Response of Leaf Protein to Ozone in Two White Clover Clones

by

Youlin Tang

Thesis submitted to the Faculty of the

Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the Degree of

Master of Science

in

Plant Pathology, Physiology, and Weed Science

APPROVED:

Dr. Boris I. Chevone, Co-Chairman	**Dr. John L. Hess Co-Chairman**

Dr. Laurence D. Moore

May, 1998
Blacksburg, Virginia

Key Words: White Clover, Ozone-Responsive Protein

Response of Leaf Protein to Ozone in White Clover Clones

Youlin Tang

(Abstract)

A white clover (*Trifolium repens*) system, based on one ozone-sensitive (NC-S) and one ozone-resistant (NC-R) clone, has been developed as an indicator to estimate the effects of tropospheric ozone on plant biomass production. A reduction in the vegetative biomass ratio (NC-S/NC-R) of the clones was correlated with increasing concentrations of ozone during a 28-day exposure period. However, the mechanism of ozone sensitivity or tolerance at the biochemical or molecular level is not known. Superoxide dismutase isozyme activities in the two clones did not respond differently to ozone treatment. However, catalase activity increased somewhat more in the leaf tissue of NC-R, compared to NC-S, after ozone treatment. Two-dimensional polyacrylamide gel electrophoresis (PAGE) showed the presence of two proteins (Ozone-Response-Proteins, ORPs) that were more responsive to ozone in the tolerant genotype than in the sensitive one. After ozone treatment for three days, the ORPs were four-fold higher in leaf tissue of NC-R compared to NC-S. Also, the amount of the ORPs was twenty-fold higher in leaf tissue of ozone-treated NC-R than in that of control NC-R. These proteins have apparent molecular weights of 21.5 kD and 23 kD and isoelectric points of 4.1-4.4 on SDS-PAGE gels. The filtrate of a 100 kD concentrator showed that the native molecular weights of the ORPs were less than 100 kD. The results obtained from a study of field samples demonstrated that protein content in leaf tissue of both NC-R and NC-S was positively correlated with ORP content.

DEDICATION

To my country, To my parents and parents- in-law, and to my wife and my daughter

ACKNOWLEDGMENTS

I would especially like to acknowledge my major co-advisors, Dr. Boris I. Chevone and Dr. John L. Hess, for their continual patience, guidance, support, and understanding and also for their helpful suggestions. I would also like to thank the other member of my committee, Dr. Laurence D. Moore, for his interest, advice throughout this project, understanding, and support. My thanks go to my friends, Dr. Dafang Bia, for his invaluable help on two-dimensional electrophoresis, Kevin Dell, for his help with computer technique and research equipment, and Carla E. Hegeman, for her help on protein transfer and FPLC technique. I also want to thank those who gave me some assistance on my studies and research, such as Dr. Thomas W. Keenan, and Dr. Elizabeth A. Grabau. Finally, my very special thanks are presented to my wife, Jun Xiao, and my lovely daughter, Jingyang Tang, for their always being there when I need them. Without their support and encouragement, I could not finish my degree.

Table of Content

Abstract----------ii

Dedication----------iii

Acknowledgments---------- ----------iv

Table of Content----------v

List of Figures---------- vii

List of Tables----------ix

1. Introduction----------1

2. Background and Literature Review- ----------4

2-A Uptake of Ozone by Plants and Effects of Ozone on Plants---------4

2-A-1 Uptake of Ozone into the Plant Leaf----------4

2-A-2 Effects of Ozone on Plants----------4

2-B Response of the Antioxidative System Induced by Ozone----------9

2-C Response of the Antipathogenic System Induced by Ozone--------13

3. Materials and Methods----------21

3-A Materials----------21

3-A-1 Plant Propagation----------21

3-A-2 Controlled Ozone Fumigation----------22

3-A-3 Sample Harvested----------22

3-A-4 Field Ozone Exposures----------23

3-B Methods----------23

3-B-1 Protein Extraction and Measurement of Protein Content--------23

3-B-2 Superoxide Dismutase Activity Assay----------24

3-B-3 Determination of Isoforms of Superoxide Dismutase----------24

3-B-4 Catalase Activity Assay----------25
3-B-5 Investigation of Soluble Protein Populations----------25
3-B-6 Separating ORPs from Rubisco Protein----------26
4. Results----------28
4-A Greenhouse Sample----------28
4-A-1 Soluble Protein Content----------28
4-A-2 Isoforms of Superoxide Dismutase----------30
4-A-3 Activity of Superoxide Dismutase Assay----------33
4-A-4 Catalase Activity----------33
4-A-5 Investigation of Soluble Protein Populations----------34
4-B Field Samples----------40
5. Discussion----------42
6. Summary----------47
Reference----------49
Vita----------56

List of Figures

Figure 1. Ozone decomposition in water----------5
Figure 2. Ozone reacts with unsaturated fatty acid----------6
Figure 3. Ozone reaction with sulfhydryls and amino acids------7
Figure 4. Antioxidative system in plant----------10
Figure 5. Molecular structure of ethylene and reaction of ozone with ethylene----------14
Figure 6. Interaction of ethylene and polyamine synthesis in plant----------14
Figure 7. Time course of arginine decarboxylase in tobacco cultivars----------17
Figure 8. The distribution of ozone concentration----------19
Figure 9. Relationship of ozone concentration and NCS/NCR foliar production----------20
Figure 10. Soluble protein content of leaf tissue----------30
Figure 11. Superoxide dismutase activity----------31
Figure 12. Identification of isoforms of SOD with KCN----------32
Figure 13. Identification of isoforms of SOD with H_2O_2----------32
Figure 14. Change of ORPs in treated plants----------35
Figure 15. Change of ORPs in controlled plants----------36
Figure 16. Change of ORPs among treated and controlled plants----------37

Figure 17. High resolution of 2-D PAGE on ORPs----------38
Figure 18. Native molecular weight of ORPs----------39
Figure 19. Change of soluble protein content in leaf tissue of field plant----------40
Figure 20. Relationship between ORP and soluble protein content----------41

List of Tables

Table 1. Ozone inhibits ATPase activity----------8
Table 2. Effect of ozone on glutathione Content----------11
Table 3. Enzyme activity changes in two *thaliana* genotypes-12
Table 4. Ozone-induced gene expression in *Arabidopsis*---------12
Table 5. Relationship between ethylene concentration and leaf injury in pea seeding----------15
Table 6. Response of ethylene, polyamine and leaf injury in tobacco to ozone----------16
Table 7. Soluble protein content in leaf tissue of greenhouse sample----------28
Table 8. Soluble protein content in leaf tissue of greenhouse sample under different season----------29
Table 9. Relative activity of SOD isoforms----------33
Table 10. Catalase activity----------34

1. Introduction

Overview

Ozone is a gas that has a dual role in the atmosphere. There is a layer of ozone in the stratosphere that protects living organisms from UV radiation, which is emitted from the sun. On the other hand, ozone levels in some locations in the troposphere are high enough to adversely affect plant and animal life. Ozone is an oxidizer of biochemical molecules. The oxidative characteristic is derived from the structure of ozone ($:O^{-}-O^{+}=O \leftrightarrow O=O^{+}-O^{-}:$); electrons move freely among the three oxygen atoms. The physical nature of ozone allows it to be broken down easily to form some reactive oxygen species and also enables direct reaction with biochemical molecules after it enters the plant leaf. Disruption of the normal metabolism of plants by ozone can result in yield loss. Annual economic losses are estimated at $2-5 billion for major agronomic crops within the United States (Heck, 1989). Plants, however, have developed defense systems that afford protection from stress environments, such as air pollution, drought and high or low temperatures. An antioxidative system has been developed in plants for protecting the chloroplast and other organelles from damage due to photooxidation or the generation of other active oxygen molecules (Alscher & Hess, 1993; Foyer, Descourvières & Funert, 1994). Some researches have demonstrated that the antioxidative system also protects plants from ozone exposure (Kangasjärvi *et al*., 1994).

The antioxidative defense system in plants functions to remove reactive oxygen species and free radicals, and consists of detoxifying enzymes (superoxide dismutase, catalase, etc.) and low molecular weight substrate (ascorbate, glutathione etc.). It is evident that after a plant is exposed to ozone, the amount of antioxidants and the activities of some detoxifying enzymes increased (Chanway & Runeckles, 1984; Castillo *et al.*, 1987; Gupta, *et al.*, 1991). However, the activity and type of enzymes induced differ among species, and even between cultivars of the same species. The mechanism of ozone induction is still poorly understood. In addition, ozone exposure can result in the same responses that pathogens elicit. These inductions include synthesis of ethylene, polyamines, enzymes of the phenylpropanoid and flavonoid pathways and pathogen-responsive proteins. Also, ozone can increase the susceptibility of plants to be attacked by some pathogens (Kangasjärvi *et al*., 1994; Schraudner *et al.*, 1996).

Because ozone causes more damage to plant than all other air pollutants combined, there is an increasing interest in developing techniques to monitor the effects of ozone on ecosystem health. A useful tool for this purpose would be a plant bioindicator system. In the past, tobacco was used for this purpose, but only provided qualitative information. At present, a system of white clover, containing resistant (NC-R) and sensitive (NC-S) clones, has been used for estimating biomass loss of sensitive plant genotypes caused by ozone (Heagle *et al.*, 1994). However, the mechanism of ozone resistance in NC-R is not known. It may be helpful for

understanding the mechanism of plant tolerance to ozone exposure to identify relationships between ozone concentration and biochemical and molecular response in white clover.

Objectives

Based on statement above, following objectives have been examined in my research.

A. Measurement of soluble protein content.

B. Antioxidative enzymes assay.

C. Investigation of soluble protein populations

2. Background and Literature Review

2-A Uptake of ozone by plants and effects of ozone on plants

2-A-1 Uptake of ozone into the plant leaf

Stomata are the main route of ozone uptake into plant leaves (Heath, 1980). According to Rich *et al*. (1970), the ingress of ozone depends on the number and size of stomata and the degree of their opening. When relative humidity was increased from 35 to 75 %, foliar uptake of ozone by red kidney beans was enhanced three-four fold (McLaughlin & Taylor, 1981), demonstrating that under high humidity, plants usually take up more air pollutant and also are more sensitive to air pollution. Abscisic acid (ABA) is a phytohormone that causes stomata closure. Adapipe & Ormod, (1972) reported that spraying ABA on leaves reduced ozone injury of the treated plants, again demonstrating that stomata are the main route for ozone to enter the plant leaf.

2-A-2 Effects of ozone on plants

Laisk *et al.* (1989) demonstrated that ozone concentration in leaf intercellular air spaces is close to zero, even though the concentration outside the leaf is high, indicating that ozone is rapidly absorbed by leaf cells or is break down to form free

oxidative radicals. Free radicals are formed in ozone-fumigated plant leaves that have been detected using electron spin resonance (ESR) spectroscopy (Mehlhorn *et al.* 1990). Once ozone enters the plant, it can either directly react with biomolecules, such as unsaturated fatty acids, proteins, and DNA or decompose to form reactive oxygen species, such as superoxide (O_2^-), hydroxyl radical ($HO^\cdot$) and hydrogen peroxide (H_2O_2). Ozone degradation (Figure 1) is favored at high pH (>8.0). The reactive oxygen species that are formed can also react with membranes, proteins and DNA (Heath, 1975).

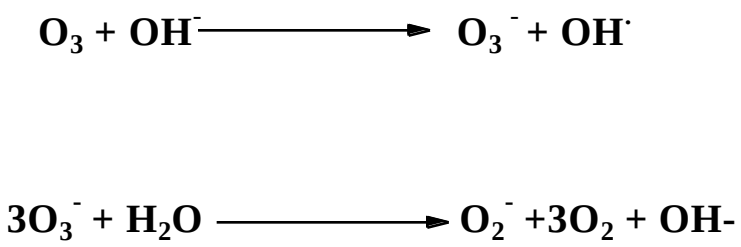


Figure 1. Ozone decomposition in water.

Ozone can react with the double bond in unsaturated fatty acids, a component of plant cell membranes, to form carbonyl and hydrogen peroxide (Figure 2). This oxidative reaction can change the physiological properties of the membrane. Unsaturated fatty acids will be shortened by this process which will increase the permeability and fluidity of the membrane (Heath, 1987). In pinto bean plant (*Phaseolus vulgaris*) exposed to 400 ppb ozone uptake of 2-deoxy glucose was twice

that of controls after five hours exposure and three times that of controls after 20 hours (Perchorowicz and Ting, 1974) These data indicate that one target for ozone is the plasma membrane, resulting in alteration in membrane permeability (Evans & Ting, 1974; Sutton & Ting, 1977).

