THE ANTIOXIDANT RESPONSE OF TULIP POPLAR (*LIRIODENDRON TULIPIFERA* L.) LEAVES TO SHORT TERM EXPOSURES OF ACUTE AND CHRONIC OZONE

A Thesis
By
Ann Gretchen Huyler

Submitted to the Graduate School
Appalachian State University
In partial fulfillment of the requirements of the degree of
Master of Sciences

August 2008
Department of Biology

THE ANTIOXIDANT RESPONSE OF TULIP POPLAR (*LIRIODENDRON TULIPIFERA* L.) LEAVES TO SHORT TERM EXPOSURES OF ACUTE AND CHRONIC OZONE

A Thesis

by

Ann Gretchen Huyler

August 2008

APPROVED BY:

Howard S. Neufeld
Howard S. Neufeld
Chairperson, Thesis Committee

Kent O. Burkey
Kent O. Burkey
Member, Thesis Committee

Annkatrin Rose
Annkatrin Rose
Member, Thesis Committee

Steven W. Seagle
Steven W. Seagle
Chairperson, Department of Biology

E D Huntley
Edelma D. Huntley
Dean, Research and Graduate Studies

ABSTRACT

THE ANTIOXIDANT RESPONSE OF TULIP POPLAR (*LIRIODENDRON TULIPIFERA* L.) LEAVES TO SHORT TERM EXPOSURES OF ACUTE AND CHRONIC OZONE

(August 2008)

Ann Gretchen Huyler, B.A., University of North Carolina Asheville

M.S., Appalachian State University

Thesis Chairperson: Howard S. Neufeld

The antioxidant ascorbic acid (AA) is a first line of defense against ozone (O_3) and O_3 generated reactive oxygen species (ROS) and plants with elevated levels of AA often exhibit greater resistance to O_3 damage. Glutathione (GSH) is a well known antioxidant that is the principle reducer of dehydroascorbic acid, or, oxidized AA. Guaiacol peroxidase (GPX) is an antioxidant enzyme whose levels are reported to increase when a plant is under oxidative stress. Few studies have investigated the presence of an immediate antioxidant response after a day or a few days of O_3 exposure, with most studies concentrating on reporting responses after a season of exposure. Likewise, measurements of the response of stomatal conductance and photosynthetic efficiency to O_3 exposure typically focus on longer timelines. This study investigated the immediate physiological and antioxidant response of AA, GSH, and GPX to short term exposures of acute and chronic O_3 on tulip poplar (*Liriodendron tulipifera*) leaves. The hourly mean acute O_3 concentration was 192 ± 2 ppb for 12 hours for one day and the

SUM00 exposure was 2347 ± 25 ppb*hrs. The hourly mean chronic O_3 exposure was 65 ± 0 ppb for 12 hours for three days and the SUM00 exposure was 2342 ± 28 ppb*hrs. The hourly mean control O_3 concentration was 14 ± 2 ppb for 12 hours per day with the SUM00 exposure being 497 ± 78 ppb*hrs. The antioxidant levels, stomatal conductance and chlorophyll fluorescence were measured a day before and during O_3 treatments, and for two days after O_3 exposure ended. In addition, leaves were stained with nitroblue tetrazolium and 3,3'-diaminobenzadine to discern the presence of superoxide anion and hydrogen peroxide, two predominant O_3-induced ROS responsible for producing stipple, the visible oxidant damage on the leaves. Neither acute nor chronic O_3 treatment elicited a response in antioxidant levels or redox values, both of which were comparable to control values. Stomatal conductance and chlorophyll fluorescence were unresponsive to acute or chronic O_3 treatment and exhibited typical daily fluctuations similar to control values. Staining also did not reveal the presence of ROS. This study suggests that tulip poplar saplings are relatively insensitive to short-term O_3 episodes even when the hourly concentrations are high, and that multiple episodes appear to be necessary to induce visible and adverse physiological and biochemical effects.

