson et al. (1976) in petunia, Cameron (1975) in sweet corn, and Saettler (1975) in beans showed that resistance to ozone is partial or completely dominant involving one or a few genes. Hucl and Beversdorf (1979) suggested that genetic control of ozone tolerance in beans is rather complex and could involve many genes.

The factors of quality, quantity, and duration of light appear to be very important in governing plant sensitivity to ozone. First, light affects stomatal opening which regulates the amount of ozone uptake by leaves. Numerous studies show that high light intensity treatment tends to protect plants against ozone injury; e.g., bean plants on 8 hour photoperiods are less sensitive to ozone at 30,000 ft.c. than at 20,000 ft.c. (Heck and Dunning, 1967). Similar results were reported by Ting and Heath (1975) and by Ting and Dugger (1971), in tobacco. To account for this, Dugger et al. (1962) claimed that high light intensity results in high soluble sugar levels which then protect against ozone injury. There is some circumstantial evidence that plant sensitivity to ozone is influenced by certain wave lengths (Heck, 1968). Temperature controls stomatal aperture, which then indirectly influences sensitivity to ozone (Ting and Heath, 1975).

Plants with adequate nitrogen levels are generally more sensitive to ozone and other oxidants than are those with deficient or excess nitrogen (MacDowell, 1965) and (Leone et

al., 1966). Brewer (1960) reported that increased phosphorous decreased spinach and mango leaf weight and reduced oxidant injury. Potassium had no effect on leaf weight but did increase injury when available phosphorous was low. When nitrogen was high, potassium reduced injury, suggesting a significant interaction among the macro-elements. Mass et al. (1973) and Hoffman et al. (1973) reported that salinity reduced both growth and ozone injury to pinto beans. The authors concluded that greater ozone tolerance was related to lower uptake of ozone.

There are a few reports concerning the effects of oxidants on plants infected with pathogens. Resh and Runeckles (1973) found that bean leaves infected and noninfected with Uromyces phaseoli did not respond differentially to low ozone levels. On the other hand, wheat leaves infected with Puccinia helcaniki were less injured by ozone than healthy leaves (Heagle and Key, 1973). The protection afforded by pathogens seems to be specific, viz. mesophyll cells directly below stomata with visible appressoria and cells adjacent to inoculated areas were protected. For these reasons, the authors suggested that protection of cells was due to a diffusible material from the fungus. Brennan and Leone (1969), observed reduced ozone injury in tobacco leaves infected with mosaic virus and suggested that virus infection may alter susceptibility to ozone by hastening maturity.

As mentioned previously, ABA application leads to stomatal closure, which then leads to reduced ozone uptake and decreased leaf injury. Certain other chemicals which

reduce stomatal aperture including phosphon D, 8-hydroxy-quinolin sulfate, and phenylmercuric acetate tend to protect against injury from ozone as well as other pollutants (Seidman et al., 1965). Unfortunately, plant productivity is reduced by these chemicals due to lack of CO₂ absorption. Attempts to offset the adverse effect of ozone in beans has been successful by using antioxidants such as ascorbic acid, and nickel-N-dibutyl dithiocarbamate (NBC) or prevention of SH bond oxidation by treatment with glutathione (Dass and Weaver, 1968). In tobacco, herbicides like isopropalin and pebulate reduced ozone sensitivity (Sung and Moore, 1979), and in turfgrasses the systemic fungicides benomyl were effective in reducing ozone injury (Papple and Ormrod, 1977). Tomlinson and Rich (1973) found that free sterol content of bean leaves decreased after ozone fumigation. However, treatment with cytokinins such as kinetin, N-6 benzyladenine, and benzimidazole resulted in less ozone injury, accompanied by higher levels of free sterol in the leaf. Howell (1974), suggested that increased phenol synthesis by ozone inhibits ATP synthesis, oxidative phosphorylation and SH-dependent enzyme activity.

MATERIALS AND METHODS

Genetic Study

Twelve bean varieties were exposed to ozone 12-14 days after planting. The ozone concentration was maintained at 0.28-0.32 ppm for 10 hours. Of the 12 varieties, the following four varieties, French Horticulture (F.H.), a cranberry type; Pink Half Runner (P.H.R.), an old dry bean variety; Nep-2 and MSU #0669, both navy bean varieties, expressed the highest stability for the studied trait, and were selected for further studies. To eliminate genetic variability within cultivars, seeds from only one mother plant per variety were utilized. Twelve crosses (including reciprocals) were made among the ozone-sensitive P.H.R. and 0669 and ozone-tolerant F.H. and Nep-2. The majority of the F_1 seeds were planted to obtain the F_2 generations. The parents, F_1 and F_2 generations were fumigated with ozone inside a plexiglas exposure chamber 12-14 days after planting (primary leaf stage) at 0.28-0.34 ppm for 10 hours. The exposure chamber was large enough to contain 36 plants. Ozone was regenerated by passing ambient air over a u.v. tube and was carried to the chamber by a flow of ambient air. The moisture content inside the chamber was adequate to maintain normal growth and photosynthesis. Plants were watered prior to fumigation and were planted in

plastic pots to decrease ozone absorption by the pots.

Each group of plants included F_1 and F_2 plants from the same cross and their two parents. Plants were arranged in a completely randomized design, since no detectable gradient in ozone injury had been found in a preliminary test. Amount of leaf injury on each plant was visually evaluated on a scale of 0 to 4, 2 days post-fumigation, where 0 represents no damage, 1 - minor damage, 2 - moderate damage, 3 - severe damage, and 4 - very severe damage.

In addition, F_1 and F_2 generations and the four varieties were planted in the field in two consecutive summers (1979 and 1980). A space of 15 cm was maintained between plants and 30 cm between rows, in order to prevent interaction between neighboring plants. Plants after anthesis were visually evaluated for presence or absence of ozone injury.

The F_2 segregants from the crosses FH x PHR, FH x 0669, PHR x 0669, and Nep-2 x PHR (30 plants per cross) were separately exposed to ozone in the chamber at 12-14 days, and injury recorded 2 days later. They were then allowed to yield F_3 seeds. Ten seeds from each plant were planted in the field in 1980, and the amount of injury was recorded after anthesis, as mentioned previously. In all field experiments, the groups of plants (group = variable number of plants from the same variety, cross or generation) were arranged in completely randomized designs.

Stomatal Densities and Conductivities

Stomatal densities were determined by the leaf impres-

sion method. A cellulose acetate film and acetone were used to obtain an accurate impression of the primary leaf surface. Primary impressions were made on 8 plants each of the four varieties at 13 days after seeding. One adaxial and two abaxial leaf surface impressions were taken on both primary leaves. The impressions were made at about the same position between major veins on all leaves. Stomata number per mm^2 was determined from the cellulose acetate replicates using a Ziess light microscope (x41).

Thirteen-day old plants of each variety (seven plants per variety) were placed inside the chamber 2 hours prior to the exposure to ozone. Plants were watered and exposed to ozone (0.28-0.34 ppm) for 8 hours. Stomatal resistance was measured with an autoporometer (Li-65 automatic diffusive resistance meter) immediately before the fumigation and at 4 and 6 hours after the beginning of fumigation. Four measurements were taken per plant; two in each primary leaf, one on the abaxial and one on the adaxial surface. The experiment was replicated three times and the stomatal resistance figures obtained were converted to their reciprocals, viz. stomatal conductivities.

Ascorbic Acid (AA) and Glutathione (GSH) Assay

Ascorbic acid assay was conducted by using the 2,6-dichloro-phenollindophenol (DCIP) photometric method described by Hanson et al. (1971). One g. fresh weight of primary leaves of uniform size were quickly detached and ground with sorvall omni mixture in 10 ml of an ice cold 0.0005 M di-sodium EDTA

solution containing 3% TCA (trichloro-acetic acid) for 1-2 minutes. The homogenate was quickly filtered through Whatman #4 paper and brought up to 20 ml with EDTA-TCA extracting solution. One ml distilled water, 2 ml DCIP reagent and 20 ml filtered leaf extract were added to each test tube and their optical densities at 600 nm were determined from a standard curve which was prepared previously, using various determined concentrations of AA. In the GSH assay, 2 g. of primary leaves were chopped and homogenized with 10 ml EDTA-TCA in an ice bath, the homogenate was quickly filtered through Whatman #4 paper and brought to 15 ml with EDTA-TCA.

Each homogenate (for each treatment) was titrated with 1.5 ml 0.1N NaOH to pH range of 6.0-8.0. Each test tube contained 0.5 ml distilled water, 2.0 ml leaf homogenate, 0.5 ml 0.2M potassium phosphate buffer pH 7.0, and 0.1 ml the reagent Dithiobis-2-nitrobenzoic acid (DTNB). The optical densities for each set of test tubes was determined at 412 nm from a standard curve of known concentrations of GSH. A test tube (one per each set) containing all the above ingredients and concentrations but without the reagent DTNB was used as blank. This was done because of the high absorption in 412 nm by the leaf homogenate prior to the reagent application. Similar assays were made for each one of the 4 varieties simultaneously at pre- and post-10 hours of ozone fumigation.