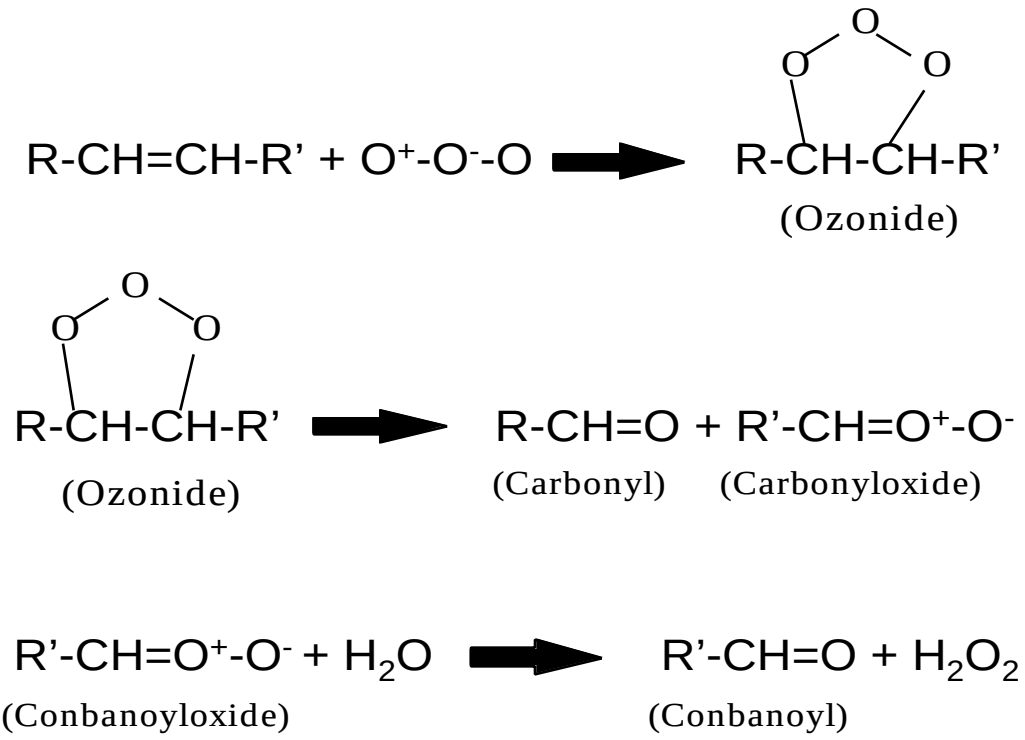


Figure 2. Ozone reacts with unsaturated fatty acid

Ozone reacts with sulfhydryls (-SH) not only to oxidize them reversibly to disulfides (-S-S-) and sulfenic acid groups ($-SO_2H$), but also to form irreversible sulfonic acid groups (SO_3H) (Figure 3). On the other hand, it has been suggested that sulfhydryls protect proteins from oxidation in biochemical systems (Mudd *et al.*, 1971). There is evidence that an ozone-resistant variety of tobacco had less sulfhydryls than that of

an ozone-sensitive variety (Tomlinson & Rich, 1968). Amino acids, such as cysteine, methionine, tryptophan, tyrosine, histidine and phenylalanine, have been reported to

Other Ozone Reaction

Sulfhydryl Oxidation

-SH	-S-S-	Disulfide
-SH	$-SO_2H$	Sulfenic acid
-SH	$-SO_3H$	Sulfonci acid

Amino acids interaction

Amino acids	Attacked Site
Cysteine	-SH
Methionine	$-SH_3$
Tyrosine, Histidine	C=C

Figure 3. Ozone reaction with sulfhydryls and amino acids

be modified by ozone (Figure 3)(Heath, 1975). These reactions will destroy enzyme activity and change protein conformation. The activities of two ATPases associated with the plasma membrane in pinto bean plants; one is Mg^{++} dependent and the other is K^+ stimulated, have been tested with ozone exposure for five hours, 500 ppb, (Dominy and Heath; 1985). Both ATPases were inactivated but their activity could be restored by dithiothreitol (DTE), a thiol reagent, if the ATPases from plant material fumigated with ozone were treated with DTE after isolation (Table 1). This

reactivation by DTE suggests that the ozone acted upon sulfhydryl groups of the proteins. Castillo and Heath (1990) also reported that ozone fumigation changed Ca^{2+} transporters' function and Ca^{2+} permeability was increased. In this experiment, pinto bean plants were exposure to ozone with 150 ppb for four hours.

Table 1. The ozone-treated inhibition of plasma membrane ATPase of pinto bean plants *in vivo*.

Addition	Activity (µmols Pi / mg protein,Hour)	
	Control	Ozone-treated
Mg^{++}[b]	1.75 ± 0.39	0.0
+DET[a]	1.66 ± 0.09	3.75 ± 0.12
$Mg^{++}+K^{+}$[c]	2.92 ± 0.09	0.0
+DET	4.00 ± 0.07	5.86 ± 0.12

a. 3 mM. b. 3 mM. c. 50 mM. Modified from Dominy & Heath, (1985)

The ozone-induced decrease of yield has been reported in number of plants. One of reasons for lower yield is a decrease in photosynthetic capacity. Ozone causes degradation of Rubisco protein, loss of Rubisco mRNA (Eckardt & Pell, 1994), a decrease in the amount of chlorophyll (Heagle *et al*., 1996) and a change in membrane components of the chloroplast (Sakaki *et al.,* 1985; Hellgren *et al*., 1995). Sakaki *et al*. (1985) reported that monogalactosyldiacyglycerol (MGDG), a major structural lipid in

thlakoids decrease substantially after ozone exposure. The triacylglycerol (TG), a component of plastoglobuli (Steinmüller and Tevini, 1985) detected at only very low levels in photosynthetically active green leaves (Harwood, 1980), increased marked by increase in ozone treated spinach. These reactions lead to the disappearance of thylakoid membrane in the chloroplast after ozone exposure.

A comprehensive review on uptake of ozone into plants and effects of ozone on plants has been described by Chappelka and Chevone (1992), Runeckles (1992), and Runeckles and Chevone (1992).

2-B. Response of the antioxidative system induced by ozone

In plants, an antioxidative system is employed to scavenge the reactive oxygen species, such as hydrogen peroxide, that are produced in electron transport chains in chloroplast and mitochondria. The system consists of enzymes such as superoxide dismutase (SOD), catalase and low molecular mass scavengers, such as ascorbate acid and glutathione (Scandalios, 1990; Bowler *et al*., 1992 and Alscher & Hess, 1993) (Figure 4). This system is also used for scavenging reactive oxygen species that may occur from the decomposition of ozone (Gupta *et al.,* 1991; Kangasjärvi *et al.,* 1994 and Schraudner *et al.,* 1996). After plants were treated with ozone, the activities of the enzymes in the antioxidative system were induced in some plants but not in other plants. The ozone-induced activity level of a specific enzyme was different

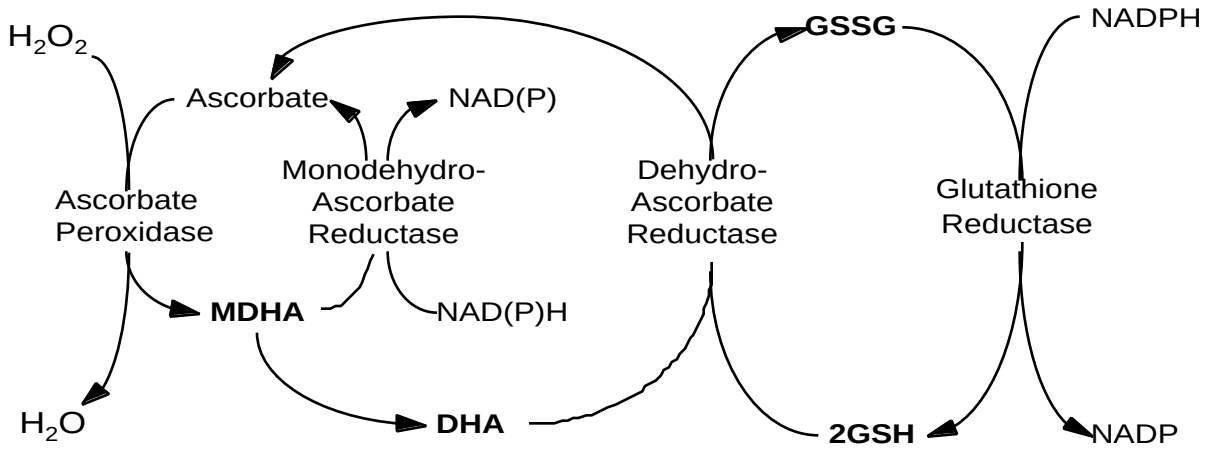
depending on the plant species or genotype and usually involved cell damage. Ozone-induced mechanism at the molecular level also have been examined in some plants.

$$O_2^- + H_2O \xrightarrow{SOD} H_2O_2 + 1/2\ O_2$$

$$2\ H_2O_2 \xrightarrow{Catalase} 2\ H_2O + O_2$$

$$HO^{\cdot} + Asc \longrightarrow H_2O + DH\text{-}asc$$

Superoxide dismutase (SOD), Ascorbate (Asc), Dehydroascorbate (DH-asc)



The ascorbate-glutathione cycle
Dehydroascorbate (DHA), reduced glutathione (GSH), oxidized glutathione (GSSG), monodehydroascorbate (MDHA)

Figure 4. Antioxidative system in plants

Gupta *et al.* (1990) fumigated *Populus* with ozone for 3 hours at 180 ppb and measured glutathione content in leaves. As shown in Table 2, total glutathione

concentration increased in ozone-fumigated plants, compared with controls. After

Table 2. Effect of ozone on glutathione content in *Populus* leaves

	GSH+GSSG	GSH	GSSG
	(nmol/mg chl.)		
Control	147	141 ± 28	6 ± 2
Fumigated	437	293 ± 40	144 ± 42
Change	+3-fold	+2-fold	+24-fold

Ozone dose: three hours / 180 ppb. Modified from Gupta *et al.* 1990

ozone exposure, enzyme activity changes have been measured in two genotypes of *Arabidopsis thaliana* (Kubo, *et al.*, 1995; Rao *et al.*, 1996). As the data shows in Table 3, the same species, but different genotypes, had a different responses to ozone exposure. In genotype, Columbia, activities of SOD, catalase, and glutathione reductase did not change before and after ozone exposure. But in genotype, Landburg Erecta, only the activity of catalase was not induced. The induced level of ascorbate peroxidase was almost the same in the two genotypes. However, the induced level of peroxidase activity in Columbia was much higher than in Landburg Erecta, compared with control plants. According to Sharma and Davis (1994), mRNA content of SOD and peroxidase in *Arabidopsis thaliana* was induced with an ozone dose for six hours/14 days with 150 -300 ppb, but that of catalase was not (Table 4). It

Table 3. Comparison of the enzyme activity changes in two genotypes of Arabidopsis thaliana after ozone exposure

	Columbia [1]	Landburg Erecta [2]
	Change	Change
SOD	None	+83%
Catalase	None	None
GR	None	+98%
Peroxidase	+4.4-fold	+90%
APX	+80%	+87%

SOD = Superoxide dismutase, GR = Glutathione Reductase
APX = Ascrobate peroxidase

1. Kubo *et al.*, 1995; ozone dose: eight hours / seven days, 150 ppb
2. Rao *et al.*, 1996; ozone dose: six hours / eight days, 200 ppb

Table 4. Ozone-induced gene expression in *Arabidopsis.*

mRNA of	Change of content
SOD	+ 2-3-fold
Catalase	None
Peroxidase	+ 5-fold

Ozone dose: six hours/14 days with 150 - 300 ppb. Modified from Sharma & Davis

is a useful tool to examine the response of enzyme activities of the antioxidative system to ozone exposure in transgenic plants. Pitcher *et al*. (1991) showed that in

transgenic tobacco (cv. W38), overproduction of SOD did not protect leaves from ozone damage. However Van Camp *et al*. (1994) demonstrated that increased levels of SOD protected transgenic tobacco (cv. PBD6) against ozone exposure. The reason for these differences still remains unknown. However, sufficient data exist to demonstrate that ozone can evoke a response of the antioxidative system in some plant genotypes.

2-C. Response of the antipathogenic system induced by ozone

Ozone induces not only the antioxidative system, but also the antipathogenic system. Ozone can elicit the same response in plants as pathogens do, including induction of the synthesis of ethylene, polyamines, enzymes of the phenypropanoid and flavonoid pathways, such as phenylalanine-ammonium lyase (PAL), chalcone synthase (CHS) and cinnamyl alcohol dehydrogenase (CAD), and pathogen-responsive proteins (PR-proteins), such as β-1,3-glucanase and chitinase (Kangasjärvi *et al*., 1994).

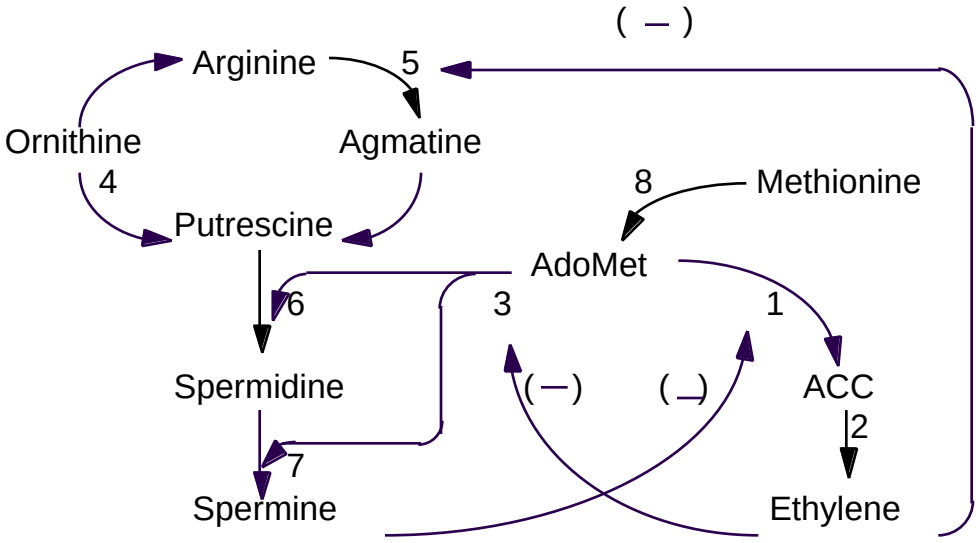
Ethylene is considered to be a key factor in increasing plant sensitivity to ozone (Kangasjärvi *et al*., 1994). This nature is dependent on the double bond in the ethylene molecule (Figure 5-1), which is readily attacked by ozone. Ethylene interaction with ozone may produce highly reactive, water-soluble free oxyradicals

$$\begin{array}{c} H - C = C - H \\ \quad | \quad | \\ \quad H \quad H \end{array} \qquad 5\text{-}1$$

$$O_3 + C_2H_4 \longrightarrow 2HCOH + H_2O_2 \qquad 5\text{-}2$$

Figure 5. Molecular structure of ethylene and reaction of ozone with ethylene

and aldehydes (Figure 5-2), which may directly damage the plasma membrane (Mehlhorn & Wellburn, 1987; Mehlhorn *et al.*, 1990). In the pathway of ethylene biosynthesis (Figure 6), 1-aminocyclopropane-1-carbocylic acid (ACC) synthase is



AdoMet = S-adenosyl methionine;
ACC = 1-amino-cyclopropane-1-carbocylic acid
1, ACC synthase; 2, ACC oxidase; 3, AdoMet decarboxylase;
4, ornithine decarboxylase; 5,arginine decarboxylase;
6, spermidine synthase; 7, spermine synthase; 8, AdoMet syntethase

Figure 6. Interaction of ethylene and polyamine synthesis in plants

the regulatory enzyme (Kende, 1993). In potato, a large increase in ACC synthase gene transcript in response to ozone exposure was detected (Schlagnhaufer *et al.*,

1993). This result showed that in plants, stress ethylene biosynthesis may be regulated by ozone. Mehlhorn *et al*. (1987) presented the relationship between ethylene concentration and leaf injury in pea seeding, using AVG (aminoethoxyvinylglycine) exogenously applied to the plant leaf. AVG is known to inhibit ACC synthase during the biosynthesis of ethylene (Yang & Hoflman, 1984). Pea seedings were treated with ozone for seven hours at 150 ppb. As the Table 5 shows, visible leaf injury area in control plants was much higher (>50%) than in AVG-treated plants (0-5%).