ACKNOWLEDGMENTS

I thank the Office of Student Research, the Graduate Student Association at Appalachian State University, and the USDA for project funding and support. In addition, nothing would have been possible without the enthusiasm, expertise, and kindness of Dr. Howard S. Neufeld who was the wonderful center of my universe for my Masters degree and project at Appalachian State University. Nor would this degree have been possible without my dearest aunt, Ms. Laverne Lorenz, whose unmitigated humor and perseverance against debilitating rheumatoid arthritis and cancer set the bar way high for my own endeavors. Dr. Kent O. Burkey and his senior lab manager, Ms. Renee Tucker, were astonishingly generous with their patience, skill, and knowledge throughout the antioxidant analysis, especially Ms. Tucker. For the writing and editing of the thesis, I am especially indebted to Dr. Burkey. My committee would have been incomplete without Dr. Annkatrin Rose who took up the mantle from a departing professor and provided invaluable thesis critiques. Major support came from two friends and masters students, Ms. Kristen Bright and Ms. Cori-Alice Holladay, who assisted in the experiments and endowed me with wonderful company over gossip sessions, BBQ excursions, fried food dinners, and old-time music outings. My work and friendship with Ms. Elizabeth Harris permitted both an income and a comrade-in-arms wielding laughter and an additional tent pole to my occasional circus-like existence.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

In the 1950s, researchers discovered that ozone (O_3) was causing widespread damage to crops from North Carolina to Ontario and was the principal human irritant in the smog that inundated Los Angeles in the 1940s (Treshow and Bell 2002). Due to its ability to generate reactive oxygen species (ROS) and its strong oxidizing capacity, slightly beneath fluorine (Long and Naidu 2002), O_3 is considered the most phytotoxic of the major air pollutants (Krupa et al. 2000).

Reactive oxygen species, also called free radicals, have unpaired electrons in the outer orbitals, giving them the ability to react with and transform non-radical molecules into radicals (Edreva 2005). Reactive oxygen species are initially generated when O_3 reacts with apoplastic solutes in the cell wall (Long and Naidu 2002, D'Haese et al. 2005). These reactions create hydrogen peroxide (H_2O_2), superoxide anion ($\cdot O_2^-$), hydroperoxide ($\cdot O_2H$) and the extremely toxic hydroxyl radical ($\cdot OH^-$) (Long and Naidu 2002). Due to the highly reactive nature of O_3, the concentration of O_3 in the sub-stomatal cavity is demonstrably zero (Laisk et al. 1989, Wang et al. 1995) and O_3's main destructive potential arises from the highly oxidative nature of these ROS (Ranieri et al. 1996, Kangasjärvi et al. 2005).

O_3 directly or indirectly impacts physiological processes by impairing stomatal conductance (g_s) (Grulke et al. 2007), reducing Rubisco content and photosynthetic activity (Pell et al. 1997), peroxidizing lipids, oxidizing sulfhydryl protein groups, accelerating

senescence of tissues, altering signaling pathways, and disrupting the regulation of ion flow across membranes (Chernikova et al. 2000). A particular ability of O_3 is to incorporate itself into carbon-carbon double and triple bonds and break them through the process of ozonolysis (Long and Naidu 2002).

The oxidizing capacity of ROS makes them vitally important as signaling molecules and as defenders against pathogen attack (Langebartels et al. 2002). Reactive oxygen species, especially $\cdot O_2^-$ and H_2O_2, are routinely generated in large amounts by electron transport chains in photosynthesis and respiration and by other physiological processes whose functionality depends upon atmospheric oxygen accepting liberated electrons (Smirnoff 2000, Edreva 2005). Finely tuned antioxidant and scavenging mechanisms manage ROS at low concentrations and prevent their toxic build up (Foyer and Noctor 2005). High concentrations of ROS supply powerful oxidative signals that induce programmed cell death (PCD), a senescence process involved in the hypersensitive response (HR) (Wohlgemuth et al. 2002, Overmyer et al. 2003).