Glutathione Reductase Activity

Prior to and immediately after ozone fumigation, 2 g. fresh weight leaves were chopped, ground and homogenized

with 25 ml 0.1M of Tris HCl buffer, pH 7.5, and 2 g. Poly Vinyl Pyrrolidone (PVP) in an ice bath. The homogenate was centrifuged at 12,500 RPM (20,000 g.) for 10 minutes and the supernatant collected and recentrifuged at 17,500 RPM (37,000 g.) for 10 minutes. Supernatants were collected and used as a crude leaf enzyme extract. To determine enzymatic activities, 0.7 ml distilled water, 0.2 ml 0.1M Tris HCl buffer pH 7.5 and 0.15 ml of the supernatant were added to one cuvette used as blank, while the same ingredients plus 0.01 ml 0.1M EDTA and 0.03 ml 0.005M NADPH were added to a second cuvette. The reading of the second cuvette at 340 nm (peak for NADPH absorbance) was registered on a chart recorder for 5 minutes. Then 0.005 ml of 0.1M oxidized glutathione (GSSG) was added as an electron acceptor as in the following equation: $\text{GSSG} + \text{NADPH} + \text{H}^+ \text{ -----} \rightarrow 2 \text{ GSH} + \text{NADP}^+$. This reaction is catalyzed by glutathione reductase and its velocity can be determined from the slope of the line (absorbance units/min) which represents the drop in absorbance at 340 nm due to conversion of NADPH to NADP^+ . The slope of the line before GSSG addition was subtracted from the slope after GSSG addition. The reaction rate was then calculated as follows.

The molar extinction of NADPH is 6.22×10^3 (mol/liter at 340 nm). Therefore, 6.22×10^3 absorbance units per minute are decreased due to the oxidation of 1 mol of NADPH by GSSG. In this case, 0.00622 absorbance units per minute were used as the extinction coefficient. This corresponds to 1 nM of NADPH oxidized to NADP^+ by GSSG. In addition, the amount of soluble proteins in each sample was determined

using 1 mg/ml Bovin serum albumin (BSA) as standard and Coomassie Blue (Bradford assay) as a reagent. Soluble protein content was determined spectrophotometrically at 595 nm from daily prepared standard curve of BSA. All the elements were inserted in the following formula:

$$\frac{A \times B}{C \times D \times E} = \text{GSH reductase specific activity rate where}$$

A* = the slope of the line represents the decrease
in 340 nm absorbance

B = the volume of all the ingredients in the 2
cuvettes = 1.095 ml

C = the volume of the plant extract in the 2
cuvettes = 0.15 ml

D* = the concentration of soluble proteins in the
plant extract

E = NADPH molar extinction coefficient = 0.00622

The GSH reductase catalyzation rate units are: nmMNADPH/min/
mg protein.

*The values are variable and depends on the cultivar and the timing (before or after fumigation with ozone) when extractions were prepared.

Application of Exogenous AA and GSH

Primary leaves of 60 plants, 30 each of PHR and 0669, were sprayed 6 and 12 hours before ozone exposure (0.28-0.34 ppm) with distilled water (control), 0.005M AA or 0.005M GSH. Ozone damage was determined visually (by classifying to 0-4 groups as mentioned above) one day after exposure.

Formation of Ethane and Ethylene

Fourteen-day old plants of each variety (24 per variety) were fumigated with ozone as described previously and when injury symptoms appeared on the leaves, a disc of approximately 1.5 cm diameter was removed from the center of each primary leaf. Discs were placed inside 25 ml flasks with 0.5 ml distilled water and sealed with rubber caps. All flasks including empty control flasks were incubated 24 hours above a source of high intensity light ($1-1.5 \text{ mv/cm}^2$). Subsequently, amounts of ethane and ethylene in each flask were measured with gas chromatography techniques whereby samples of flask gases were inserted by syringe into a 0.318 cm by 100 cm stainless steel column packed with Porapak R and held at 80°C in a Varian model 2400 gas chromatograph. Gas flows were: N_2 , 35 ml min^{-1} ; air, 300 ml min^{-1} ; H_2 , 30 ml min^{-1} . Ethylene and ethane could be detected down to 5 ml liter^{-1} , ethylene appearing as the first peak. Identities of ethylene and ethane were based on order of appearance and retention time.

RESULTS

Genetic Analysis

Results from the ozone exposure chamber indicate that the varieties FH (tolerant) and 0669 (sensitive) in this experiment showed more stability in the expression of the trait than the other two varieties, Nep-2 (tolerant) and PHR (sensitive) (Tables A1-A6). The data of the F_1 plants implies that cytoplasmic factors are not involved in the genetic control of tolerance or sensitivity to ozone "attack", since no differences have been found between F_1 progenies from reciprocal crosses (Tables A1-A6). The fact that F_1 progenies from crosses between tolerant and sensitive varieties were, in the majority of the cases, ozone tolerant (Tables A1-A4) implies that most of the alleles which condition the expression of tolerance to ozone are partially or completely dominant. The fact that most of the F_1 progenies from the cross between the two sensitive varieties (Table A6) were unexpectedly ozone tolerant, indicates that alleles of different genes interact with each other in a complementary way.

It is possible that the trait is controlled by several genes since F_2 segregants have shown a wide assortment of reaction classes in all the crosses, excluding the cross between the two tolerant varieties (Table A5) in which a

Table A1. Pattern of segregation for ozone injury score in the cross (PHR x Nep-2), as observed in results obtained from the ozone chamber.

<u>Varieties and Crosses</u>	<u>Number of Plants</u>	<u>Level of Ozone Injury</u>				
		<u>0</u>	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>
Nep-2	56	27	19	10		
PHR	69	3	6	25	28	7
a (PHR x Nep-2) F ₁	17	11	4	2		
b (PHR x PHR) F ₁	15	12	1	2		
(PHR x Nep-2) F ₂	233	133	50	21	27	2
Observed		183		50		
Expected		174.75		58.25		

$$\chi^2_{1df} =$$

$$\frac{[183 - 174.75 (-0.5)]^2}{174.75} + \frac{[50 - 58.25 (-0.5)]^2}{58.25} = 1.96 \text{ N.S.}$$

$$\alpha = 0.05$$

Table A2. Pattern of segregation for ozone injury score in the cross (FH x PHR), as observed in results obtained from the ozone chamber.

<u>Parents and Crosses</u>	<u>Number of Plants</u>	<u>Level of Ozone Injury</u>				
		<u>0</u>	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>
PHR (sensitive)	53	1	4	19	12	17
FH (tolerant)	38	24	9	5		
(PHR x FH) F ₁	16	9	6	1		
(FH x PHR) F ₁	15	9	4	1		
(PHR x FH) F ₂	201	136	21	15	20	9
Observed		157		44		
Expected		150.75		50.25		

$$\chi^2_{1df} =$$

$$\frac{[157 - 150.75 (-0.5)]^2}{150.75} + \frac{[44 - 50.25 (-0.5)]^2}{50.25} = 0.877 \text{ N.S.}$$

$$\alpha = 0.05$$

Table A3. Pattern of segregation for ozone injury score in the cross (FH x 0669), as observed in results obtained from the ozone chamber.

<u>Parents and Crosses</u>	<u>Number of Plants</u>	<u>Level of Ozone Injury</u>				
		<u>0</u>	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>
0669 (sensitive)	40		2	7	12	19
FH (tolerant)	43	34	9			
(0669 x FH) F ₁	18	8	8	2		
(FH x 0669) F ₁	16	10	5	1		
(0669 x FH) F ₂	224	105	48	30	28	13
Observed		153		71		
Expected		168		56		

$$\chi^2_{1df} =$$

$$\frac{[153 - 168 (-0.5)]^2}{168} + \frac{[71 - 56 (-0.5)]^2}{56} = 5.004$$

$$\alpha = 0.05$$

Table A4. Pattern of segregation for ozone injury score in the cross (Nep-2 x 0669), as observed in results obtained from the ozone chamber.

<u>Parents and Crosses</u>	<u>Number of Plants</u>	<u>Level of Ozone Injury</u>				
		<u>0</u>	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>
Nep-2 (tolerant)	48	19	16	5		
0669 (sensitive)	43	1	2	13	18	9
(Nep-2 x 0669) F ₁	21	12	9	2		
(0669 x Nep-2) F ₁	17	9	5	3		
(Nep-2 x 0669) F ₂	181	100	31	27	16	7
Observed		131		50		
Expected		135.75		45.25		

$$\chi^2_{1df} =$$

$$\frac{[131 - 135.75 (-0.5)]^2}{135.75} + \frac{[50 - 45.25 (-0.5)]^2}{45.25} = 0.532 \text{ N.S.}$$

$$\alpha = 0.05$$

Table A5. Pattern of segregation for ozone injury score in the cross (FH x Nep-2), as observed in results obtained from the ozone chamber.