Table 5. Relationship between ethylene concentration and leaf injury in pea seeding

Treatment	C_2H_4 evolved (nmol g^{-1} dry Wt h^{-1})	visible leaf injury (%)
Control	2.6 (± 0.4)	>50
10^{-3} M AVG	0.4 (± 0.1)	0-5

AVG = Aminoethoxyvinylglycine, ozone dose: seven hours and 50 - 150 ppb
Modified from Mehlhorn *et al.* 1987

Ozone also induces biosynthesis of polyamines in plants, which are considered to have two functions in decreasing ozone damage (Kangasjärvi *et al*., 1994). First is the inhibition of ethylene synthesis and second is the stabilization of membranes and scavengers of oxygen radicals. As Figure 6 shows, spermine is an inhibitor of ACC

synthase. Langebartels *et al*. (1991) demonstrated the relationship between ethylene biosynthesis and polyamine production using Bel W3 (ozone-sensitive) and Bel B (ozone-resistant) tobacco (Table 6). The plants were exposed to ozone for 7 hours

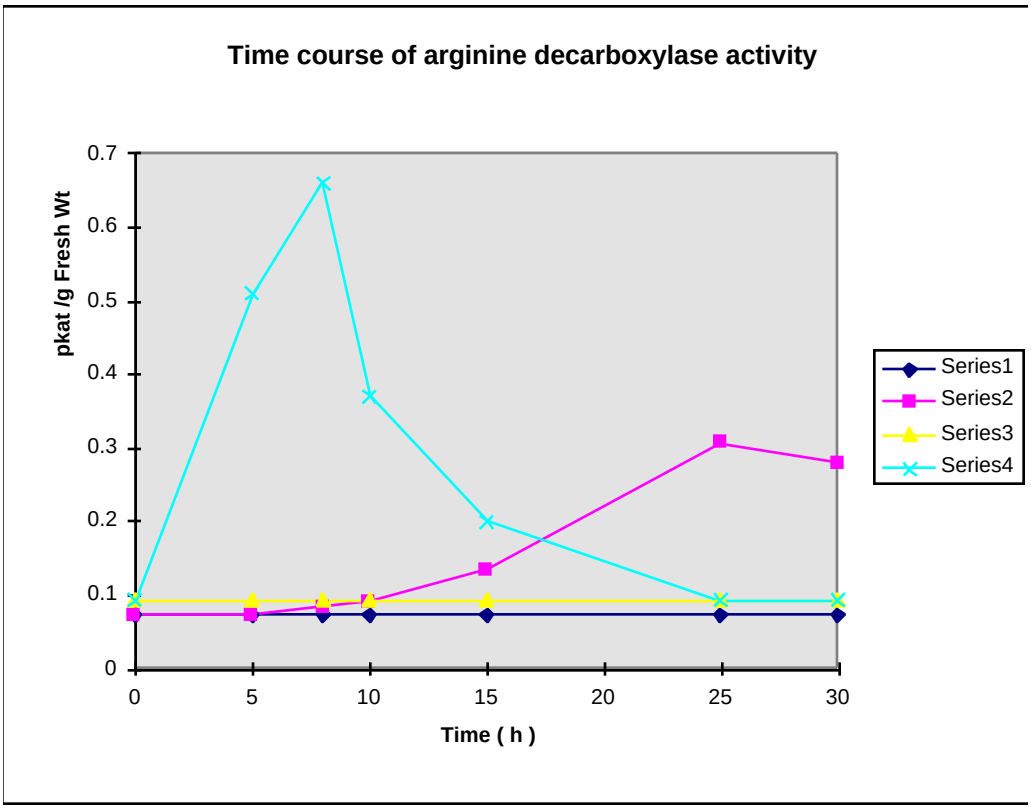
Table 6. Response of ethylene, polyamine and leaf injury in tobacco to ozone.

	Ethylene		Putrescine		Spermidine		Leaf injury	
ozone	Bel W3*	Bel B**	Bel W3	Bel B	Bel W3	Bel B	Bel W3	Bel B
ppb	(nmol g^{-1} fresh wt h^{-1})		(nmol g^{-1} fresh wt)				%	
0	0.14	0.13	51	77	21	38	0	0
100	0.16	0.09	131	223	31	52	12	0
200	0.48	0.16	118	225	28	44	52	0

* = Ozone-sensitive; ** = Ozone-resistant. Modified from Langebartels *et al.* 1991

with different ozone concentrations. Ethylene increased more quickly in Bel W3 (ozone-sensitive) than in Bel B (ozone-resistant); however, polyamine concentration increased more slowly in Bel W3 (ozone-sensitive) compared with that in Bel B (ozone-resistant). Leaf injury area was higher in Bel W3 (ozone-sensitive) than in Bel B (ozone-resistant). Exogenous application of polyamines preserved chlorophyll retention in thylakoid membranes of barley chloroplasts (Popovic *et al*., 1979), stabilized oat leaf protoplasts against lysis (Fuhere *et al*., 1982) and reduced membrane permeability in discs of beet root storage tissue (Naik *et al*., 1978). In the

polyamine biosynthetic pathway (Figure 6), arginine decarboxylase (ADC) is a regulatory enzyme. Langebartels *et al*., (1991), using Bel W3 (ozone-sensitive) and Bel B (ozone-resistant) tobacco treated by ozone for 7 hours with 150 ppb, demonstrated that ADC activity increased faster in Bel B (ozone-resistant) than in Bel W3 (ozone-sensitive) (Figure 7).



1 = Control Bel W3 2 = Ozone-treated Bel W3

3 = Control Bel B 4 = Ozone-treated Bel B

Bel W3 (Ozone-sensitive); Bel B (ozone-resistant).

Modified from Langebartels *et al*. (1991)

Figure 7. Time course of arginine decarboxylase in tobacco cultivars

In parsley, all pathogen-induced genes that were examined were induced by ozone (Eckey-Kaltenbach *et al*., 1994). In addition, a pathogen-sensitive wheat genotype was sensitive to ozone exposure (Sah *et al*., 1993). The data accumulated to date suggest that reactive oxygen species, and ethylene could be possible signals to induce a general stress response signaling pathway (Kangasjärvi *et al*., 1994; Schraudner *et al*., 1996).

Because ozone causes more damage to plants than all other air pollutants combined (Heagle, 1989; Heck *et al*., 1986), there is an increasing interest in developing techniques to monitor effects of ozone on ecosystem health. A useful tool for this purpose would be a plant system that responds to ambient levels of ozone and can be calibrated to estimate ozone concentrations and effects on other plant species. The most extensively used plant system as an indicator has been tobacco cultivars Bel W3 (ozone-sensitive) and Bel B (ozone-resistant). At present, a white clover system, composed of one ozone-resistant (NC-R) and one ozone-sensitive (NC-S) genotype, has been developed as an indictor to estimate effects of tropospheric ozone on plants (Heagle *et al*., 1994). In this indictor system, the index of plant injury caused by ozone exposure is the ratio of forage biomass production of NCS/NCR rather than leaf injury area. Figure 8 shows the ozone concentration, averaged over 24 hours, at eight locations in the United States that were used in a field study in 1993-1994. Even through plants were grown under different weather conditions and ozone concentrations, the ratio of NCS/NCR was negatively correlated with ozone

concentration (Figure 9) (Heagle *et al.*, 1995). However, the mechanism of tolerance of NC-R plants to ozone exposure is not known. Investigations with this system may be helpful to understanding mechanisms of plants that function to protect themselves from ozone damage.

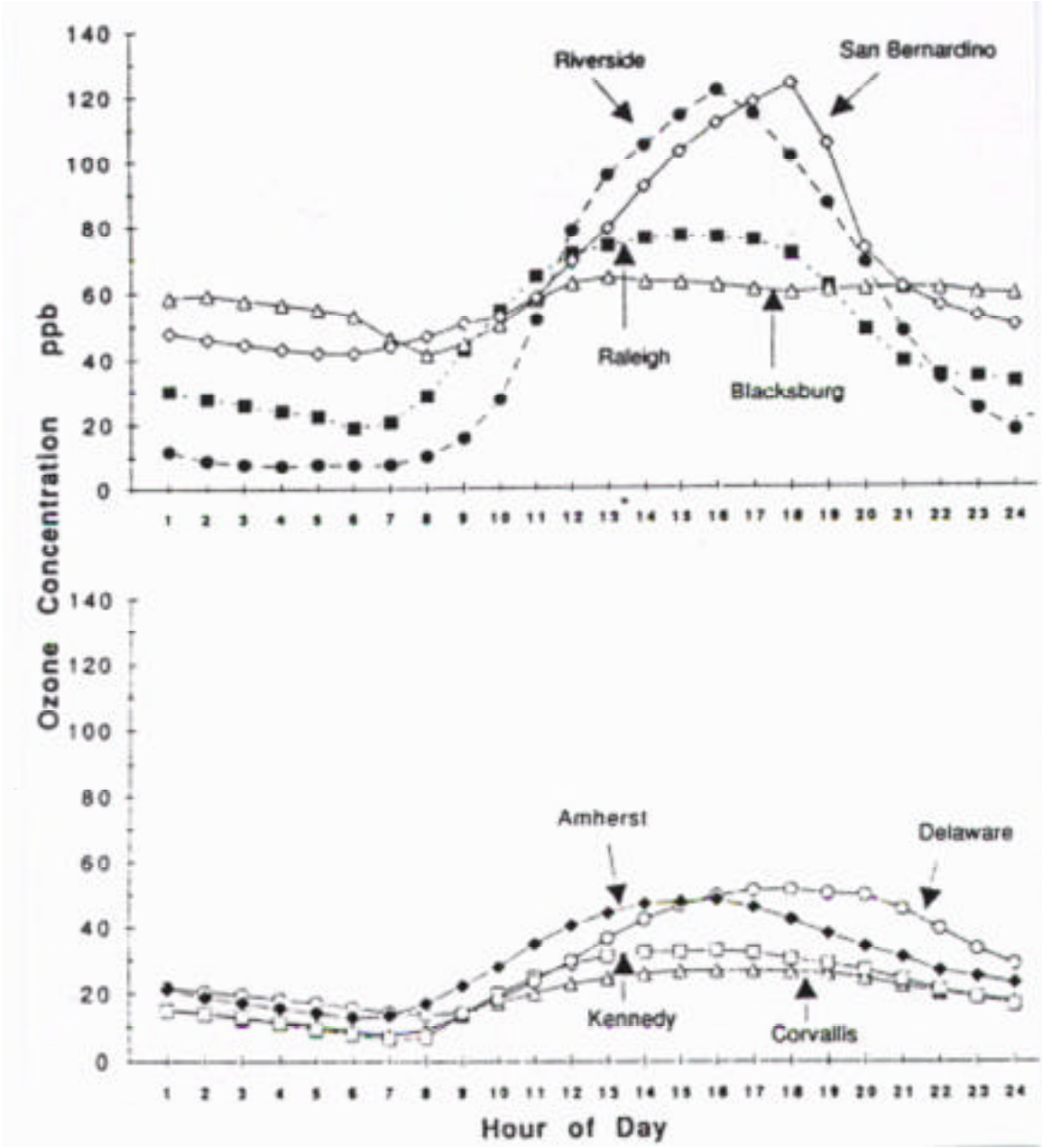


Figure 8. The distribution of ozone concentration within 24 hours period at eight locations in the United States (Heagle *et al.*, 1995)

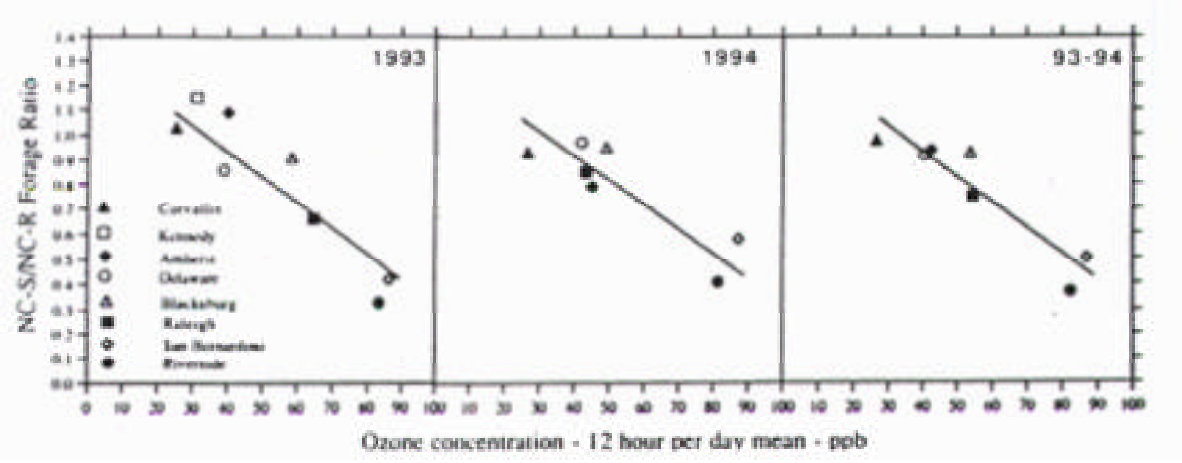


Figure 9. Relationship of ozone concentration and NCS/NCR foliar production (Heagle *et al.*, 1995)

3 Materials and Methods:

3-A Materials:

A white clover system, which consists of an ozone-sensitive (NC-S) clone and an ozone-resistant (NC-R) clone, was utilized in my experiments. The clones were obtained from A.S. Heagle, USDA/ARS, Raleigh, NC. They are well-suited for biochemical studies of ozone resistance because they are easy to propagate from cuttings and provide genetically uniform individuals.