The HR is a defensive system triggered by pathogen attack and inadvertently by prolonged exposure to O_3 (Schraudner et al. 1998, Langebartels et al. 2002). When pathogens, or O_3, infiltrate a leaf, the HR stimulates cells to flood the apoplast with ROS, primarily $\cdot O_2^-$ and H_2O_2, in an 'oxidative burst' designed for direct antimicrobial action and stimulus of PCD in order to surround the attack site with dead cells and inhibit further pathogen ingress (Langebartels et al. 2002). The rise in ROS concentrations also initiates the development of systemic acquired resistance (SAR) which produces increased pathogen resistance and O_3 tolerance in non-infiltrated tissue (Lee and Hwang 2005).

Both H_2O_2 and $\cdot O_2^-$ are consistently found at sites before the HR initiated cell death takes place (Wohlgemuth et al. 2002) and elevated concentrations of H_2O_2 and $\cdot O_2^-$ elicit lesion formation in tobacco (Ranieri et al. 2003) and *Arabidopsis*, respectively (Jabs et al. 1996). Oxidative bursts are a common signaling tool plants use to recognize pathogens (Fuhrer and Booker 2003) and to transmit information about environmental impacts (Foyer and Noctor 2005).

The HR is reported to consist of two separate oxidative bursts; the initial ROS discharge is delivered for general stresses and involves a quick and temporary rise in ROS, while the second burst lasts for hours and stimulates lesion formation around the burst site (Schraudner et al. 1998). Prolonged O_3 exposure can stimulate the development of the biphasic burst in sensitive plants (Schraudner et al. 1998), but in O_3-tolerant plants only the initial ROS burst occurs. Unlike a pathogen attack, which is highly localized at the site of infection, O_3 diffuses throughout the entire mesophyll and generates oxidative bursts across the entire leaf (Overmyer et al. 2003).

Plants have a number of defensive mechanisms that deal with inappropriately high levels of ROS (Mittler 2002). The term antioxidant refers to any molecule that can detoxify ROS without itself becoming a radical species (Noctor and Foyer 1998). Some of the main non-enzymatic antioxidants are membrane-bound tocopherol, ascorbic acid (AA), and glutathione. Both AA and glutathione are found in the cytosol, chloroplasts, mitochondria, peroxisomes, and apoplast (Mittler 2002).

The main antioxidant enzymes are superoxide dismutase (SOD), which reduces $\cdot O_2^-$ to H_2O_2, and ascorbate peroxidase (APX) and catalase (CAT), both of which reduce H_2O_2 to H_2O (Long and Naidu 2002). Both SOD and APX are found in a variety of cellular

compartments, including the cytosol, chloroplasts, mitochondria, peroxisomes and apoplast, whereas CAT is found primarily in the peroxisomes (organelles that scavenge or produce peroxides), cytosol, and mitochondria (Mittler 2002).

Peroxidases are heme-containing proteins that utilize H_2O_2 as a substrate and have been shown to respond to O_3 exposure in plants (Prasad et al. 1995). Guaiacol peroxidases (GPX) are sited in the cytosol, vacuole, and apoplast and are integral for routine plant processes including lignification, cross-linking of cell wall polymers, injury repair, ethylene production, and protection against pathogen attack (Prasad et al. 1995). Studies have linked a rise in GPX activity with the occurrence of oxidative stress (Burkey et al. 2000).

The present study focuses upon the total content and reduced form of AA and glutathione (GSH), and the activity of GPX, and their response to short term hourly episodes of chronic and acute O_3 exposure in tulip poplar (*Liriodendron tulipifera* L.) saplings.

Research investigating short term hourly exposures to O_3 has discovered significant responses in photosynthetic parameters, foliar injury, gas exchange, and membrane and protein stability (Gupta et al. 1991, Held et al. 1991, Pino et al. 1995, Ranieri et al. 2000 and 2003, Guidi et al. 2002, Moraes et al. 2004, Guéra et al. 2005, Degl'Innocenti et al. 2007, Francini et al. 2007). Short term O_3 episodes are often used to mimic sudden peak O_3 concentrations and to discern the immediate response of plants to the oxidative stress in relation to late stage responses. Plant response to peak O_3 concentrations can be different than to low chronic O_3 concentrations, even with the same cumulative uptake, and the visible damage can be more severe (Lefohn and Runeckles 1987, Musselman et al. 1994, Hildebrand et al. 1996)