<u>Parents and Crosses</u>	<u>Number of Plants</u>	<u>Level of Ozone Injury</u>				
		<u>0</u>	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>
Nep-2 (tolerant)	28	13	9	4	2	
FH (tolerant)	35	19	13	2	1	
(Nep-2 x FH) F ₁	16	11	5			
(FH x Nep-2) F ₁	16	13	3			
(Nep-2 x FH) F ₂	61	33	16	9	3	

Table A6. Pattern of segregation for ozone injury score in the cross (PHR x 0669), as observed in results obtained from the ozone chamber.

<u>Parents and Crosses</u>	<u>Number of Plants</u>	<u>Level of Ozone Injury</u>				
		<u>0</u>	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>
PHR (sensitive)	55	2	6	15	20	12
0669 (sensitive)	54			13	15	25
(PHR x 0669) F ₁	22	15	3	4		
(0669 x PHR) F ₁	9	4	5			
(PHR x 0669) F ₂	197	41	75	53	13	15
Observed		116		81		
Expected		110.8		86.187		

$$\chi^2_{1df} =$$

$$\frac{[116 - 110.8 (-0.5)]^2}{110.8} + \frac{[81 - 86.187 (-0.5)]^2}{86.187} = 0.452 \text{ N.S.}$$

$$\alpha = 0.05$$

narrow range of genetic variation probably exists.

The F_3 families analysis (Tables A8-A11) indicated that in the majority of the cases (approximately 85%), visual classification in the F_2 was essentially correct. In addition, it was judged acceptable for genetic analysis that the line between tolerance and sensitivity lies between classes 1 and 2 (Table A12), inasmuch as the ratio of tolerants to sensitives, comparing F_3 plants with F_2 parents, changes sharply at that point. Therefore, in order to facilitate genetic evaluation, plants with a degree of injury of 0 to 1 were considered as ozone tolerant while the remainder (classes 2 to 4) were considered ozone sensitive. As a result of this rearrangement, segregation in F_2 from 3 crosses between tolerant and sensitive varieties (Tables A1, A2, and A4) suggests that only a single dominant allelic difference is involved. In one cross (Table A3), the χ^2 value for monogenic inheritance was significantly greater than zero. The ratio 9:7 (tolerant:sensitive) in the F_2 generation of the cross between the two sensitive varieties (Table A6), suggests that two co-dominant complementary alleles in two different loci are involved in the regulation of tolerance to ozone.

Results from the field nursery (Table A7) indicate that the majority of the alleles that express tolerance to ozone injury in bean plants are partially to completely dominant. Since results from the chamber implied nonexistence of maternal effects, F_1 and F_2 seeds from reciprocal crosses were composited and analyzed together. The test ratio 9:7 (tolerant:sensitive) in the F_2 generation of the cross

Table A7. The field results in 1979-1980.

<u>Varieties and Crosses</u>	<u>Total Number Plants</u>	<u>Number Ozone Tolerant Plants</u>	<u>Number Ozone Sensitive Plants</u>	<u>x² Value</u>
PHR	146	10	136	
Nep-2	126	108	18	
0669	118	16	102	
FH	122	118	4	
(0669 x Nep-2) F ₁	16	14	2	
(FH x PHR) F ₁	18	15	3	
(0669 x PHR) F ₁	17	13	4	
(PHR x Nep-2) F ₁	22	16	6	
(FH x 0669) F ₁	15	14	1	
(FH x Nep-2) F ₁	19	19		
(0669 x Nep-2) F ₂	199	159	40	2.68 N.S.
(0669 x FH) F ₂	206	172	34	S.
(Nep-2 x FH) F ₂	175	164	11	
(PHR x 0669) F ₂	279	153	126	2.06 N.S.
(Nep-2 x PHR) F ₂	292	185	107	S.
(PHR x FH) F ₂	226	153	73	S.

Table A8. Test field of F₃ families of the cross PHR x FH.

F ₂ Plants		F ₃ Plants		
Serial Number	Level of Injury	Number Ozone Tolerant	Number Ozone Sensitive	Tolerant Total
1	0	5	5	0.5
2	1	7	3	0.7
3	2	4	6	0.4
4	0	7	3	0.3
5	0	8	2	0.8
6	2	5	5	0.5
7	1	6	4	0.6
8	1	7	3	0.7
9	3	1	9	0.1
10	0	10	0	1.0
11	3 died	--	-	---
12	0	8	2	0.8
13	4 died	--	-	---
14	1	6	4	0.6
15	2	3	7	0.3
16	0	7	3	0.7
17	1	9	1	0.9
18	0	7	3	0.7
19	1	8	2	0.8
20	4 died	--	-	---
21	0 died	--	-	---
22	3	5	5	0.5
23	0	7	3	0.3
24	4 died	--	-	---
25	2	4	6	0.4
26	2	3	7	0.3
27	3	2	8	0.2
28	1	6	4	0.6
29	2	1	9	0.1
30	0	9	1	0.9

Table A9. Test field of F₃ families of the cross Nep-2 x PHR.

F ₂ Plants		F ₃ Plants		
Serial Number	Level of Injury	Number Ozone Tolerant	Number Ozone Sensitive	Tolerant Total
1	0	6	4	0.6
2	1	8	2	0.8
3	2	5	5	0.5
4	0	10	0	1.0
5	1	7	3	0.7
6	3	3	7	0.3
7	1	7	3	0.7
8	1	7	3	0.7
9	4 died	--	-	---
10	0	9	1	0.9
11	1	6	4	0.6
12	4	3	7	0.3
13	1	5	5	0.5
14	1	7	3	0.7
15	4 died	--	-	---
16	1	8	2	0.8
17	0	9	1	0.9
18	1	7	3	0.7
19	3	4	6	0.4
20	2	2	8	0.2
21	0	8	2	0.8
22	1	8	2	0.8
23	0	8	2	0.8
24	3	3	7	0.3
25	0	9	1	0.7
26	1	7	3	0.7
27	0	8	2	0.8
28	2	5	5	0.5
29	1	6	4	0.6
30	3	2	8	0.2

Table A10. Test field of F_3 families of the cross FH x 0669.

<u>F₂ Plants</u>		<u>F₃ Plants</u>		
<u>Serial Number</u>	<u>Level of Injury</u>	<u>Number Ozone Tolerant</u>	<u>Number Ozone Sensitive</u>	<u>Tolerant Total</u>
1	1	7	3	0.7
2	3	3	7	0.3
3	0	8	2	0.8
4	2	6	4	0.6
5	1	5	5	0.5
6	2	3	7	0.3
7	0	9	1	0.9
8	1	8	2	0.8
9	2	4	6	0.4
10	0	10	0	1.0
11	0	9	1	0.9
12	0	8	2	0.8
13	0	8	2	0.8
14	0	8	2	0.8
15	0	9	1	0.9
16	0 died	--	-	---
17	1	5	5	0.5
18	3	2	8	0.2
19	1	7	3	0.7
20	0	8	2	0.8
21	2	2	8	0.2
22	2	4	6	0.4
23	0	7	3	0.7
24	2	1	9	0.1
25	2	3	7	0.3
26	0	10	0	1.0
27	1	9	1	0.9
28	1	9	1	0.9
29	1	6	4	0.6
30	0	9	1	0.9

Table All. Test field of F_3 families of the cross 0669 x PHR.

<u>F₂ Plants</u>		<u>F₃ Plants</u>		
<u>Serial Number</u>	<u>Level of Injury</u>	<u>Number Ozone Tolerant</u>	<u>Number Ozone Sensitive</u>	<u>Tolerant Total</u>
1	1	8	2	0.8
2	3 died	--	-	---
3	1	9	1	0.9
4	4	2	8	0.2
5	3	1	9	0.1
6	1	6	4	0.6
7	1	7	3	0.7
8	1	7	3	0.7
9	3	6	4	0.6
10	2	2	8	0.2
11	1	6	4	0.6
12	1	9	1	0.9
13	1	7	3	0.7
14	2	1	9	0.1
15	3	3	7	0.3
16	1	7	3	0.3
17	0	9	1	0.9
18	3	2	8	0.2
19	1	7	3	0.7
20	1	7	3	0.7
21	1	8	2	0.8
22	3	1	9	0.1
23	0	7	3	0.7
24	2	5	5	0.5
25	3 died	--	-	---
26	1	6	4	0.6
27	0	10	0	1.0
28	3	0	10	0.0
29	0	8	2	0.8
30	0	9	1	0.9

Table A12. Segregation of the F₃ plants in the field.