3-A-1 Plant Propagation

Clover plants were propagated by initially rooting cut stolons in tap water, then placing the rooted cutting in plastic pots containing Metromix 200. The cuttings were fertilized with Osmocote (10:10:10, N:P:K) at bimonthly intervals and watered as necessary. Clover was grown in a charcoal-air filtered greenhouse, under ambient light conditions (1250 μmols m^{-2} s^{-1} maximum PAR, 400-700 nm). Air temperature in the greenhouse was 30°C-35°C. Day / night R.H was 50 ± 10% / 70 ± 10 % and ozone concentrations were < 15 ppb.

3-A-2 Controlled Ozone Fumigation

Clover plants were exposed to ozone in four continuously stirred tank reactor (CSTR) (Heck, *et al.*, 1978) chambers. Ozone was generated by UV discharge using O_2 and monitored with a UV analyzer. Plants were exposed to a step function ozone profile that simulated episodic, diurnal ambient patterns that occur during the summer months. Daily exposures occurred for eight hours from 0900 to 1700 with a minimum concentration of 50 ppb, step functions of 30-40 ppb, and maximum concentrations of 150 ppb (maintained for three hours). Treatments were continuous for three days and conducted only during the period April through October to ensure a light intensity above photosynthetic saturation. Environmental conditions during ozone exposures were similar to greenhouse conditions: 1100 ± 150 μ mols m^{-1} s^{-1} PAR, 50 ± 15 % R.H., and 28 ± 3 °C. Pots were watered to dripping daily, prior to placing plants in the CSTR chambers.

3-A-3 Sample Harvested

Leaf tissue was sampled before the start of any treatment period and daily (depending on the experiment) after each fumigation ended. Mature tissue at leaf position three and four from the growing tip was used. Each leaf was cut into three leaflets. The leaflets were collected randomly and weighed. At each collection period two to three plants of each clone were sampled. Each Sampling period represented a

replicate. Samples were frozen in liquid N_2 and stored at -70°C before processing.

3-A-4 Field Ozone Exposures

Clover plants were grown in 10 L plastic pots in Metro mix 200 in plots at the Horton Research Center, Giles County, VA. Clover was grown under ambient ozone concentration, fertilized monthly with osmocote (10:10:10, N:P:K) and watered as required to prevent moisture stress. Foliar leaf tissue from leaf position one through five or six from the apex of the growing shoot, was harvested after exposure to ambient ozone for 28 days. Leaf position one was the youngest, fully expanded leaf on a stolon. Samples were weighted and frozen in liquid N_2 within one minute after harvest.

3-B Methods:

3-B-1 Protein Extraction and Measurement of Protein Content

Leaf tissue samples were ground in liquid N_2 in a chilled mortar with a pestle and the fine powder was added to phosphate buffer (100 mM NaH_2PO_4, 10 mM ascorbate, 1mM EDTA and 10 mM DTT, pH 7.5) at a ratio of 10 ml buffer/g leaf tissue to extract protein. The homogenate was transferred to a 30 ml centrifuge tube and was centrifuged at 22,000×g for 15 minutes at 2-4°C to remove insoluble debris. One ml of

the resulting supernatant was desalted through Sephadex G-25 (1 cm × 7 cm column) to remove small molecular mass material (Neal and Florini, 1973). Desalting occurred by centrifugation at 500×g for 15 minutes at 2-4°C. The columns were equilibrated with phosphate buffer (100 mM NaH_2PO_4, 0.2 mM EDTA, pH 7.8) prior to addition of 1 ml protein extract. The filtrate was collected and protein content was determined by the protein-dye binding assay (Bradford, 1976) using BSA as a standard.

3-B-2 Superoxide Dismutase Activity Assay

Superoxide Dismutase activity was assayed on 12 % native polyacrylamide mini-gels. Desalted leaf extracts were diluted 1:2 with electrophoresis sample buffer (1.52 g Tris base, pH 6.8, 20 ml glycerol and one mg bromophenol blue to 45 ml with ddH_2O). A total of 30 µg protein was loaded on each lane. Gels were run at a constant current of 10 mA during the stacking gel phase and 15 mA during the separating gel phase using tap water as coolant in a Hoefer-gel system. Activity of SOD was visualized using the nitroblue tetrazolium staining procedure (Beauchamp & Fridovich, 1971). Gels were scanned with a Shimadzu Model 3 densitometer at 560 nm.

3-B-3 Determination of Isoforms of Superoxide Dismutase

The isoforms of superoxide dismutase were determined using the method described by Bridges *et al*. (1981). Following electrophoresis, the gels were immersed in NBT

solutions containing either two mM H_2O_2 or one mM KCN and incubating for 30 minutes. After incubation, the stained gels were exposed to light for 30 minutes.

3-B-4 Catalase Activity Assay

Catalase activity was examined in freshly desalted leaf extracts by monitoring decomposition of hydrogen peroxide at 240 nm using the spectrophotometric method (Beers and Sizer, 1952) at 25^{o}C. The 3 ml reaction mixture contained phosphate buffer (0.026 M KH_2PO_4, 0.041 M Na_2HPO_4, pH 7.0) and 18 mM H_2O_2. Nonenzymatic H_2O_2 decomposition was assayed using boiled, desalted clover leaf extracts.

3-B-5 Investigation of Soluble Protein Populations

Soluble proteins were separated and analyzed using the two-dimensional gel electrophoresis method of O'Farrell (1975). The desalted leaf extract (500 µl) was precipitated by an adding equal volume of 20 % TCA (Trichloroacetic acid), cooling on ice for 30 minutes, and then centrifuging at 11,000×g for 10 minutes. The supernatant was discarded. The pellet was washed with acetone, dried by air, centrifuged at 11,000×g for 10 minutes again, and dissolved in lysis buffer (9.5M of urea, 2% V/V of NP-40, 5% V/V of ampholyte, 5% V/V of 2-mercaptoethanol, pH 9.0) to yield a final concentration of 2 µg protein μl^{-1}. Isoelectric focusing gels (4%) were prepared using ampholytes with a pH range of 4-8. IEF electrophoresis was carried

out at 250V for 30 minutes for pre-focusing followed by 500V for 3-4 hours with tap water as coolant. A total of 20 μg protein was loaded on top of each IEF gel after pre-focusing. After running, extruded IEF gels were immersed in 2 ml equilibration buffer (3.75 g Tris base, pH 6.8, 25 ml glycerol, 5.25 g SDS, 333 mg dithiothreitol in 250 ml dd H_2O) for 10 minutes. The equilibrated gels were either layered on 12 % SDS polyacrylamide slab gels, and run at 20 mA for stacking gels, and 30 mA for separating gels or stored at -20°C for future use. Silver or Commassie blue stains were used to visualize protein. For high resolution IEF gels, the ampholyte range was reduced to pH 3.7 to 6.

The molecular weight of ozone-induced protein subunits was estimated on SDS gel, using the standard molecular marker proteins (Weber & Osborn, 1975).

The change of ozone-responsive proteins was determined by using a densitometor. 31 kD standard market protein was chosen as a reference because the amount of standard market protein was controlled.

3-B-6 Separating ORPs from Rubisco Protein

A Centricon® 100 (Amicon INC, Bevery, MA) concentrator was used for separating native soluble proteins into high and low molecular weight fractions. Proteins in the filtrate and retentate were examined by one- and two-dimensional gel electrophoresis.

The concentrators were treated with clover leaf extract overnight to prevent proteins from binding to the membrane. The pre-treated concentrators were washed with dd H_2O and centrifuged at 800×g for 10 minutes. One ml of desalted leaf tissue extract was loaded in concentrators and centrifuged at 950×g for 24 minutes twice. The filtrate and retentate were collected separately for further use.

4 Results

4-A Greenhouse Sample

Fumigation of both clover clones for three days at a maximum O_3 concentration of 150 ppb (three hours / day) did not result in any necrotic lesions, visible chlorophyll loss, or other obvious foliar symptoms.

4-A-1 Soluble Protein Content

The soluble protein content of control leaf tissue in NC-R and NC-S was 19.7 ± 3.8 mg protein/g leaf fresh weight and 21.5 ± 7.0 mg protein/g leaf fresh weight, respectively (Table 7). After the clover plants were exposed to ozone for three days, soluble

Table 7. Soluble protein concentration (mg P/g fresh leaf weight) in leaf tissue of O_3-sensitive (NC-S) and O_3-resistant (NC-R) clover clones before and after O_3 fumigation for three days (maximum concentration of 150 ppb for three hours/day)

	NC-R[1]	NC-S
Control	19.7± 3.8 [2]	21.5 ± 7.0
O_3 treatment	20.9± 3.8	23.6± 4.8

1. Sample size for protein determination was a minimum of n = 15 (see Table 8)
2. Values represent one standard deviation of the mean

Table 8. Soluble protein concentration (mg P/g fresh leaf weight) in leaf tissue of O_3-sensitive (NC-S) and O_3-resistant (NC-R) clover clones before and after O_3 fumigation for three days (maximum concentration of 150 ppb for three hours/day) under different seasons

Season[1]	n[2]	NCS-C	n	NCS-O_3	n	NCR-C	n	NCR-O_3
Winter	11	14.6 ± 1.8	6	17.2 ± 1.8	9	17.4 ± 2.6	6	16.5 ± 1.7
Spring	5	23.5 ± 4.1	5	27.2 ± 0.5	13	21.1 ± 5.9	13	27.3 ± 2.4
Summer	16	21.2 ± 2.5	4	20.9 ± 0.7	30	21.8 ± 3.1	7	22.8 ± 1.7

1. A year is divided into three seasons; Spring = March, April and May, Summer = June, July and August, and Winter = September, October, November and December.
2. n = sample size. The samples were collected in different days under either control or fumigated conditions.

protein content did not change in leaf tissue of NC-R (20.9 ± 3.8 mg protein g^{-1} fresh leaf weight) and increased only slightly in leaf tissue of NC-S (23.8 ± 4.8 mg protein g^{-1} fresh leaf weight) (Figure 10).

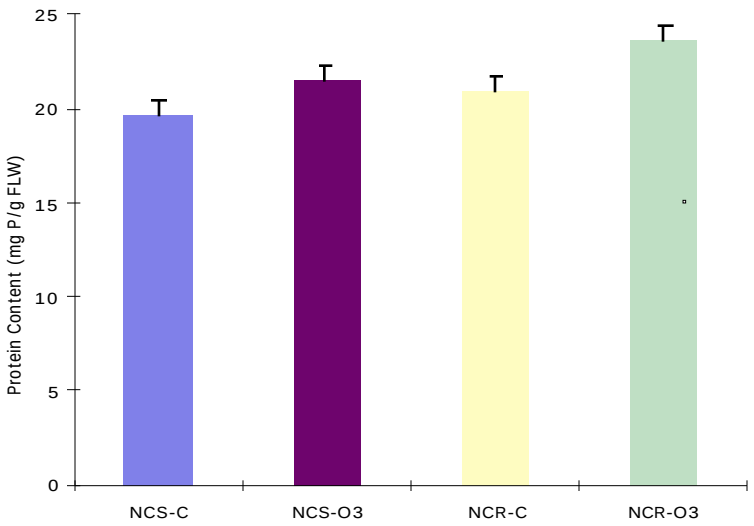


Figure 10. Soluble protein concentration in leaf tissue of control plants (NCR-C and NCS-C) and fumigated plants (NCR-O_3 and NCS-O_3). The plants were treated as described in section 3-A-2.

4-A-2 Isoforms of Superoxide Dismutase

Three isoforms of superoxide dismutase were present in both clover clones (Figure 11). Activity of the most anionic two isozymes was inhibited by potassium cyanide (KCN) (Figure 12) and hydrogen peroxide (H_2O_2) (Figure 13) indicating that these proteins contained the Cu/Zn ions. The least mobile isoform was distinguished as the putative mitochondrial Mn SOD because of its insensitivity to either KCN or H_2O_2 (Figure 12-A and 13-A). There was no Fe SOD in the extract of leaf tissue of white clover clones, because Fe SOD is sensitive to H_2O_2 and is insensitive to cyanide (Kanematus and Asada, 1990; Van Camp *et al.*, 1990). The absence of the most mobile band from root tissue indicating that this isozyme was the chloroplastic Cu/Zn SOD (data no show). The plastidic SOD was the predominate isozyme in the both

clover clones and comprised 41% and 47% of the total activity in NCR and NCS, respectively.