Ascorbic acid is a powerful antioxidant containing a double carbon bond which allows AA to react rapidly with O_3 (van Hove et al. 2001), reduce OH^- at diffusion-limited rates (Foyer 1993) and make AA a principal scavenger of H_2O_2 in the apoplast (Long and Naidu 2002). By itself or in conjunction with ascorbate peroxidase, ascorbic acid oxidizes a variety of ROS and precludes ROS formation by keeping α-tocopherol reduced and by sustaining the xanthophyll cycle (Smirnoff 1996).

The total amount of apoplastic AA in leaf tissue has been correlated with O_3 tolerance across a great range of plant species (Conklin and Barth 2004). Ascorbic acid content and redox values have been associated with greater O_3 tolerance in both woody and herbaceous species (Ranieri et al. 1996, Conklin et al. 1996, Wieser et al. 1998, Zheng et al. 2000, Pasqualini et al. 2001, Scebba et al. 2003, Sanmartin et al. 2003, Chen and Gallie 2005, and Burkey et al. 2003 and 2006). However, numerous experiments have also contested the role of AA in determining the O_3 sensitivity of a species (Castillo and Greppin 1988, Guzy and Heath 1993, Ranieri et al. 1996, Turcsányi et al. 2000, van Hove et al. 2001, Strohm et al. 2002, D'Haese et al. 2005, Herbinger et al. 2002 and 2005).

Glutathione is primarily cytoplasmic, and one of its main functions is to regenerate high levels of reduced AA in the apoplast (Mittler 2002). The reduced form of GSH has a higher oxidative potential than AA and scavenges a variety of ROS, notably H_2O_2 (Polle 2001, Foyer 1993). The accumulation of GSH has been correlated with levels of H_2O_2 (Sheng et al. 1997).

As researchers have found a variety of responses of AA to O_3, responses of glutathione to O_3 exposure are equally variable. Wieser et al. (1998) tested the response of glutathione in Norway spruce (*Picea abies* (L.) Karst.) that were either exposed for 49 days

to a gradual increase in O_3 concentrations to 100 ppb or a sudden and sustained exposure to 100 ppb O_3. After 30 days, total glutathione content (GSH + GSSG) increased significantly only in the sudden O_3 exposure treatment, immediately after which total glutathione content declined to a level commensurate with the total glutathione content response in the gradually increasing O_3 treatment and in the control treatment. Conversely, total glutathione concentrations in field grown 60 year old beech (*Fagus sylvatica* L.) and potted beech seedlings were reported to be significantly elevated after an entire growing season's exposure to 2x O_3 levels (Herbinger et al. 2005). Under 100 ppb O_3 fumigation, O_3-tolerant trembling aspen (*Populus tremuloides* Michx.) clones increased total glutathione content while O_3-sensitive clones reduced their content, leading Sheng et al. (1997) to suggest that GSH exerts a protective role against O_3. However, in five cultivars of snap bean (*Phaseolus vulgaris* L.), exposed to O_3, total glutathione content failed to distinguish O_3-sensitive and O_3-tolerant plants (Burkey et al. 2000).

The stimulation of GPX activity has been associated with the HR, tissue injury, and the onset of abiotic stresses, including O_3 exposure (Chernikova et al. 2000). Burkey et al. (2000) viewed GPX activity as an early gauge for ROS-induced stress but not as a differentiator to categorize O_3 sensitivity. In contrast, Ranieri et al. (1996) and Kronfuss et al. (1996) found no response in GPX activity in pumpkin (*Cucurbita pepo* L. cv. Ambassador), or Norway spruce needles, respectively. However, Chernikova et al. (2000) were able to use differences in the levels of GPX activity to discriminate between the O_3 sensitivities in the soybean (*Glycine max* L.) cultivars Essex and Forrest, and similar differences were found for white clover (*Trifolium repens* L.) clones and birch (*Betula pendula* Roth.) (Nali et al. 2005, Pellinen et al. 1999, respectively).