<u>Level of Injury of the Parental Plants (F₂)</u>	<u>Number F₃ Ozone Tolerant Plants</u>	<u>Number F₃ Ozone Sensitive Plants</u>	<u>Tolerant: Sensitive</u>
0	288	62	4.65:1
1	270	112	2.4:1
2	62	118	1:1.9
3	38	112	1:2.9
4	5	15	1:3

between the two sensitive varieties produced a χ^2 value not significantly different from zero. Only in one cross (out of four) between tolerant and sensitive varieties (Nep-2 x 0669) the Mendelian monogenic ratio of 3:1 (tolerant:sensitive) was statistically (χ^2 N.S. from 0) acceptable.

Stomatal Density

The data in Table B5 suggests that 0669, a sensitive variety, has significantly fewer stomata per mm^2 on the abaxial (lower) leaf surface than do the other 3 varieties. FH, a tolerant variety, has significantly higher stomata per mm^2 on the adaxial (upper) leaf surface in comparison to the other 3 varieties.

Stomatal Conductivity

With reference to the data in Tables B1 and B2, the abaxial stomatal conductivity is about 8 to 10-fold greater than the adaxial conductivity, which implies that most ozone uptake occurs through the lower surfaces of the leaf canopy. In general, both abaxial and adaxial conductivity decreased significantly in all 4 varieties as ozone exposure time increased (Tables B1-B4). The two tolerant varieties exhibited higher stomatal conductivity (both abaxial and adaxial) prior to ozone fumigation and in the two periods of fumigation in contrast to the two sensitive varieties (Tables B1 and B2). The two main factors; variety and timing (period of fumigation) were significant in both abaxial and adaxial analyses of variance (Tables B3 and B4). The interaction between the

Table B1. Means of abaxial stomatal conductivity (cm/sec) of the four bean varieties at different durations of ozone exposure.

<u>Length of Ozone Fumigation (Hours)</u>	<u>FH</u>	<u>PHR</u>	<u>Nep-2</u>	<u>0669</u>	<u>Mean</u>
0	0.420	0.417	0.437	0.347	0.405
4	0.290	0.192	0.225	0.190	0.224
6	0.086	0.070	0.082	0.047	0.071
Mean	0.266	0.227	0.248	0.195	

Table B2. Means of adaxial stomatal conductivity (cm/sec) of the four bean varieties at different durations of ozone exposure.

<u>Length of Ozone Fumigation (Hours)</u>	<u>FH</u>	<u>PHR</u>	<u>Nep-2</u>	<u>0669</u>	<u>Mean</u>
0	0.050	0.020	0.044	0.037	0.038
4	0.057	0.014	0.023	0.018	0.028
6	0.020	0.008	0.013	0.006	0.012
Mean	0.042	0.014	0.026	0.020	

Table B3. Analysis of variance for abaxial stomatal conductivity for the four bean varieties.

<u>Source</u>	<u>d.f.</u>	<u>SS</u>	<u>MS</u>
Total	503	16.2	
Block	2	0.286	0.146
Variety	3	0.861	0.287
Timing	2	9.1	4.55
Variety x Timing	6	0.463	0.077
Error	22	0.335	0.015
Sample	216	3.691	
Det.	252	1.464	

Table B4. Analysis of variance for adaxial stomatal conductivity for the four bean varieties.

<u>Source</u>	<u>d.f.</u>	<u>SS</u>	<u>MS</u>
Total	503	0.294	
Block	2	0.0072	0.0036
Variety	3	0.063	0.021
Timing	2	0.058	0.029
Variety x Timing	6	0.019	0.003
Error	22	0.0348	0.016
Sample	216	0.09	
Det.	252	0.022	

Table B5. *Stomatal density in primary leaves (No./mm²) for the four bean varieties.

	FH		0669		Nep-2		PHR	
	<u>abaxial</u>	<u>adaxial</u>	<u>abaxial</u>	<u>adaxial</u>	<u>abaxial</u>	<u>adaxial</u>	<u>abaxial</u>	<u>adaxial</u>
218		73	178	44	214	55	217	35
200		62	166	38	223	49	208	34
192		55	132	32	244	42	219	44
225		66	147	44	216	47	196	49
245			169		197		205	
194			154		212		198	
233			163		198		218	
228			167		215		195	
$\bar{x}$ 217		64	159.5	39.5	215	48	207	40

*Means of four measurements per plant.

variety and timing was significant only in abaxial conductivity (Table B3).

Ascorbic Acid and Glutathione Assay

When the 4 varieties were considered together, there was a significant decline in the amounts of ascorbic acid (AA) detected after ozone fumigation. On the other hand, no differences in AA content were detected among the varieties before and after exposure to ozone (Tables C1 and C2). Glutathione (GSH) contents among the four varieties prior to fumigation were not significantly different. However, after ozone treatment, there was a significant decline in the amount of GSH in the varieties PHR, 0669 (both sensitive) and FH (ozone tolerant), while Nep-2 (ozone tolerant) showed a significant increase.

GSSG Reductase, Specific Activity, and Protein Content

GSH reductase activity was significantly greater in primary leaves of the two tolerant varieties than the activity in the two sensitives (Tables C5 and C6). Also, it appears that ozone within the leaves significantly altered GSH reductase activity. Enzymatic activity recovered slightly in both Nep-2 (tolerant) and 0669 (sensitive), and decreased in FH (tolerant) and PHR (sensitive).

Ozone treatment decreased the amount of soluble protein in the primary leaves of all varieties (Table C7).

Table Cl. Ascorbic acid content (mg/100 gr fw.) in primary leaves of the four bean varieties.

<u>Variety and Treatment</u>	<u>Nep-2</u>	<u>0669</u>	<u>PHR</u>	<u>FH</u>	<u>Average</u>
Pre-fumigation with ozone	560.4	554.8	548.6	542.5	551.6
Post-fumigation with ozone	515.2	520.7	508.5	528.2	518.1
Average	537.8	537.7	528.6	535.3	

Table C2. Analysis of variance for ascorbic acid content differences among four bean varieties prior to and after ozone fumigation.

<u>Source</u>	<u>d.f.</u>	<u>SS</u>	<u>MS</u>
Treatment	7	31,439	
Q1	1	26,773	26,773
Q2	1	1,246	1,246
Q3	1	1,608	1,608
Q4	1	684	684
Q5	1	630	630
Q6	1	223	223
Q7	1	273	273
Error	16	65,759	4,110
Sample	72	12,912	180

Q1 = contrast between pre and post fumigation with ozone.

Q2 = within pre contrast between Nep-2 and the other three varieties.

Q3 = within post contrast between FH and the other three varieties.

Q4 = within pre contrast between 0669 and the average of PHR and FH.

Q5 = within post contrast between 0669 and the average of PHR and Nep-2.

Q6 = within pre contrast between PHR and FH.

Q7 = within post contrast between PHR and Nep-2.

Table C3. Glutathione (GSH) content (mg/100 gr fw.) in primary leaves of the four selected varieties of dry beans.

<u>Variety and Treatment</u>	<u>Nep-2</u>	<u>0669</u>	<u>PHR</u>	<u>FH</u>	<u>Average</u>
Pre-fumigation with ozone	4.67	4.69	4.82	4.66	4.71
Post-fumigation with ozone	7.24	2.71	2.13	2.28	3.59
Average	5.95	3.70	3.47	3.47	

Table C4. Analysis of variance for glutathione differences among four bean varieties prior to and after ozone fumigation.

<u>Source</u>	<u>d.f.</u>	<u>SS</u>	<u>MS</u>
Treatment	7	466.55	
Q1	1	145.64	145.64
Q2	1	0.194	0.194
Q3	1	301.02	301.02
Q4	1	0.003	0.003
Q5	1	4.56	4.56
Q6	1	0.002	0.002
Q7	1	0.135	0.135
Error	16	336.33	22.9
Sample	72	130.11	1.8

Q1 = contrast between pre and post fumigation with ozone.

Q2 = within pre contrast between PHR and the average of the other three varieties.

Q3 = within post contrast between Nep-2 and the average of the other three varieties.

Q4 = within pre contrast between FH and the average of Nep-2 and 0669.

Q5 = within post contrast between 0669 and the average of PHR and FH.

Q6 = within pre contrast between Nep-2 and 0669.

Q7 = within post contrast between PHR and FH.

Table C5. Glutathione reductase specific activity (nmM NADPH/min/mg protein) in primary leaves of the four bean varieties before and after ozone exposure.

<u>Number</u>	<u>Variety</u>	<u>GSH Reductase Activity Before Fumigation</u>	<u>GSH Reductase Activity After Fumigation</u>
1	Nep-2	7.29	8.31
2	Nep-2	5.55	9.66
3	Nep-2	6.92	9.44
Average		6.59	9.04
1	FH	8.05	6.82
2	FH	6.59	5.85
3	FH	7.31	6.02
Average		7.32	6.23
1	PHR	4.43	3.49
2	PHR	4.32	3.22
3	PHR	4.12	3.20
Average		4.29	3.30
1	0669	3.18	4.35
2	0669	2.06	3.40
3	0669	2.91	3.65
Average		2.71	3.80

Table C6. Analysis of variance for the activity of leaf glutathione reductase in four bean varieties prior to fumigation with ozone.