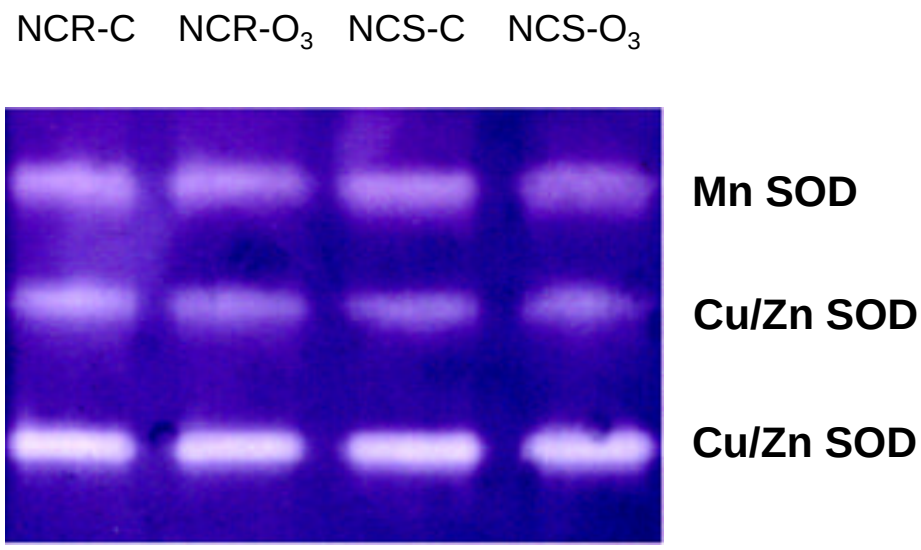


Figure 11. Enzyme activity gel indicates isoforms of SOD in tolerant (NR-R) and sensitive (NC-S) clover after a three-day fumigation (120 ppb, three hours, maximum O_3 concentration/day). Three isoforms are present: mitochondrial (Mn SOD), cytosolic (least mobile Cu/Zn SOD) and plastidic (most mobile Cu/Zn SOD). Ozone treatment does not causes an increase in enzyme activity in any SOD isoform. 20 μg protein was loaded on top of each lane. Gel stained with nitroblue tetrazolium.

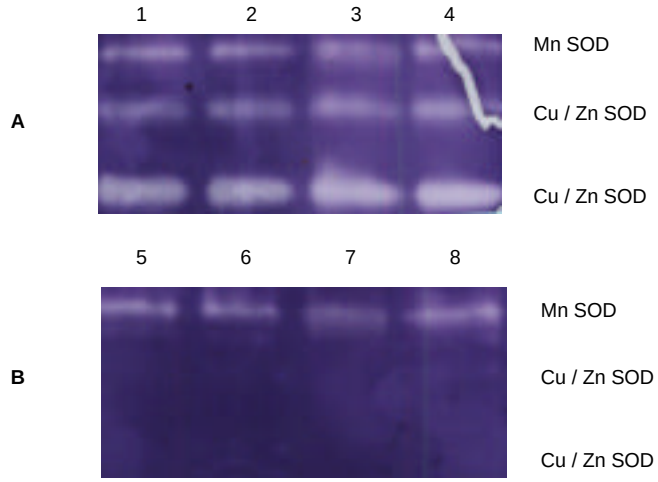


Figure 12. Isoforms of SOD were identified in native gels using KCN. Lanes 1, 2, 5, 6 were loaded with 10 µg protein and lanes 3, 4, 7, 8 were loaded with 20 µg protein. After electrophoresis, the gel was cut in half. One half was immersed in a NBT solution (A), and other half was immersed in a NBT solution containing 1 mM KCN (B). SOD activity of lower two isoforms were inhibited, indicating that these SODs contain Cu and Zn ions.

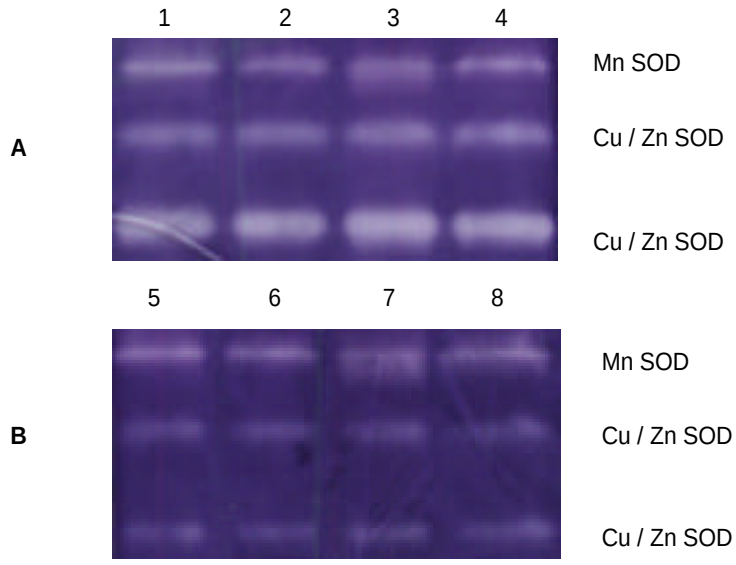


Figure 13. Isoforms of SOD were identified using H_2O_2. Lanes 1, 2, 5, 6 were loaded with 10 µg protein and lanes 3, 4, 7, 8 were loaded with 20 µg protein. After electrophoresis, the gel was cut in half, one half was immersed in a NBT solution (A), and other half was immersed in a NBT solution containing 2 mM H_2O_2 (B). SOD activity of bottom two isoform was inhibited. This indicates the SODs contain Cu and Zn ions. Gels stained with nitroblue tetrazolium.

4-A-3 Activity of Superoxide Dismutase Assay

In leaf tissue of both NC-S and NC-R control plants, the activity of Mn SOD was similar with a ratio of relative activity (NC-S/NC-R) of 93% (Table 9). However, both the cytosolic and chloroplastic SODs had higher activity in NC-R than in NC-S with a ratio of 82% and 89%, respectively. After plants were exposed to ozone, SOD activity in both clones did not increase (Table 9). In NC-R plants, the activity ratio of NCR-O_3/NCR-C was lowest (82%) for the chloroplastic SOD and similar for the other two isoforms (87% and 86%). In NC-S, the mitochondrial and chloroplastic SODs were unaffected by O_3 (relative activity ratio of 96 % and 97 %, respectively), whereas the cytosolic SOD activity decreased by 13% in treated tissues.

Table 9. Relative activity of superoxide dismutase (SOD) isoforms in foliar tissue of O_3-sensitive (NC-S) and O_3-Resistant (NC-R) clover clones after O_3 fumigation for three days.

	SOD isoforms		
Ratio of Relative Activity[1]	Mn SOD (mitochondrial)	Cu/Zn SOD (cytosolic)	Cu/Zn SOD (chloroplastic)
NCS-C/NCR-C	0.93	0.82	0.89
NCR-O3/NCR-C	0.87	0.86	0.82
NCS-O3/NCS-C	0.96	0.87	0.97

1. Ratios determined from a densitometer scan of a representative activity gel

4-A-4 Catalase Activity

The activity of catalase in non-fumigated leaf tissue of NC-R and NC-S plants was basically similar and ranged from a mean of 270 units mg^{-1} protein in NC-R to 285 units mg^{-1} protein in NC-S (1 unit = 1µmole H_2O_2 decomposed / minute). After O_3 treatment, catalase activity increased to about 330 units mg^{-1} protein in both clones, a 23% rise in NC-R and a 15% rise in NC-S (Table 10).

Table 10. Catalase activity in leaf of O_3-sensitive (NC-S) and O_3-resistant (NC-R) clover clones after O_3 fumigation for three days.

Treatment	n	Protein	Catalase Activity	Change in	Change in
		mg P/g F.W.	units / mg P	Protein	Catalase Activity
NCR-C	9	22.0 ± 1.4[1]	270 ± 40		
NCR-O_3	5	22.1 ± 0.9	333 ± 20	+ 0.3 %	+ 23 %
NCS-C	6	19.1 ± 0.5	285 ± 40		
NCS-O_3	8	20.9 ± 1.1	327 ± 10	+ 9.4 %	+ 15 %

1. Values represent the mean and one standard deviation

4-A-5 Investigation of Soluble Protein Populations

Two-dimensional gel electrophoresis of foliar soluble proteins revealed the accumulation of two, low molecular weight proteins in both clones after ozone treatment (Figure 14). These O_3-responsive proteins (ORPs) were not visible on silver-stained PAGE gels of both control leaf tissues extracts (Figure 15). The

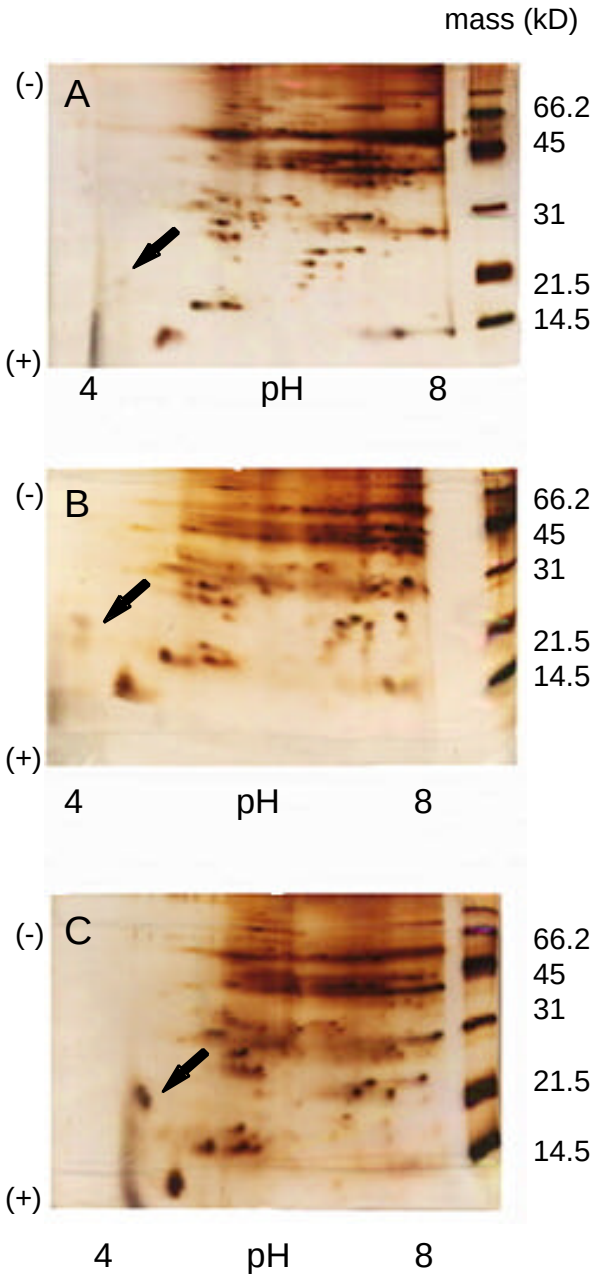


Figure 14. Two dimensional gel electrophoresis of soluble proteins in an O_3-tolerant clover clone during a three-day fumigation (one day for A, two days for B and three days for C, 120 ppb, three hours, maximum O_3 concentration/day). Two proteins (designated by the arrows) increase substantially in concentration over the treatment period. Gel stained with silver.

molecular weights of these proteins were 21.5 and 23 kD determined by the method described in 3-B-5. The ORPs have an apparent isoelectric point between pH 4.1 - 4.4. After ozone treatment for three days, the protein concentration in NC-R plants had increased about 20-fold compared to the initial day of treatment (Figure 16).

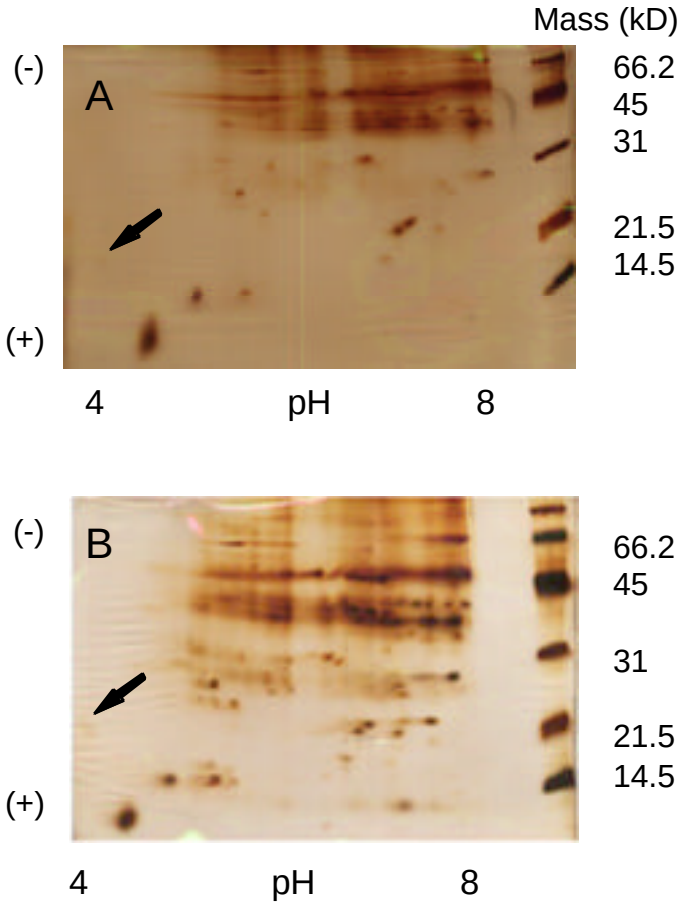


Figure 15. Two dimensional gel electrophoresis of soluble proteins in an O_3-sensitive clover clone (A) and an O_3-tolerant clover clone (B) under control condition for three-days. Two proteins (designated by the arrows) can't be seen over the treatment period. Gel stained with silver.