The tulip poplar tree (*Liriodendron tulipifera* L.) is an important early successional tree in the southern Appalachians, and a major timber tree (Beck 1990). Tulip poplar has been the subject of numerous studies concerning its response to O_3, many of which have generated conflicting data concerning its sensitivity to this pollutant. Tulip poplar's fast growth and indeterminate leaf production would suggest a strong O_3 sensitivity due to elevated uptake of O_3 (Harkov and Brennan 1981). The fast growing black cherry (*Prunus serotina* L.), is a well studied example of a highly O_3-sensitive tree (Neufeld et al. 1993 and 1995, Hildebrand et al. 1996, Chappelka and Samuelson 1998, Chappelka et al. 1999) compared to the slow growing and O_3-insensitive chestnut oak (*Quercus rubra* L.) (Neufeld, unpublished manuscript), and hemlock (*Tsuga canadensis* (L.) Carr.) (Neufeld et al. 2000).

Tulip poplar has been shown to develop stippling, pigmented dead cells (Brace et al. 1999) in response to O_3 exposure, both in the field and in controlled exposure studies (Duchelle et al. 1982, Tjoelker and Luxmoore 1991, Huang 1992, Hildebrand et al. 1996, Simini et al. 1992, Chappelka and Samuelson 1998, Somers et al. 1998, Davis and Skelly 1992, Rebbeck 1996). However, Rebbeck and Loats (1997) reported negligible lesion formation with potted tulip poplar saplings exposed to two growing seasons of 1.7x O_3 and suggested that the saplings may have originated from resistant populations.

Photosynthesis in tulip poplar is either unresponsive to O_3 exposure (Loats and Rebbeck 1999, Tjoelker and Luxmoore 1991), or declines (Chappelka et al. 1988, Huang 1992, Rebbeck and Loats 1997, Rebbeck and Scherzer 2002,). Reductions in photosynthesis, if they do occur, appear only late in the season (Huang 1992) and are often not correlated with a similar decline in g_s (Huang 1992, Rebbeck and Loats 1997, Rebbeck and Scherzer 2002).

Little work has been done on tulip poplar's antioxidant defenses in response to O_3 exposure. The only prior research was by Woo et al. (2004), who reported elevated APX activity in saplings exposed to 150 ppb O_3 for 8 hrs/day for 34 days. Additionally, there are essentially no studies on the impacts of very short term exposures to O_3 on leaf level processes, particularly in trees. Considering that typical O_3 episodes (periods when the ozone is greater than 60 ppb) in the southern Appalachian mountains last for only a few days at a time (Neufeld, unpublished data), it would be important to know if trees are responding to individual, short-term episodes, or only showing measurable responses after prolonged exposures to multiple, extended O_3 episodes.

This study sought to determine whether (1) tulip poplar saplings responded in terms of leaf level physiological processes, such as photosynthetic electron transport as measured by chlorophyll fluorescence, and g_s to short-term O_3 episodes, and (2) whether the biochemical antioxidant defenses to O_3 also respond to these short exposures. In this study, I exposed saplings to either chronic levels of O_3 (~60-70 ppb) for up to three days or acute levels (~190-210 ppb) for one day, with equal total O_3 exposures for both treatments, and I then compared their responses both during and up to 48 hrs after exposure.

MATERIALS AND METHODS

PLANT MATERIAL

One hundred second-year tulip poplar saplings were purchased from the North Carolina Forest Service in Goldsboro, NC and kept in a refrigerator at 5°C for six weeks. These saplings originated from trees in the mountains of eastern TN or western NC. On May 1st, saplings were potted in 19.4 L PVC pots (12.5 cm diameter, 39.5 cm depth), containing Sun Gro Metro-mix 360 growing medium and maintained outdoors at the ASU greenhouse in Boone, NC, until August 2006. All seedlings were hand watered to field capacity and fertilized once in May with Osmocote Plus (15:9:12) at 1.8 kg m^{-3}. Leaves were sprayed with Cleary's (Cleary Chemical, Dayton, NJ), MilStop (BioWorks, Inc., Geneva, NY) or Rhapsody with Biotune (AgraQuest Inc., Davis, CA) on an as need basis in attempts to control powdery mildew (*Erysiphe polygoni* or *E. liriodendri*, not tested) (Hepting 1971).