<u>Source</u>	<u>d.f.</u>	<u>SS</u>	<u>MS</u>
Total	11	43.68	
Varieties	3	40.24	13.41
Error	8	3.44	0.43

According to Duncan's New Multiple Range Test = 0.05

Nep-2 FH PHR 0669

Analysis of variance for the activity of leaf glutathione reductase in four bean varieties after fumigation with ozone.

<u>Source</u>	<u>d.f.</u>	<u>SS</u>	<u>MS</u>
Total	11	64.16	
Varieties	3	62.17	20.72
Error	8	1.99	0.25

According to Duncan's New Multiple Range Test = 0.05

Nep-2 FH PHR 0669

Table C7. Soluble proteins (mg/ml) content in the leaves of four bean varieties prior to and after fumigation with ozone.

<u>Number</u>	<u>Variety</u>	<u>Proteins in the Plant Leaf Pre Fumigation</u>	<u>Proteins in the Plant Leaf Post Fumigation</u>
1	FH	1.149	1.054
2	FH	1.145	1.069
3	FH	1.212	1.046
1	Nep-2	1.278	1.228
2	Nep-2	1.313	1.252
3	Nep-2	1.291	1.241
1	PHR	1.149	0.906
2	PHR	1.158	0.921
3	PHR	1.193	0.972
1	0669	1.149	0.697
2	0669	1.063	0.683
3	0669	1.114	0.745

Application of Exogenous AA and GSH

Application of either AA or GSH to leaves significantly increased tolerance to ozone in the two sensitive varieties (Table C8). Generally, GSH appeared to increase tolerance to ozone more efficiently. Treatment with AA and GSH 6 hours prior to fumigation reduced ozone injury to a greater degree than did treatment 12 hours before fumigation.

Formation of Ethane and Ethylene

In general, leaf discs taken from ozone fumigated plants produced higher amounts of ethane and ethylene as compared with discs from untreated plants (see Appendices III-VI). Sensitive PHR and 0669 varieties produced much more ethylene after exposure to ozone than tolerant FH and Nep-2 varieties (Table D2). There were no significant differences between the two sensitive or the two tolerant varieties.

Although ethane is a natural product of plant metabolism, it is usually produced in smaller quantities as compared to ethylene. Unlike ethylene, there were no significant differences in ethane production among the four varieties after fumigation with ozone (Table D1).

Table C8. Application of exogenous GSH and ascorbic acid to the primary leaves of the two sensitive varieties.

<u>Variety</u>	<u>Treatment</u>	<u>Injury Level 6 Hour Pre Fumigation</u>	<u>Injury Level 12 Hour Pre Fumigation</u>
0669	H ₂ O	4	4
0669	H ₂ O	4	4
0669	H ₂ O	4	4
0669	H ₂ O	4	4
0669	H ₂ O	4	4
0669	A.A. (0.005M)	2	3
0669	A.A. (0.005M)	1	4
0669	A.A. (0.005M)	1	3
0669	A.A. (0.005M)	2	4
0669	A.A. (0.005M)	2	3
0669	GSH (0.005M)	0	2
0669	GSH (0.005M)	1	1
0669	GSH (0.005M)	1	1
0669	GSH (0.005M)	1	2
0669	GSH (0.005M)	0	1
PHR	H ₂ O	4	4
PHR	H ₂ O	4	4
PHR	H ₂ O	4	4
PHR	H ₂ O	4	3
PHR	H ₂ O	3	3
PHR	A.A. (0.005M)	0	0
PHR	A.A. (0.005M)	2	2
PHR	A.A. (0.005M)	1	3
PHR	A.A. (0.005M)	0	2
PHR	A.A. (0.005M)	1	3
PHR	GSH (0.005M)	0	0
PHR	GSH (0.005M)	0	2
PHR	GSH (0.005M)	0	2
PHR	GSH (0.005M)	1	0
PHR	GSH (0.005M)	0	2

Table D1. Analysis of variance for ethane production from ozone injured leaves of four bean varieties.

<u>Source</u>	<u>d.f.</u>	<u>SS</u>	<u>MS</u>
Total	99	166,222	
Variety	3	3,619	1,206 N.S.
Error	96	162,603	1,694

Table D2. Analysis of variance for ethylene production from ozone injured leaves of four bean varieties.

<u>Source</u>	<u>d.f.</u>	<u>SS</u>	<u>MS</u>
Total	99	215,490,000	
Variety	3	184,440,000	61,466,667
Error	96	31,050,000	323,428

According to Duncan's New Multiple Range Test = 0.05

<u>FH</u>	<u>Nep-2</u>	<u>PHR</u>	<u>0669</u>
a	a	b	b

DISCUSSION

Genetic Analysis

Previous studies which have dealt with genetic regulation of tolerance to ozone injury in plants are very contradictory. This lack of agreement among previous workers can probably be explained as due to their research being conducted in many different crops in which overall genetic backgrounds were different, and in which experimental factors such as ozone concentration, length of exposure, and plant age were variables. Because ozone apparently enters leaves only through stomata, variables such as temperature, humidity, soil moisture, and others which directly affect stomatal aperture, therefore, may affect amount of ozone uptake into the leaves. This situation, therefore, adds to the difficulty of reaching unanimous interpretations of the genetic basis involved. In fact, there is no fundamental reason why ozone tolerance in a large array of species has to be based upon a single genetic interpretation.

There is probably more than one way in which plants can be protected from ozone injury. Rapid stomatal closure in response to ozone exposure could decrease ozone uptake by the leaves (Engel and Gabelman, 1966). A compact arrangement of cells within the leaf could reduce ozone flecking by reducing the volume of intercellular space available for flow of ozone

molecules. Close spacing of cells could imply an increase in the number of chloroplasts per unit area which may increase the number of still functional chloroplasts in the presence of ozone (Uharing, 1978).

Many believe that ozone alters the permeability of membranes in the cell due to oxidizing properties (Ting et al., 1974; Beckerson and Hofstra, 1980; and Heath, 1980). Sulfhydryl groups, unsaturated fatty acids and aromatic residues of amino acids are primary targets of ozone and its by-products in the cell membranes (Heath, 1980). The ability to chemically reduce rapidly the oxidized components in the membrane in the presence of ozone, and thus to repair membrane injury, endows plant with ozone tolerance (Sutton and Ting, 1977). Tingey et al. (1975), have found that ozone affects numerous metabolic enzymes in leaf tissues of sensitive soybean cultivars. Therefore, tolerance may be associated with adaptive enzymes which function normally in the presence of ozone.

Different reactions to ozone activity may be found in different developmental stages of the leaf. Dugger et al. (1962), already showed that older, more mature leaves were injured more easily than younger leaves. Uharing (1978) suggested that stomata in young leaves are not fully developed and functional. Therefore, ozone uptake is higher in mature leaves. In addition, the greater intercellular volume in mature leaves is more favorable for flow of ozone. In contrast, Dugger et al. (1962) and Ting and Mukerji (1971), found that stomatal conductivity for gas exchange in pinto

beans and in cotton leaves remained constant throughout leaf maturation, and the period of maximum sensitivity was correlated with low soluble sugar and amino acid content. Evans and Ting (1973) concluded that leaf sensitivity throughout the developmental period was a function of internal conditions rather than to variation in stomatal resistance.

The results obtained in this research and others suggest that the trait "tolerance-to-ozone-flecking" in higher plants is controlled by several genes, and that it is highly affected by the environment to which the plants are subjected. Due to its physiological complexity it was impossible in this study to assess the genetic basis of ozone tolerance rigorously. As a result of the F_2 - F_3 data (Tables A8-A12), I grouped the 5 injury classes (0 to 4) obtained in the chamber study into two major classes, tolerant (0 to 1) and sensitive (2 to 4). According to this rearrangement, it is suggested that basically at least two major interacting dominant alleles (in two different loci) arbitrarily denoted as A and B, account for ozone tolerance in the varieties of this study. Assuming that the four varieties used in this study were 100% homozygous, the two sensitive varieties, PHR and 0669, are assigned genetic symbols AAbb and aaBB, respectively. On the other hand, the two tolerant varieties, FH and Nep-2, must both be AABB. Such genotypes would account for ozone tolerant F_1 plants in crosses between the two sensitive varieties, and for the 9:7 (tolerant:sensitive) ratio among the F_2 segregants. The proposed system would also account for the appearance of tolerant F_1 plants in the crosses between

tolerant and sensitive varieties, and for a 3:1 (tolerant:sensitive) ratio, in three of the four crosses.