The accumulation of the ORPs was much lower in NC-S with the same treatment, that is only 25% of the protein concentration observed in NCR-O_3 or a five-fold increase

compared to the initial day of treatment (Figure 16). A greater separation of the ORPs

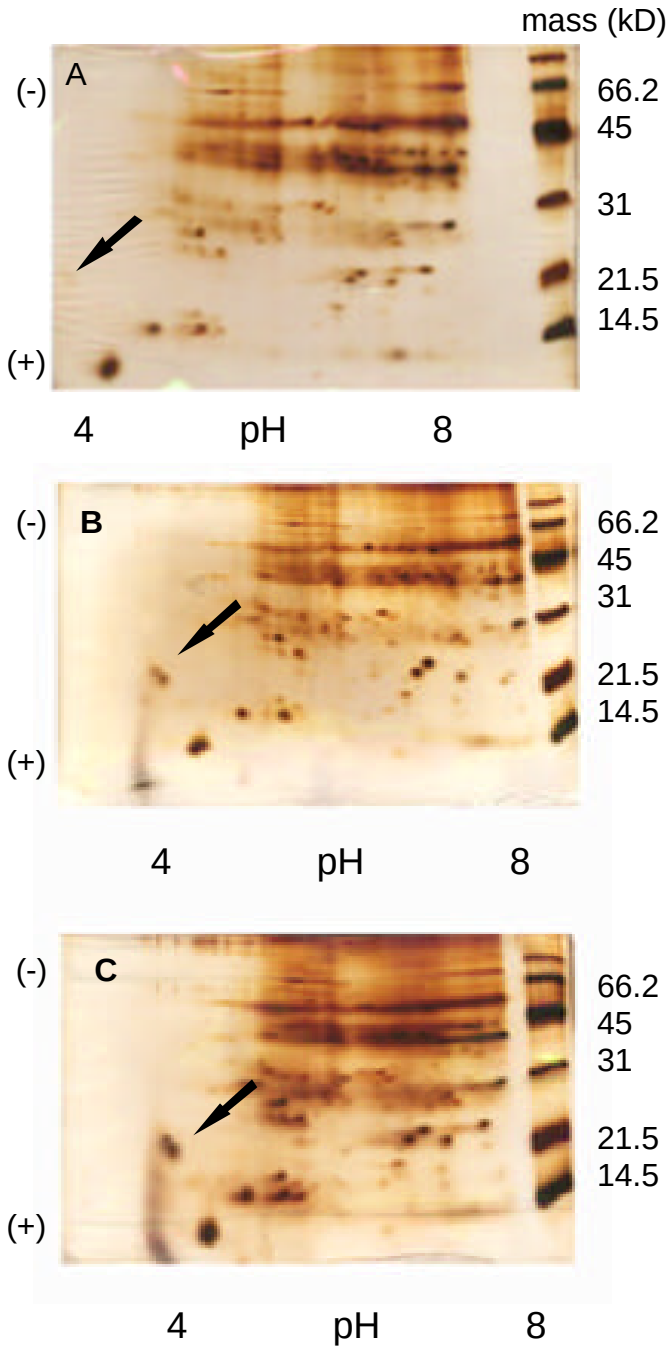


Figure 16. Two dimensional gel electrophoresis of soluble proteins in an O_3- tolerant clover clone (A) under control condition for three-days, an O_3-sensitive clover clone (B), and an O_3-tolerant clover clone (C) during a three-day fumigation (120 ppb, three hours, maximum O_3 concentration/day). Two proteins (designated by the arrows) increased 20-fold and 4-fold in NC-R leaf tissue treated with ozone, compared to control plant and NC-S plant over the treatment period, respectively,. Gel stained with silver.

in the IEF dimension was obtained by changing the pH range from 4.0-8.0 to 3.7-6.0 (Figure 17). This result demonstrated that there were at least four inducible proteins instead of two.

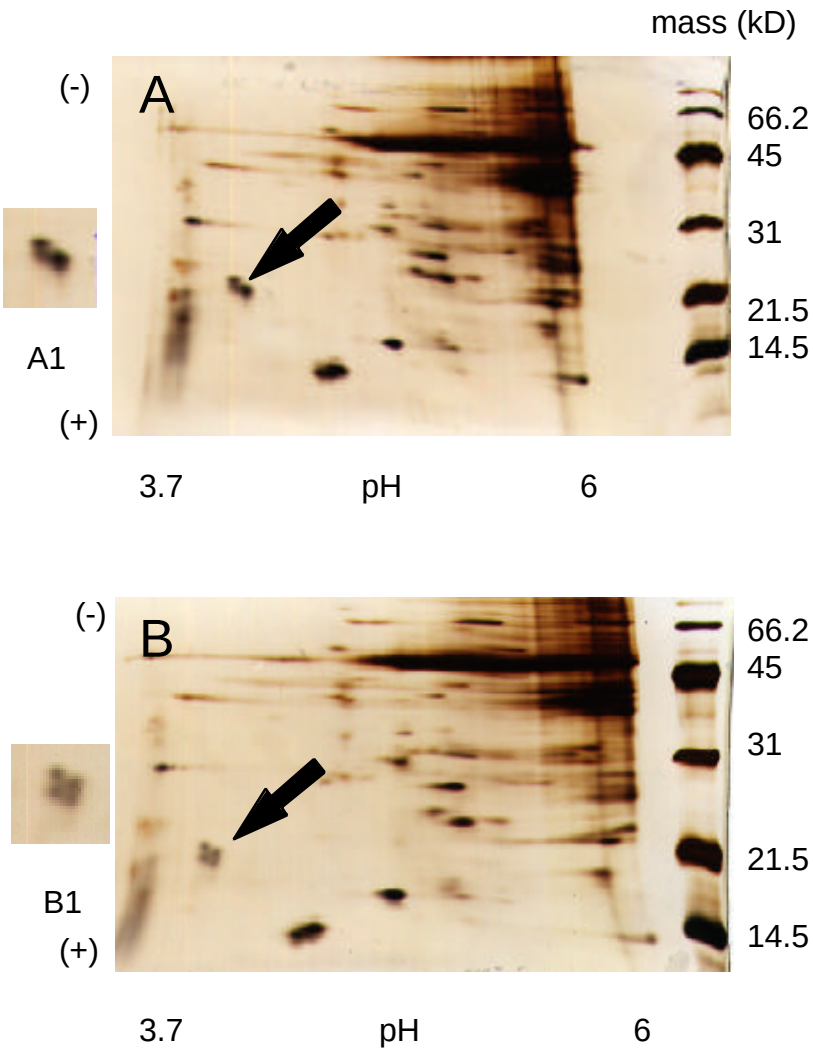


Figure 17. High resolution two-dimensional gel electrophoresis of soluble proteins in leaf tissue of fumigated O_3-tolerant clover. Isoelectric focusing is from pH 3.7 to 6.0. The inducible proteins are indicated by arrows. At a protein loading concentration of 20 µg gel^{-1}, four distinct polypeptides are resolved (insert B1). Gel stained with silver.

By using a centricon 100 concentrator to separate crude leaf extracts, the ORPs appeared in the filtrate (Figure 18-A) indicating their native molecular mass was less then 100 kD. The ORPs concentration was increased in the filtrate because Rubisco and other large, native proteins were retained by the filter (Figure 18-B).

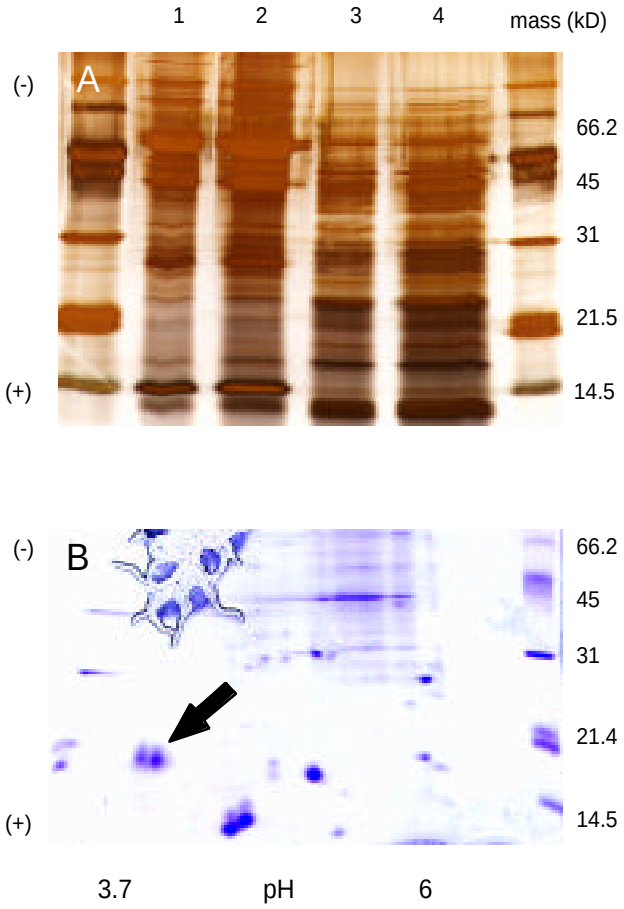


Figure 18. A. A single dimensional SDS gel (silver stain) of soluble proteins in fumigated NC-R clover leaves. Lane one (2.5 µg protein) and two (5 µg protein) indicate proteins retained by a Centricon 100 filter (native MW >100 kD), lanes three (2.5 µg protein) and four (5 µg protein) indicate proteins passing through a Centricon 100 filter (MW < 100 kD). B. A two dimensional gel (Commassie blue stain) indicating O_3-inducible proteins (arrow) in the Centricon 100 filtrate. Note the absence of the Rubisco LSU at 55 kD.

4-B Field samples

Soluble protein content in NC-R leaf tissue ranged from 24-26.5 mg g^{-1} fresh weight in leaf 1 through 4 and then declined to 21 mg g^{-1} fresh weight in leaf 5 (Figure 19). The ORPs were prominent in leaves 1 to 4, but decreased considerably in leaf 5. In NC-S, mean protein content in leaf one and two was about 22-25 mg g^{-1} fresh weight (Figure 20) and declined in leaf three through five to a concentration of about 16 mg g^{-1} fresh weight. The ORPs were barely apparent on two-dimensional gels in younger leaves, but became more visible in leaf positions four and five (Figure 20).

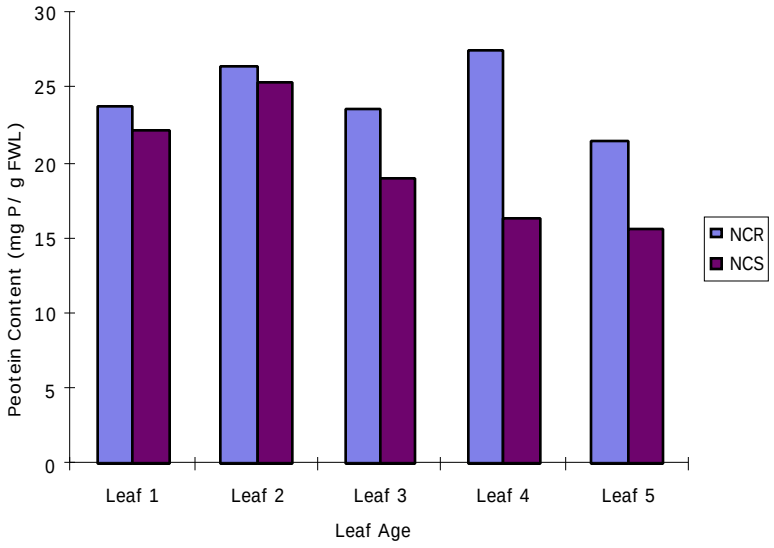


Figure 19. Soluble Protein content in NC-S and NC-R leaves exposed to ambient O_3 concentrations. Leaf positions are from the growing tip of a stolon (one is the youngest leaf and five is the oldest).