OZONE EXPOSURE SYSTEM

Ozone was supplied by a Griffin ozone generator (Griffin Technics Corporation, Lodi, NJ) using pure oxygen and dispensed through teflon tubing into three O_3 exposure chambers (2.05 m x 0.75 m x 0.77 m) located in the greenhouse (Figure 2). Chambers were constructed of aluminum framing with flexible PVC sidewalls. An exhaust fan pulled air through the chambers and vented outside the greenhouse. Chamber air turn over rates (although not measured) were fast enough to keep chamber temperatures close to greenhouse

temperatures. Air being drawn into the chambers was first passed through a charcoal filter to remove the majority of ambient O_3. Ozone was then added back using a plenum at the top of the chamber to insure mixing, before being delivered to the plants in the main part of the chamber. A small cage fan was used to stir the air inside the chambers.

Ozone concentrations for each chamber were monitored by a TECO Model 49 ozone analyzer (Thermo Fisher, Waltham, MA). Relative humidity was monitored by a Vaisala Humicap sensor (Vaisala Inc., Finland) located in each of the O_3 chambers while photosynthetically active radiation (PAR) was monitored by Hamamatsu GaAsp diodes (G1118, Hamamatsu Corp., Hamamatsu City, Shizuoka Pref., 431-1202, Japan) placed on poles in the chambers above the leaf canopy and calibrated against a Li-Cor 190sb quantum sensor (Li-Cor Inc., Lincoln, NE). In addition, relative humidity, PAR and O_3 were recorded in ambient air near where the saplings were growing. Temperatures both outside and in the exposure chambers were monitored with shielded Type T thermocouples. All data were recorded by a Campbell 21x data logger (Campbell Scientific, Inc., Logan, UT) and then downloaded automatically each night to a portable laptop computer.

OZONE TREATMENTS

There were three ozone treatments: (1) chronic ozone with a SUM00 O_3 exposure (sum of all hourly concentrations) of ~2342 ppb*hrs given over three days, 12 hrs daily, at ~65 ppb, (2) acute ozone with a SUM00 O_3 exposure of ~2347 ppb*hrs given over one day for 12 hrs, at ~200 ppb, and (3) a control treatment that received only charcoal-filtered (CF) air at ~15 ppb for a total exposure of 540 ppb*hrs over 36 hrs. The mean SUM60 (sum of all concentrations 60 ppb or greater) for the three replications of the chronic exposure treatment was 2198 $\pm$ 66 ppb*hrs, 2345 $\pm$ 24 ppb*hrs for the acute exposure treatment, and 0 ppb*hrs

for the control treatment. During fumigations, O_3 levels were monitored and flow rates adjusted by hand to maintain constant square wave exposures.

EXPERIMENTAL TREATMENTS

Treatments were rotated among the three O_3 chambers, with one replication of each treatment at any particular time, and treatments were replicated three times. For each replication, two randomly chosen saplings with a minimum of five viable and mature leaves were allocated to each O_3 chamber. A total of 18 saplings were used for the entire experiment. Two leaves each were chosen out of a set of five leaves from each sapling for chlorophyll fluorescence and stomatal conductance (g_s) measurements. The leaves selected for g_s measurements were the oldest and the youngest in the five leaf set for each repetition. The difference in leaf ages was kept constant by moving up one leaf position on the stem for each repetition. This procedure allowed for separate analysis of leaf response by age (young vs. old) and to obtain the full mean response available to a sapling leaf cohort. New leaves flushed out in less than a week and leaves were defined as mature upon completion of expansion.

Each replication of the experiment ran for six days and consisted of one full day of acclimation, three days of chronic O_3 fumigation or one day of acute O_3 fumigation, which occurred on the 3rd day of the chronic exposure, plus two additional 'recovery' days when the saplings received only CF air. The experiment began on June 24th and the last replicate ended July 14th.