Handling the situation in this way allowed some clarification of the ambiguity represented by this trait. However, in the F_3 generation there was not complete genetic fixation viz. the F_3 progenies for almost every F_2 parental plant still appeared to continue to segregate. In addition, there was some difference between the two tolerant varieties in the level of response to ozone (FH was somewhat more tolerant than Nep-2 and more stable). Similar differences were found between the two sensitive varieties. Such phenomena may be explained by introducing in addition to the two major genes, several genes with minor effect and non-genetic factors into the picture. These two circumstances may account also for the variability within each of the two main classes, namely, tolerant and sensitive. The available data do not permit us to determine how many minor genes are involved in the expression of the trait, whether the minor alleles (which increase the expression of ozone tolerance) are partially or fully dominant, or strictly additive, whether non-allelic interaction exists between some of the minor genes, and whether or to what extent they might be affected by external factors of the environment.

Non-genetic factors definitely affect the phenotypic expression because of the observed variability within each parent and among F_1 plants from the same cross. Such non-genetic factors could be due to: environmental variables such as temperature, soil moisture, humidity, etc., which

alter plant response to ozone, and developmental non-uniformity within fumigated plants, unequal distribution of ozone inside the chamber, and human error in estimating scores on plants subjected to ozone damage.

The four cultivars apparently possess slightly different minor alleles, therefore exhibit slightly different responses to ozone, and are affected and interact differently by and with the various environmental variables. This can explain the slight difference in response to ozone within the two tolerant and the two sensitive varieties, and the difference in phenotypic stability. The segregation in the F₂ generation in the cross between the two tolerant varieties (Table A5) may be due partially to the segregation of these minor alleles for which the parents differ. Continuous segregation within F₃ families strengthens the assumption that the minor alleles are still segregating, although the effect of environmental variables in addition cannot be eliminated. The presence of both minor genes and environmental variables may explain why at least 5 response classes were necessary to visually classify ozone-treated plants. My attempts to find basic genetic interpretation by grouping the 5 classes into the two main categories were not fruitful in the cross FH x 0669 (Table A3). It is possible that this particular cross is one out of 20 cases in which probability theory states that the 3:1 ratio will be acceptable. At any rate, this suggestion should be rechecked.

The results in this study agree with those of previous studies (Hanson et al., 1976; Cameron, 1975; and Butler

et al., 1979) that cytoplasmic genes or extra-chromosomal factors are probably not involved as a determinant of ozone tolerance.

Environmental conditions in the field during this experiment were not under close experimental control. According to Taylor (1974), the complex interaction between climatic factors, ozone concentrations, exposure duration, soil conditions, and physiological characteristics of the plant determine the reaction of plant tissues. Therefore, the phenotype as observed in the field will be an imperfect indicator of the genotype.

Ambient air pollution levels were not measured during the experiment. While ozone is the primary pollutant involved in the bio-injury of many beans, the effect of other pollutants such as SO₂, NO₂, and peroxyacetyl nitrate (PAN) cannot be ignored.

Under field conditions it was impractical to classify the whole plant (due to large variability of ozone injury within each plant) into more than two categories; tolerant and sensitive. Nevertheless, the χ^2 for testing the goodness of fit of the 9:7 ratio within the F₂ generation of the cross between the two sensitive varieties (PHR x 0669) was statistically not significantly different from zero and similarly the 3:1 ratio in just one of four crosses between tolerant and sensitive varieties (Nep-2 x 0669). It is plausible that under the uncontrolled conditions in the field, and because of the imprecise evaluation of the whole plant reaction it was impossible to identify successfully

the genetic segregation of such a trait as ozone tolerance. However, the fact that the two major alleles (A and B) which express ozone tolerance, are dominant, and the presence of complementary interaction between them were demonstrated even under uncontrolled conditions.

It is customary to perform progeny tests in breeding for traits with low heritability. Since ozone tolerance is affected to a great extent by environmental factors, its heritability is probably low. Information on the F_2 plants gained via their F_3 families increases the value of the heritability in this case which leads to an increase in the precision of genetic determination.

Previous analysis of the inheritance of tolerance to ozone has been conducted primarily in a quantitative fashion. Hanson et al. (1976) crossed seven petunia inbreds (ozone tolerant and ozone sensitive) in all possible combinations to yield a complete 7 x 7 diallel set. In addition, they transformed the visual injury levels (from 1 to 5) into number of hours prior to appearance of each particular level of injury. Hence, class 1 = no visible damage was transformed into 4.1 hours, whereas, the highest level of leaf damage corresponded to 6.8 hours. Working in this way, they constructed an analysis of variance to test for presence of additivity, dominance, and maternal effect. They concluded that the alleles which contribute to ozone tolerance seem to act primarily in an additive manner. Furthermore, they estimated component of variation including D, H, F, E, the dominance ratio, and heritability. In many papers like

Engel and Gabelman (1966) in onion, Papple and Ormrod (1977) in turfgrasses, Rasput and Ormrod (1976) in eggplant, Huang et al. (1975) in tobacco, and Butler et al. (1979) in beans, the visual scores of ozone injury were transformed into percentages of injury area of leaf surface. By using such quantitative transformed data in typical quantitative genetic assays they determined also in what fashion (additivity, dominance, etc.) alleles for ozone tolerance act. Knudson et al. (1977) suggested correlating the amount of ozone injury with decreasing chlorophyll concentration.

In my opinion, the above three methods do not faithfully represent overall ozone injury. Injured area on different leaf surfaces can vary greatly from each other in regard to the severity of the damage, e.g., the number of dead cells in cross section. On the other hand, chlorophyll concentration does not tell us how much of the leaf area was damaged. Perhaps a combination of both methods, while tedious, would give a better estimation. The method which was used by Hanson et al. (1976), is subjected to a great amount of error in its measurements, and in addition, they checked only one sensitive variety and then used the results on the other varieties, which probably increased the amount of imprecision. In addition, the term ozone-resistant which is widely used is not accurate, since even so-called ozone-resistant plants exhibit typical ozone injury symptoms under appropriate conditions.

Stomatal Conductivity

Flecking on the upper surface (adaxial) of bean leaves is a common symptom of ozone injury on dicotyledonous plants although most of ozone uptake by the leaf occurs in the lower surface (abaxial) (Rich and Tomlinson, 1974). The authors assumed that the random pattern of stomata in the lower surface, the different geometry of the air passages through the spongy parenchyma, and the greater tolerance of spongy parenchyma cells than palisade cells to ozone make it unlikely that ozone injury would appear on the abaxial surface of the leaf. Often the first visible symptom of ozone toxicity is the death of the palisade parenchyma cells that line the cavities directly beneath the adaxial stomata (Heath, 1980).

Stomatal conductivity has always been considered directly related to ozone injury, since ozone enters leaves through the stomata (Ting and Heath, 1975). According to research with different species including onion (Engel and Gabelman, 1966), tobacco (Turner et al., 1972), petunia (Thorne and Hanson, 1976), and beans (Butler and Tibbitts, 1979), ozone-tolerant varieties, due to their lower stomatal conductivity during ozone exposure, absorbed much less ozone in their leaves than sensitive varieties, and therefore, avoided leaf injury. Engel and Gabelman (1966), suggested that rapid regulation of the membranes of guard cells accounts for tolerance to ozone.

In addition, differences in stomatal density which were found in tobacco by Dean (1972) and in beans by Butler and

Tibbitts (1979) between tolerant (lower density) and sensitive (higher density) varieties, according to the authors, may be part of the mechanism that protects plants from ozone injury. In this study, such differences in stomatal conductance and density between tolerant and sensitive varieties have not been found. On the contrary, the two tolerant varieties had larger values of both abaxial and adaxial stomatal conductance than the two sensitive varieties. Similar findings were reported in beans by Hucl and Beversdorf (1979), and in cowpea by Adepipe and Tingey (1979). Although in all the varieties stomatal conductance was significantly decreased due to ozone exposure, conductivity of the tolerant varieties was less affected by ozone after four hours of exposure, in contrast to the sensitive varieties. In view of the fact that alteration of the electric potential of guard cell membranes and permeability due to ozone could affect stomatal closure, it is reasonable that the guard cell membranes of the tolerant varieties were less affected by ozone than membranes in the sensitive varieties. This may be due to biochemical differences, or due to a better ozone injury repair mechanism.

The lower stomatal density mainly in the abaxial surface of the sensitive variety 0669 may partially account for its low stomatal conductance values. FH, on the other hand, had the highest stomatal density which can partially account for its high stomatal conductance. It is important to say that the total variability in the values of stomatal conductance was high mainly due to its dependence on many environmental variables. In conclusion, the above-suggested mechanism,

although appealing because of its simplicity, is not supported by the data of this study. The possibility that differences between tolerant and sensitive varieties are due to changes in the capacity to repair biochemical ozone injury was suggested already by Dugger et al. (1962), Evans and Ting (1973), and Sutton and Ting (1977), and others.

Physiological Study

Foyer and Halliwell (1976), found that high concentrations of ascorbic acid (AA) in chloroplasts can react with oxidant radicals like superoxide ($O_2^{\cdot-}$), and hydrogen peroxide (H_2O_2). One would assume that AA might react with ozone as well, and possibly prevent or alleviate the damage caused by the ozone. Such an interpretation was suggested by Freebairn and Taylor (1960), and by Dass and Weaver (1968).