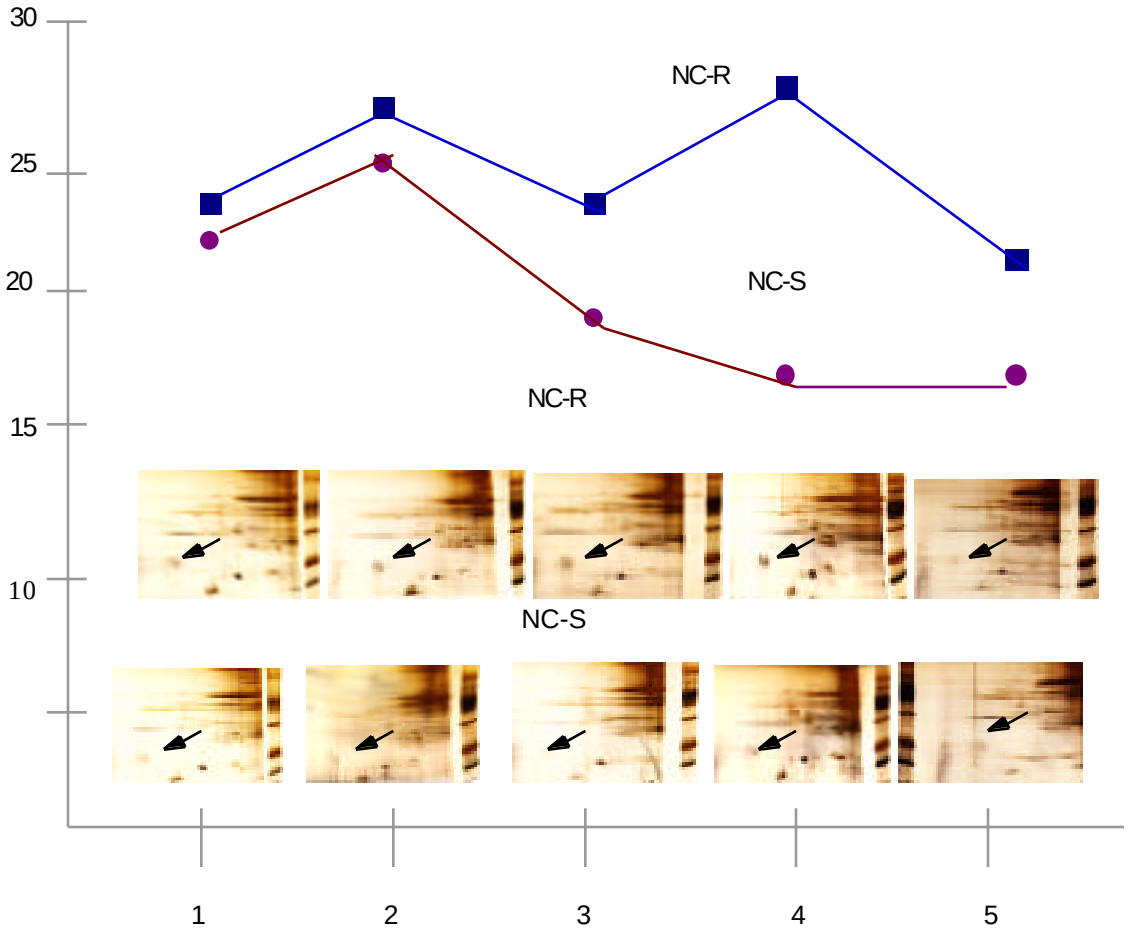


Figure 20. Soluble protein content in NC-S and NC-R leaves exposed to ambient O_3 concentrations [ordinate represents protein content (mg P/g FLW)]. Leaf positions are from the growing tip of a stolon (one is the youngest leaf). The upper graph represents soluble leaf protein which declines substantially in NC-S at leaf position three. The lower figures are 2-D gels of soluble protein content in NC-R and NC-S at each leaf position (arrows indicate O_3-inducible proteins). These proteins are barely apparent in NC-S and become more visible in leaf four, whereas they are prominent in NC-R in leaf one through four.

5. Discussion

The white clover system, consisting of ozone-resistant (NC-R) and ozone-sensitive (NC-S) clones, have useful characteristics for investigation mechanisms of ozone resistance because of their different response to ozone exposure based on biomass accumulation (Blum *et al.,* 1993; Rebbeck *et al.,* 1989 Heagle *et al.,* 1992 and Heagle *et al.,* 1994). In the two-year field study (1993-1994) at eight locations in the United States, the ratios of foliar biomass production of NC-S/NC-R clones were negatively corrected with ozone concentration under different ambient ozone concentration and environmental conditions (Figure 9)(Heagle *et al.,* 1995).

This system was used in my experiments for investigating resistant mechanisms to ozone in white clover clones because reports of the response of white clover clones to ozone at the biochemistry and molecular level are quite limited. Treatment of sensitive clover for three days at an ozone concentration of 120 ppb (three hours/day) did not result in any necrotic lesions, visible chlorophyll loss or other obvious foliar symptoms. The phenomenon of no apparent foliar injury demonstrated that the level of ozone stress applied to the clover plants was not enough to cause irreversible disruption in physiological function. At these exposure concentrations, cellular defense processes presumably could operate for repair of any reversible, oxidative lesions that may have occurred within the leaf cells or apoplast.

Stomatal conductance (regulation of pollutant flux to the leaf interior) is not involved in ozone resistance in the white clover clones. Under ambient CO_2 concentrations stomatal resistance in the NC-R leaf was lower than in the NC-S leaf after both clover clones were exposed to ozone for four weeks with a concentration from 50 - 82 ppb (Heagle, 1993). When plants were exposed to higher ozone levels for eight weeks, the stomatal resistance was almost identical.

Soluble protein content was not affected by ozone fumigated for three days and was around 22 mg protein g^{-1} fresh leaf weight (Figure 10). It has been estimated that nearly 70 % of soluble leaf protein is in the form of ribulose-1,5-bisphosphate carboxylase protein (Rubisco) (Miller and Huffeker, 1982). As mentioned above, ozone exposure has caused degradation of Rubisco protein and loss of Rubisco mRNA (Eckardt and Pell, 1994). Our results indicated that under the ozone concentrations used in my experiments, net protein degradation did not occur in either clover clones. The cellular metabolism would be normal and cell damage did not occur. Under these conditions, a cellular defense system against oxidative stress could function in the same manner as in non-fumigated clover clones.

In recent years, the antioxidant system has received much attention as a possible mechanism against active oxygen species that may be generated during pollutant exposure. This system consists of superoxide dismutase (SOD), catalase, and glutathione reductase (GR). The function of this system in defense of ozone exposure

has been tested in some plant species, such as *Arabidopsis*. Research has been directed toward plastidic forms of these enzymes, since ozone is known to cause Rubisco protein degradation and chlorophyll loss.

Superoxide dismutases have been identified as an essential component in an organism's defense mechanism (Bonler *et al.*, 1992). As described above (Figure 4), SOD converts superoxide to hydrogen peroxide. The induction of SOD activity by ozone has been reported in some species. *Arabidopsis thaliana* was exposed for six hours/day, eight days with an ozone concentration 200 ppb. The activity of SOD was increased (Rao, *et al.*, 1996). Other report on *Arabidopsis thaliana* response to O_3 fumigation comes from Kubo *et al.* (1995), who fumigated plants was for 14 hours/day seven days with ozone at a concentration from 100 - 150 ppb. Ozone treatment had increased on the activity of SOD. The genetic enhancement of chloroplastic SOD activity provided protection against ozone injury in tobacco (Van Camp *et al.*, 1994). Overexpression of cytosolic SOD also afforded moderate protection against foliar ozone injury in tobacco (Pitcher and Zilinskas, 1996).

Gene expression of SOD isoforms and other antioxidant enzymes in response to ozone has been investigated in *Nicotiania* species and *Arabidopsis thaliana*. The mRNA of the cytosolic antioxidant proteins, glutathione S-transferase, Cu/Zn SOD and APx, increased substantially after ozone exposure in wild-type *Arabidopsis*, however, mRNAs of chloroplastic SOD and GR, as well as catalase, decreased

(Conklin and Last, 1995; Sharma and Davis, 1994). In *Nicotiania plumbaginifolia*, transcript production of two catalase genes and glutathione peroxidase increase or were not affected. In *Nicotiania tabacum* cv. PBD6, an ozone sensitive variety, cytosolic SOD and APx mRNA increased only after visible ozone injury was apparent (Willekens, *et al.,* 1994).

In my experiment, after three days of ozone fumigation, the activity of SOD did not increase in the two white clover clones (Figure 11 and Table 9). There was more SOD activity in the control resistant clone than in the control sensitive clone before ozone treatment. The SOD activities decreased after ozone exposure within a range of 13%-18% among the three SOD isoforms in the resistant clone, indicating that ozone fumigation did not induce activity of this enzyme. A 13% decrease of SOD activity in the cytosol after ozone fumigation and similar SOD activities in the mitochondria and chloroplast in the sensitive clone before and after ozone treatment demonstrated that ozone did not alter enzyme activity, except in the cytosol. Hydrogen peroxide (H_2O_2) is a product of the SOD catalyzed reaction. Hydrogen peroxide can be decomposed to H_2O and O_2 in a reaction that is catalyzed by catalase (Figure 4). Ozone fumigation caused a slight increase in catalase activity of 23 % in the resistant clone, and 15% in sensitive clone (Table 10). This result suggests that ozone may contributes to H_2O_2 production in the leaf interior. The mechanism of ozone resistance in the white clover clones could be different from that in *Arabidopsis* plants.

O_3-responsive proteins (ORPs) may play a role in resistance to ozone in white clover. The results of greenhouse studies demonstrated that the ORPs content is ozone-concentration dependent. The result of two-dimensional gel electrophoresis of the filtrate from 100 concentrator showed that the ORPs were present in the filtrate (Figure 18), indicating a native molecular weight of less then 100 kD. These polypeptides appear not to belong to the small heat-shock protein family whose native molecular weights range from 240 to 400+ kD (Jinn *et al.*, 1995; Helm *et al.*, 1997). The study of the field samples clearly showed a relationship between soluble protein content and O_3-responsive protein content. Mean soluble protein concentration in resistant clone remained high in leaf 1 through 4 and then declined in leaf 5 as early senescence became evident (Figure 20). The ORPs were prominent in leaves 1 to 4, but decreased considerably in leaf 5. In the sensitive clone, mean soluble protein content was high in leaves 1 and 2, but declined substantially in leaves 4 and (Figure 20). The ORPs were barely apparent on two-dimensional gels in the younger leaves, but became more visible in older leaves in position 4 and 5. These results demonstrated that, at ambient ozone concentrations, maintenance of high soluble leaf protein (mainly Rubisco) in the resistant clone was correlated with the presence of the inducible polypeptides, whereas a loss of leaf protein in sensitive clone was correlated with very low concentrations of the ORPs.

6. Summary

Based on previous studies and on the results from my experiments, the following conclusions can be made concerning the response of white clover clones to ozone..

1. Two clover clones (NC-R and NC-S) have useful characteristics for investigating mechanisms of ozone resistance because of different responses to ozone based on biomass accumulation.
2. The soluble protein content in leaf tissue of both resistant and sensitive clones did not change after ozone exposure.
3. The activities of three isoforms of superoxide dismutase did not increase in leaf tissue of the resistant clone after ozone treatment, indicating that enhanced SOD activity is not associated with resistance in the white clover system.
4. Catalase activity increased slight in resistant (22%) and sensitive clones (15%) after fumigation with ozone, suggesting a minimal effect of ozone on H_2O_2 production within the leaf tissue.
5. The ozone-responsive protein (ORP) concentration increased five-fold in leaf tissue of the sensitive clone, and twenty-fold in leaf tissue of the resistant clone after a three-days ozone fumigation, compared with control plants. This differential pattern of accumulation suggests that the ORPs function in ozone resistance. The ORPs have native molecular weight of less 100 kD, and subunit molecular weights of 21-23.5 kD.

Isoelectric points are from pH 4.1 to 4.4.

6. In field studies, the ORP concentration was positively correlated with the retention of soluble leaf protein in the two clones again indicting a possible function in ozone resistance by delaying the onset of senescence.

Reference

Adepipe, N. O. and Ormrod, D. P. (1972) Hormononal control of ozone phytotoxity in *Raphaus sativus.* ***Zeitschrift für Pflanzenphysiologie*** **68**:254-258

Alscher, R. G. and Hess J. L. (1993).Antioxidants in higher plants. Publisher: Boca Raton CRC Press

Beauchamp, C. and Fridovich, I. (1971) Superoxide dismutase: Improved assays and assay application to acrylamide gels. **Analytical Biochemistry 44:**276-287

Beer, R.F., Jr. And Sizer, I.W. (1952) A specitrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. **Journal Biological Chemistry 195**:133-140

Blum, U., Heagle, A.S., Burns, J.C. and Linthurest, R.A. (1983) The effects of O_3 on fescue-clover forage: regrowth, yield and quality. **Environ. Expt. Bot. 23:**123-132

Bowen, J.E. (1972) Suger transport in immature internodal tissue of sugarcane. I. Mechanism and kinetics of accumulation. **Plant Physiology 49**:82-86

Bowler, C., van Montagu M. and Inzé D. (1992) Superoxide dismutase and stress tolerance. **Annual Review of Plant Physiology and Plant Molecular Biology 43**:83-116

Bradford, M. (1976) A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of protein-dye binding. **Anal. Biochem.72**:248-254

Bridges S.M. and Salin M.L. (1981) Distribution of iron-containing superoxide dismutase in vascular plants. **Plant Physiology 68(2):**275-278

Castillo F. J. and Heath, R.L. (1990) Ca^{2+} transport in membrane vesicies from Pinto bean leaves and its alteration after ozone exposure. **Plant Physiology 94**:788-795

Castillo F. J., Miller P.R. & Greppin H. (1987) Extracellular biochemical markers of photochemical oxidant air pollution damage to Norway spruce. **Experimentia 43:**111-220

Chanway C.P. and Runeckles V.C (1984) The role of superoxide dismutase in the susceptibility of bean leaves to ozone injury. **Canadian Journal of Botany 62:**236-240

Chappelka, A. H. and Chevone B. I. (1992) Crop responses to ozone. In **surface level**

ozone exposures and their effects on vegetation, edited by Lefohn A. S., Lewis publishers. pp 271- 324

Conklin, P and Last, R. (1995) Differential accumulation of antioxidant mRNA in Arabidopsis thaliana exposed to ozone. **Plant Physiology 109:**203-212

Dominy, P.J. and Heath, R.L. (1985) Inhibition of the K^+-stimulated ATPase of the plasmalemma of Pinto bean leaves by ozone. **Plant Physiology 77:**43-45

Eckardt, N. A. and Pell, E. J. (1994) O_3 - induced degradation on Rubisco protein and loss of Rubisco mRNA inrelation to leaf age in potato. **New phytologist 127**:741-748

Eckey-Kaltenbach, H. Ernst, D. Heller, W. and Sandermann, H. Jr. (1994) Biochemical plant responses to ozone. IV. Cross-induction of defensive pathways in parsley (Petroselinum crispum L.) Plants. **Plant Physiology 104 (1):**67-74.