Hanson et al. (1971), reported that ozone tolerant petunia varieties had higher AA concentrations than sensitive varieties on an area basis. On the fresh weight basis however, no such differences were found. Later, Thorne and Hanson (1976) concluded that the correlation of variety sensitivity to ozone with AA concentrations per unit area was not significantly different from zero. In the present study, AA concentrations were determined on a fresh weight basis and were much greater (10-fold) than concentrations in petunia leaves. Although a significant reduction of AA concentrations occurred after ozone exposure the remaining concentrations were still high. This implies that in beans perhaps only a small amount of AA reacts directly with ozone or with its by-products, possibly

because endogenous AA in beans is not in a highly mobilized form. In addition, AA content of each of the four varieties (in comparison) was not significantly different prior to, and after ozone fumigation. These two facts indicate that AA plays a minor role in protection from ozone injury.

Mountain (1963) demonstrated that ozone exposure caused an in vivo oxidation of reduced glutathione (GSH) in mouse lung. The simple tripeptide GSH is found in almost all living cells and takes part in numerous biochemical reactions, as well as helping stabilize certain enzymes by preventing oxidation of thiol groups (Jocelyn, 1972). Most of the GSH in beans and other legumes is structurally different from ordinary GSH since it contains the amino acid β -alanine instead of glycine (Carnegie, 1963). However, the catalytic behavior of both is similar. It is possible that GSH, which can reduce oxidized thiol groups such as disulfide bonds or sulfonic acid ($\text{—SO}_2\text{H}$) back into sulfhydryl groups (Ting and Heath, 1975), could play an important role in an ozone injury repair mechanism. Similar to previous studies with AA, application of exogenous GSH to different plant organs prior to ozone fumigation significantly reduced the level of injury. The amount of GSH in the four varieties of the present study was 100-fold smaller than AA. No differences in GSH concentrations were detected among the four varieties prior to ozone fumigation; however, after fumigation, GSH concentrations of Nep-2 (tolerant) rose significantly, while the concentrations in the remaining varieties significantly decreased. Therefore, it is possible that GSH takes part in

ozone injury repair. Furthermore, Foyer and Halliwell (1976) found that GSH will non-enzymatically reduce dehydroascorbate (the oxidized state of AA) back to AA. This implies that GSH may play an important role, too, in the limited contribution of endogenous AA to tolerance against ozone injury.

Quite surprisingly, FH, the highly ozone tolerant variety, unlike the other tolerant variety Nep-2, exhibited reduction in GSH content after exposure to ozone. There are two possibilities for this outcome:

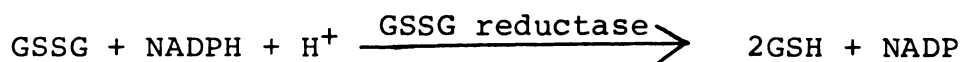
- a) that FH is endowed with another mechanism to tolerate chronic ozone concentrations, and
- b) that the extraction of GSH from FH leaves was not complete.

Leaves of FH are substantially thicker than leaves of the other varieties (this was verified by using the electronic leaf area meter) which implies that perhaps FH after exposure to ozone had more GSH per unit area than the sensitive varieties or possibly more mesophyll cells and chloroplasts per unit area than the other three varieties, which would enable it to tolerate high concentrations of ozone.

Dass and Weaver (1968) and Freebairn and Taylor (1960), significantly reduced ozone injury in beans by spraying leaves and roots with exogenous AA and GSH prior to ozone fumigation. The data in the present study confirmed their results; however, it was found here that GSH sprays are more efficient than AA sprays in reducing injury which again suggests that GSH is a major component in ozone flecking repair mechanism. In addition, application of

both compounds 6 hours prior to ozone fumigation was more efficient than application 12 hours prior to fumigation, which suggests that the exogenous AA and GSH were subjected to an overall oxidation after application.

Due to the oxidation, GSH is converted to its oxidized form, abbreviated GSSG, which can be converted back to the reduced form, GSH, by the enzyme glutathione reductase (GSSG reductase) in the following reaction:



In the present study, specific activity of GSSG reductase was much higher in the two tolerant varieties than in the two sensitives. Since GSH conversion to GSSG is due to the reduction of S — S- bonds (bonds previously oxidized due to ozone) by GSH back to SH- groups, the higher GSSG reductase activity in the two tolerant varieties can be interpreted as higher ability to repair ozone damage of cell membranes. Sutton and Ting (1977), based on previous experiments by Dugger et al. (1962) and Dugger and Palmer (1969), found that dipping bean leaves in glucose solution immediately following ozone fumigation significantly reduced ozone injury. They suggested that glucose probably acts by providing the necessary energy to repair oxidized cell components. It is possible that glucose utilization via the pentose phosphate pathway which, according to Tingey et al. (1975), is activated by ozone, yields NADPH molecules which increase the rate of GSSG reduction.

The different activities of GSSG reductase between tolerant and sensitive varieties could be:

- a) due to the presence of two isozymes that differ in rates of catalysis, which means that the isozymes which are formed by the structural genes of the tolerant varieties have higher rates of catalysis than the isozymes formed by the sensitive varieties, and/or
- b) due to quantitative differences in the formation of GSSG reductase, viz., the regulatory genes of tolerant varieties produce more GSSG reductase molecules than the sensitive varieties.

Schedle and Bassham (1977) found that the activity of glutathione reductase is inhibited by Zn^{+2} ,; therefore, higher Zn^{+2} concentration in leaves of the sensitive varieties in contrast to the tolerant varieties also could account for their low GSSG reductase activity. In general, the activity of GSSG reductase will vary due to alterations of the $\text{NADPH}/\text{NADP}^+$ ratio, which increases during illumination (Lendzian and Bassham, 1975). This may explain why response to ozone flecking as reported by Heck (1968), and Taylor (1974) was affected by the length of the photoperiod prior to fumigation with ozone.

The results from this study showed that exposure to ozone induced changes in GSSG reductase activity. Such changes were not consistent among susceptible and tolerant varieties; however, these changes did not alter the overall picture, viz., the tolerant varieties always exhibit (prior to and after fumigation) significantly higher levels of GSSG

reductase activity. The study of GSSG specific activity supports the hypothesis that the two tolerant varieties, FH and Nep-2, during ozone "invasion", due to their higher enzymatic activity, regenerate GSH faster from GSSG, and therefore, are able to more quickly reduce essential ozone-oxidized compounds in the leaf. This possibility led me to believe that probably the method which was used to extract GSH (quantitative assay) which was originally used in spinach leaves was only partially effective with FH leaves; viz., not all the GSH from FH leaves was released. Therefore, it is possible that FH leaves had a high or the highest GSH content prior to ozone fumigation. This condition is plausible considering the fact that FH is also the most ozone-tolerant variety; however, a conclusive answer to this matter may only be obtained through further studies.

As reported by workers in cotton (Ting and Mukerji, 1971) and in beans (Cracker and Starbuck, 1972), it was found in this study, too, that there is a significant decrease in soluble protein levels within the leaf immediately after exposure to ozone. This suggests that ozone may affect protein metabolism by promoting protein hydrolysis. Generally, it seems that a good correlation may exist between the content of soluble protein in the leaf and a visual estimation of ozone injury. A continuous study of this aspect, which will yield scientific support for such correlation, will increase the precision in ozone injury determination.

Ethane and Ethylene Production

John and Curtis (1977) showed that the unsaturated fatty acid, linolenic acid, is the main precursor of ethane in bean plants. Ethane production by plants was found to be associated with tissue injury, which may be related to membrane destruction and peroxidation of linolenic acid (Liberman, 1979). Ting and Heath (1975) mentioned that exposure to ozone may cause peroxidation (ozonation) of double bonds in unsaturated fatty acids within membrane lipids. Since linolenic acid is very prevalent in leaf tissues, it probably serves as a target for ozone "attack". Therefore, quantitative differences in linolenic acid content in the narrow sense or unsaturated fatty acids content in the broad sense between cell membranes of tolerant versus sensitive varieties may account for the differences in ozone injury. If this assumption is correct, sensitive varieties (with higher linolenic acid content) will be expected to produce more ethane after ozone fumigation than the tolerant varieties with lower content of linolenic acid.

Methionine, the sulfur-containing amino acid, is generally the accepted precursor for ethylene, although both ethylene and ethane can be produced from linolenic acid hydroperoxide (Dumelin and Tappel, 1977). Wilson et al. (1978), reported that an analogue of rhizobitoxin, an inhibitor of ethylene from methionine, partially inhibited the emission of ethylene but not the emission of ethane from curcubit leaf tissues maintained in bisulfite solution. This observation supports the idea that the two gases are

produced largely by different pathways; however, Wilson's results do not exclude the possibility that some ethylene may arise from peroxidized linolenic acid. Tingey et al. (1976), found in a wide array of plant species that plants exposed to ozone produced more ethylene in the dark than in the light. On the other hand, Wilson et al. (1978), reported that light enhanced both ethylene and ethane production. In full agreement with the results obtained by Wilson et al. (1978) in cucurbits, Filner (personal communication) in cucumber, and Peisner and Young (1979) in alfalfa in which the production rates of both ethylene and ethane increased after exposure to SO_2 , in this study production of ethane and ethylene was increased due to exposure to ozone (in comparison with non-fumigating plants). However, unlike cucurbits (Bressan et al., 1978), the two ozone sensitive bean varieties in the current study did not produce significantly higher amounts of ethane than the two tolerant varieties after ozone exposure. In spite of the fact that the leaf discs were incubated (after ozone fumigation) under high light intensity, which was claimed by Tingey et al. (1976), to inhibit or slow ethylene production, ethylene production was significantly higher in leaf discs from sensitive varieties.

In conclusion, the lack of consistency between ethane production and ozone injury suggests that in beans ozone-tolerant and sensitive varieties have similar concentrations of linolenic acid or in general similar unsaturated fatty acids content in the cell membrane. Therefore, tolerance to ozone in beans is probably not due to reduced content of

linolenic acid in particular or unsaturated fatty acids in general. Since ethylene production is promoted by almost any type of plant tissue injury, including ozone flecking, it is hard to propose a mechanism for ozone tolerance based on ethylene evolution. However, this burst of evolved ethylene following an ozone exposure suggests that ethylene is involved in the trigger mechanism of plant responses to ozone injury. Abeles (1973) found that in the presence of ethylene there is an increase in the activity of phenylalanine ammonia lyase, polyphenol oxidase, and peroxidase which are involved in the synthesis of phenolic compounds, and which are known to be activated during ozone exposure (Howell, 1974). The increase in the activity of these enzymes could be mediated through an ozone enhancement in the rate of ethylene production. In spite of the fact that one of the primary objectives of this study was to propose a mechanism for ozone tolerance, a distinctive positive correlation between levels of visual ozone injury and ethylene production has been observed in this study. Such a correlation was studied by Tingey et al. (1976), and as a result they suggested that ethylene measurements provide a more sensitive and accurate measure of plant response to ozone than the common visual injury determination.

CONCLUSION

The idea in this study has emerged that glutathione (GSH) and glutathione reductase (GSSG reductase) might be involved as a repairing mechanism of ozone injury. Halliwell and Foyer (1978), using affinity chromatography, found that spinach GSSG reductase, similar to animal and yeast GSSG reductase, is a dimer with subunits of similar size; therefore, it is possible that GSSG reductase in bean plants has a similar structure. This fact could be associated with the results from the genetic analysis in which at least two interacting major genes appear to be involved in the response of plants tolerant to ozone. Hence, by this hypothesis, the two dominant alleles A and B which increase ozone tolerance, code for the two "active" polypeptides of GSSG reductase, resulting in higher enzymatic activity in Nep-2 and FH (both tolerant and both AABB). In contrast, the lower enzymatic activity in the two sensitives, PHR and 0669 (AAbb and aaBB) in which dominant alleles of only one gene are present, is due to the formation of only one active polypeptide. The appearance of one dominant allele per each one of the two major genes (AaBb), as exists in F₁ plants from the cross between the two sensitive varieties, would endow these plants with the active GSSG reductase isozyme.

The role of the minor genes in the expression of ozone tolerance is undefined, and they might be involved in determining the: number of mesophyll cells and chloroplasts per unit of leaf area, intercellular space in the leaf, carriers involved in GSH and GSSG mobilization within leaf cells, structural entities in cell membranes, mainly in the plasmalemma, and a large array of regulatory genes which control in leaf tissues the amount of soluble proteins, SH dependent enzymes, structural proteins, saturated and unsaturated fatty acids, glycolipids, phospholipids, etc.

The environmental variables which affect the expression of ozone tolerance can be classified into two major categories:

- a) affecting directly or indirectly stomatal aperture which control ozone uptake into the leaf.
- b) affecting within the leaf reaction or processes which are associated with the presence of ozone or its by-products.

In fact, the activity of GSSG reductase can be affected by environmental variables such as length of photoperiod (Lendzian and Bassham, 1975), and temperature (Esterbauer and Grill, 1978).

The other suggested mechanisms for tolerance to ozone injury which were examined in this study, namely, differences in response of guard cells to ozone, stomatal density, and variation in the amount of the unsaturated fatty acid linolenic acid, were not supported by the data from this

study. However, it is possible that these mechanisms may account for the variability (in regard to ozone tolerance) which exist among different species and genera.

APPENDICES

APPENDIX I: Concentration of GSH and ascorbic acid in the primary leaves (mg/100g fw.)
post fumigation with ozone.

Variety	mg of GSH		Variety	mg of A.A.		mg of GSH		mg of A.A.	
	100g fresh wt.			100g fresh wt.		100g fresh wt.		100g fresh wt.	
Nep-2	7.2	547	PHR	1.9	512				
	7.0	533		1.8	511				
	7.6	529		2.2	483				
	7.1	521		2.3	442				
	6.6	506		2.2	495				
	7.8	499		2.7	492				
	7.4	483		2.4	516				
	7.2	466		2.9	523				
	7.3	501		2.6	543				
	7.9	532		2.6	525				
	7.0	517		2.6	552				
	6.8	549		2.0	500				
0669	2.0	562	FH	1.8	512				
	2.6	544		2.0	560				
	2.5	507		2.1	493				
	2.4	519		2.4	488				
	2.6	462		2.3	506				
	2.9	516		2.5	519				
	3.2	488		2.6	531				
	3.4	434		2.7	549				
	3.1	510		2.3	555				
	2.6	581		1.9	532				
	2.5	555		2.3	571				
	2.7	571		2.5	522				

APPENDIX II: Concentration of GSH and ascorbic acid in primary leaves (mg/100g fw.)
pre fumigation with ozone.

Variety	mg of GSH		Variety	mg of A.A.		mg of GSH		mg of A.A.	
	100g fresh wt.			100g fresh wt.		100g fresh wt.		100g fresh wt.	
Nep-2	5.1		PHR	580		5.8		563	
	4.8			597		6.0		572	
	4.6			541		5.2		571	
	4.4			610		5.5		549	
	4.4			506		4.3		544	
	4.3			532		4.8		539	
	4.0			515		4.6		566	
	4.2			589		4.4		592	
	4.7			531		4.3		515	
	5.2			579		4.2		516	
	5.3			564		4.0		551	
	5.1			581		4.7		506	
0669	5.2		FH	594		4.0		583	
	5.0			577		4.6		517	
	5.0			586		4.9		542	
	4.9			553		4.5		550	
	4.6			591		4.9		573	
	4.2			567		4.3		521	
	4.5			551		5.2		563	
	4.8			513		5.1		598	
	4.2			521		4.4		514	
	4.6			551		4.9		519	
	4.4			552		4.7		504	
	4.9			532		4.5		526	

APPENDIX III: Ethylene production of ozone injured leaf discs (mm²).

Nep-2	FH	PHR	0669
254	249	1515	1813
474	346	3966	1905
236	280	2865	2384
385	156	3747	2286
466	417	1817	2452
249	258	2953	3113
354	321	2073	4022
218	292	3251	4221
349	415	1929	3120
265	353	2003	2525
374	243	1816	3081
115	575	4312	4715
500	110	2677	3219
425	315	3233	4160
332	496	3870	3510
174	311	4022	2872
256	222	3021	2466
440	205	2866	4155
502	255	3032	4432
557	480	2843	3190
383	293	3611	4154
470	512	3416	2656
492	503	2430	3819
508	410	2931	2726
610	376	3115	2934
$\bar{x} = 375$	335	2932	3197

APPENDIX IV: Ethane production of ozone injured leaf discs (mm²).

Nep-2	FH	PHR	0669
126	141	211	293
144	132	129	116
132	205	130	177
188	214	130	184
100	250	144	206
115	181	155	131
132	116	229	115
199	173	181	219
171	244	136	125
111	155	123	127
130	221	104	211
125	113	229	185
116	122	191	109
184	167	157	166
153	155	166	174
132	164	138	218
114	139	174	130
176	211	215	219
210	166	212	147
225	174	163	172
230	209	207	211
242	188	136	150
133	145	252	166
158	125	202	145
191	183	166	118
$\bar{x} = 157$	172	171	169

APPENDIX V: Ethylene production of non ozone treated leaf discs (mm²).

Nep-2	FH	PHR	0669
175	96	162	85
92	65	74	76
115	114	65	110
135	102	122	55
102	116	101	80
84	131	88	131

APPENDIX VI: Ethane production of non ozone treated leaf discs (mm²).

Nep-2	FH	PHR	0669
0	0	0	0
0	0	0	0
0	0	0	0
0	0	0	0
26	32	0	0
0	0	0	0

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