Ernst, D., Schraudner, M., Langebartels, C. and H. Sandermann, Jr. (1992) Ozone-induced changes of mRNA levels of β-1,3-glucanase, chitinase and pathogenesis-related protein 1b in tobacco plants. **Plant Molecular Biology 20**:673-682

Evans, L.S. and Ting, I.P. (1974) Effect of ozone on 86Rb-labeled potassium transport in leaves of *Phaseolus Vulgaris* L. **Atmospheric Environment 8** 855-861

Foyer C.H., Descourvières P. & Funert K. (1994) Protection against oxygen radicals: an important defense mechanism studied in transgenic plants. **Plant, Cell and Enviroment 17:** 507-523

Fuhere, J., Kaur-Sawhney, R., Shih, L. M. and Galston, A. W. (1982) Effects of xegenous 1,3-diaminopropane and spermidine on senescence of Oat leaves. **Plant Physiology 70:**1597-1600

Gupta, A. S., Alscher, R. G. and McCune, D. (1990) Ozone exposure, glutathione levels and photosynthesis in hybrid poplar. In **sulfur nutrition and sulfur assimilation in higher plants**. pp 195-197

Gupta, A. S., Alscher, R. G. and McCune, D. (1991) Response of photosynthesis and cellular antioxidants to ozone in *Populus* leaves. **Plant Physiology 96**:650-655

Harwood, J.L. (1980) Plant acyl lipids: structure, distribution and analysis. In **the Biochemistry of Plants**. Vol. 4. Lipids: Structure and Function. Edited by Stumpf, P.K. pp 1-55 Academic Press, New York.

Heagle, A. S. (1989) Ozone and crop yield. **Annual Review of Phytopathology 27:**397-423

Heagle A.S., McLaughlin, M.R., Miller, J.R. and Joyner, R.L (1992) Response of two white clover clones to peanut stunt virus and ozone. **Phytopathology 82:**248-258

Heagle A. S., Miller, J. E., Chevone B. I., Dreschel, T. W., Manning, W. J., McCool, P. M., Morrison, C. L., Neely, G. E. and Rebbeck, J. (1995) Response of a white clover inducators system to tropospheric ozone at eight locations in the Unite States. **Water, Air and Soil Pollution 85:**1373-1378

Heagle, A. S., Miller, J. E. and Sherrill, D. E. (1994) Atmospheric pollutants and Trace gases. A white clover system to estimate effects of tropospheric ozone on plants. **Journal Environmental Quality 23:**613-621

Heagle, A. S., Miller, J. E., Sherrill, D. E. and Rawlings (1993) Effects of ozone and carbon dioxide mixtures on two clones of white clover. **New Phytologist 123:**751-762

Heagle, A. S., Richard, A., Reinert, A. and Miller, J. E. (1996) Response of white clover to ozone in different environments. **Journal Environmental Quality 25**:273-278

Heath, R. L. (1975) Ozone. In **Responses of Plants to Air Pollution**. Edited by Mudd, J. B. and Kozlowski T. T. 23-51

Heath, R. L. (1980) Initial events in injury to plants by air pollutants. **Annual Review of Plant Physiology. 31**:395-431

Heath, R. L. (1987) The biochemistry of ozone attack on the plasma membrane of the plant cells. In **Recent Advances in Phytochemistry**. Phytochemical Effects of Environmental Compounds, 21 (eds J.A. Saunders, L. Kosak-Channing & E. E. Conn). pp 29-54. Plenum Press. New York. N.Y.

Heck, W.W. (1989) Assessment of crop losses from air pollutants in the United States. In **Air Pollution's toll on Forests and Crops**. Edited by MacKenzie, J.J. and El-Ashry, M.T. p 235-351. Yale University Press

Heck, W. W., Heagle, A. S. and Shriner, D. S. (1986) Effects on vegetation: native, crops, forests. p. 247-350. In Stern, A. S. (ed.) **Air Pollution**. Vol. 6 Academic Press, New York.

Heck, W. W., Philbeck, R.B. and Dunning J.A. (1978) A continuous stirred tank reactor system (CSTR) for exposing plants to gaseous air contaminants. Principles, specifications, construction and operation. **Agric.Res.Ser.,** USDA,ARS-S-181

Hellgren, L.I, Carlsson, A.S., Selldèn, G. and Sandelins, A.S. (1995) *In situ* leaf lipid metabolism in garden pea (*Pisum Sativum* L.) exposed to moderately enhanced levels

of ozone. **Journal of Experimental Botany:46(283):**221-230

Helm, K.W., Lee, G.J. and Vierling, E. (1997) Expression of native structure of cytosolic class II small heat-shock proteins. **Plant Physiology 114**:1477-1485

Jinn, T.L., Chen, Y.M. and Lin, C.Y. (1995) Characterization and physiological function of class I low-molecular-mass, heat-shock protein complex in soybean. **Plant Physiology 108:** 693-701

Kanematsu, S. and Asada, K. (1990) Characteristic amino acid sequences of chloroplast and cytosol isozymes of Cu/Zn superoxide dismutase in spinach, rice and horsetail. **Plant Cell Physiology 31**:99-112

Kangasjärvi, J., Talvinen, J., Utriainen, M. and Karjalainen (1994) Plant defense system induced by ozone. **Plant Cell and Environment 17:**783-794

Kende, H. (1993) Ethylene biosynthesis. **Annual Review of Plant Physiology and Plant Molecular Biology 42**:529-551

Kubo, A., Saji, H., Tanaka, K. and Kondo N. (1995) Expression of *Arabidopsis* cytosolic ascorbate peroxidase gene in response to ozone or sulfur dioxide. **Plant Molecular Biology 29**:479-489

Laisk, A., Kull, O. and Moldau, H. (1989) Ozone concentration in leaf intercellular air spaces is close to zero. **Plant physiology 90**:1163-1167

Langebartels, C., Kristina, K. Leonardi S., Schraudner, M., Trost M., Heller, W. and Sandermann, H. Jr. (1991) Biochemical plant responses to ozone. I. Differential induction of polyamine biosynthesis in tobacco. **Plant physiology 95**:882-889

McLaughlin, S. B. and Taylor, G. E. (1981) Relative humidity: Important modifier of pollutant uptake by plants. **Science 211**:167-169

Mehlhorn, H. (1990) Ethylene-promoted ascorbate peroxidase activity protects plants against hydrogen peroxide, ozone and paraquat. **Plant, Cell and Environment 13**:971-976

Mehlhorn, H. and Wellburn A. R. (1987) Stress ethylene formation determines plant sensitivity to ozone. **Nature 327**:417-418

Mehlhorn, H., Tabner, B. and Wellburn, A. R. (1990) Electron spin resonance evidence for the formation of free radicals in plants exposed to ozone. **Physiologia Plantarum 79**:377-383

Mudd, J. B., McManus, T. T. and Ongun, A. (1971) Inhibition of lipid metabolism in chloroplasts by ozone. Proc., Int. Clean air Congr., 2nd, 1970. Academic Press, New York. pp 256-260

Naik, B. I., and Srivastava, S. K.(1978) Effect of polyamines on tissue permeability (beet root cells). **Phytochemistry 17 (11):**1885-1887

Neal, M. W. and Florini, J. R. (1973) A rapid method for desalting small volumes of solution. **Analytical Biochemistry 55:**328-330

O'Farrell, P.H. (1975) High resolution two-dimensional electrophoresis of proteins. **Journal Biological Chemistry 250:**4007-4021

Perchorowicz, J.T. and Ting, I.P. (1974) Ozone effects on plant cell permeability. **American Journal of Botany 61(7)** 787-793

Pitcher, L. H., Brennan, E., Hurley, A., Dunsmuir, P., Tepperman J. M. and Zilinskas B. A. (1991) Overproduction of petunia chloroplastic copper/zinc superoxide dismutase does not confer ozone tolerance in transgenic Tobacco. **Plant Physiology 97:**452-455

Pitcher, L. H. and Zilinskas (1996) Overexpression of copper/zinc superoxide dismutase in cytosol of transgenic tobacco confers partial resistance to ozone-induced foliar necrosis. **Plant Physiology 110:**583-588

Popovic, R. B., Kyle, D. J., Cohen, A. S. and Zalik, S. (1979) Stabilization of thylakoid membranes by spermine during stress-induced senescence of Barley leaf discs. **Plant Physiology 64:**721-726

Rao, M. V., Paliyath, G. and Ormrod D. P. (1996) Ultraviolet and ozone-induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*. **Plant Physiology 110:**125-136

Rebbeck, J., Blum, U. and Heagle A.S. (1989) Effects of ozone on the regrowth and energy reserves of a ladino clover-tall fescue pasture. **J. App.Ecol. 25:**658-681

Rich, W., Wagoner P. E., and Tomlinson, H. (1970) Ozone uptake by bean leaves. **Science 169**:79-80

Runeckles, V. C. (1992) Uptake of ozone by vegetation. In **surface level ozone exposures and their effects on vegetation**, edited by Lefohn A. S., Lewis publishers. pp 157- 188

Runeckles, V. C. and Chevone B. I. (1992) Tree responses to ozone. In **surface level**

ozone exposures and their effects on vegetation, edited by Lefohn A. S., Lewis publishers. pp 189 - 270

Sah, D. N., von Tiedemann A. and Fehrmann H. (1993) Effect of ozone exposure on resistance of wheat genotypes to *Pyrenophora tritici-reppentis*. **Journal of Phytopathology 138:**249-256

Sakaki, T., Ohnishi, J.I., Kondo, N. and Yamada, M. (1985) Polar and neutral lipid changes in spinach leaves with ozone fumigation: triacylglycerol synthesis from polar lipids. **Plant Cell Physiology 26(2):**253-262

Scandalios, J.C. (1990) Response of plant antioxidant defense genes to environmental stress. **Adv. Gen. 28**:1-40

Schlagnhaufer, C. D., Arteca, R. N. and Pell, E. J. (1993) Ozone stress induces the expression of ACC synthase in potato plants. **Plant physiology 102(S):**131

Schraudner, M., Ernst, D., Langebartels, C. and H. Sandermann, Jr. (1992) Biochemical Plant responses to ozone. III. Activation of the defense-related proteins _-1,3-glucanase and chitinase in Tobacco leaves. **Plant physiology 99:**1321-1328

Schraudner, M., Langebartels, C. and H. Sandermann, Jr. (1996) Plant defense systems and ozone. **Biochemical Society Transactions 24(2):**456-461

Sharma, Y. K. and Davis K. R. (1994) Ozone-induced expression of stress-related genes in *Arabidopsis thaliana*. **Plant physiology 105:**1089-1096

Steinmüller, D. and Tevini, M. (1985) Composition and function of plastoglobuli. I. Isolation and purification from chloroplasts and chromoplasts. **Planta 163**:201-207

Sutton, R. and Ting, I.P. (1977) Evidence for repair of ozone induced membrane injury: alteration in sugar uptake. **Atmospheric Environment 11**:273-275

Tomlinson, H. and Rich, S. (1968) The ozone resistance of leaves as related to their sulfhydryl and ATP content. **Phytopathology 58:**808-810.

Weber K. and Osborn (1975) in **The Proteins**, Vol. 1 3rd ed. Academic Press, p 179

Yang, S. F. and Hoffman, N. E. (1984) Ethylene biosynthesis and its regulation in higher plants. **Annual review of plant physiology 35**:155-189.

Van Camp. W., Bowler, C., Villarroel, R., Tsang, E.W.T., Van Montagu, M. and Inzé, D. (1990) Characterization of iron superoxide dismutase cDNA from plants obtained by genetic complementation in *Escherichia coli*. **Proc. Natl. Acad. Sci. USA 78:**9903-

9907

Van-Camp, W., Willekens, H., Bowler, C., Montagu, M. V., Inzé, D., Reupold-Popp, P., H. Sandermann Jr and Langbartels, C. (1994) Elevated levels of superoxide dismutase protect transgenic plants against ozone damage. **Bio/Technology 12**:165-168

Willekens, H., Van Camp, W., Montagu M.Van, Inze, D., Langebartels C. and Sandermanm (1994) Ozone, sulfur dioxide and ultraviolet B have similar effects on mRNA accumulation of antioxidant genes in *Nicotiana plumbaginifolia* L. **Plant Physiology 106:** 1007-1014

VITA

Youlin Tang was born on October 25th, 1956 in Zhenzhou, China. He had been a worker in the Chengdu Cable Factory for six years. He received a bachelor degree in major of Biology at Chongqing Teacher's College in July, 1984 in China. After graduation from the college, he worked in the Education Committee of Chengdu and Chongqing University. He came the Virginia Tech as a visiting scholar in 1993 and worked in Dr. John L. Hess's lab for two and half years. He has been a candidate for the M.S degree in plant physiology in the Department of Plant Pathology, Physiology and Weed Science of Virginia Polytechnic Institute and State University since spring semester, 1996. His research focuses on effects of air pollutants on plants. His paper, Antioxdant Activity and Photosynthesis in Two White Clover Genotypes with Different Ozone Sensitivities, was presented at the Air & Waste Management Association's 90th Annual Meeting & Exhibition on June 11th, 1996 at Toronto, Canada. The other presentation, Response of Leaf Protein to Ozone in Two White Clover Clones, was presented at the meeting of Southern Section American Society of Plant Physiologists on March 22nd, 1998 at Roanoke, Virginia. The paper, Ozone-Responsive Proteins in a Tolerant and Sensitive Clone of White Clover (*Trifolium repens* L.) has been submitted for publication.