PLANT RESPONSE MEASUREMENTS

Stomatal Conductance

Stomatal conductance was measured with a Model 1600 Li-COR Steady State Porometer (LI-COR Biosciences, Inc., Lincoln, NE) at approximately 8am, 12 pm, 3 pm, and 6 pm, except on the last day of the treatment period, when measurements ended at 3 pm. The period of time the chamber door was open was minimized during measurements (< 3 minutes for fluorescence measurements, < 5 minutes for g_s measurements) to avoid major changes in chamber O_3 concentrations.

Chlorophyll Fluorescence

Chlorophyll fluorescence measurements (F_v/F_m and Area (F_a)) were made with a Hansatech Handy PEA Fluorometer (Hansatech Instruments Ltd., Norfolk, England). Measurements were made each day at approximately 7:30 am and 5:30 pm, when enough light was available for the leaves to be photosynthetically active. The portion of the leaf to be measured was acclimatized in the dark with clips for 30 minutes prior to measurement. The duration of signal was two seconds and the intensity of light was 3000 μmol m^{-2} s^{-1}.

Antioxidant Assay Procedures

Leaves were sampled for antioxidants on acclimation day, the last day of O_3 exposure, and the two rest days. Each of these samplings occurred in the early afternoon at ~3:00 pm, to mitigate confounding by a possible diurnal pattern of AA content (Guzy and Heath 1993, Burkey et al. 2003). Leaves were bagged in aluminum foil packets, immediately immersed in a Dewar container containing liquid nitrogen and then placed in a -80°C freezer for a maximum of three months until analyzed. Leaves were then transported in liquid nitrogen to the USDA/ARS Plant Science Research Lab in Raleigh, NC where the

antioxidant assays were performed. All samples were stored in a -80°C freezer until analysis. For all assays, the frozen leaf tissue was ground with liquid nitrogen using a mortar and pestle pre-cooled with liquid nitrogen. For all assay tissue extracts, ~200 mg of ground tissue was used in a 1:10 ratio with the appropriate extraction buffers (see below). A Shimadzu Model UV-1601 spectrophotometer was used to measure absorbances. All antioxidant assay measurements for an individual extract were repeated twice and the results averaged.

Ascorbic Acid and Dehydroascorbic Acid Protocol

Tissues were extracted in a buffer comprised of 6% (w/v) meta-phosphoric acid (3 grams per 50 ml ddH_2O) and 0.2 mM diethylenetriaminepentaacetic acid (DTPA) (3.9 mg per 50 ml ddH_2O). Tissues were then incubated in extraction buffer for 10 minutes before being centrifuged at 20,000 x g for 10 minutes at 4°C. The supernatant was removed, centrifuged again for two minutes and the clearer supernatant removed for analysis.

Spike procedure

The stability of reduced AA during extraction was determined by placing a known amount of commercial reduced AA into an extract and measuring the recovery. Comparisons included a spiked extract solution, a spiked buffer solution, and a control extract solution. The spiked extract solution contained 2 μmoles AA, tissue, and extraction buffer. The spiked buffer solution contained only the AA and extraction buffer, while the control extract solution contained only tissue and extraction buffer. The stability of ascorbic acid was demonstrated by the equivalence of the sum of the ascorbic acid values in the spiked buffer and the control extract compared to the ascorbic acid values for the spike extract.

Assay

For the assay, 990 µl of potassium phosphate buffer (100 mM K_2PO_4 at pH 7.0) and 10 µl tissue extract were placed in a disposable plastic UV-transparent cuvette. Ascorbic acid values were measured by adding 2 µl of 1 U/µl ascorbate oxidase, which oxidized the AA and lowered the A_{265}. Dehydroascorbic acid values were measured after adding 2 µl dithiothreitol (DTT) to reduce the DHA resulting in an increase in A_{265}. Changes in A_{265} were used to calculate AA and DHA values according to Beer's Law using an extinction coefficient at 265 nm of 14.3 $mM^{-1}cm^{-1}$.

Reduced and Oxidized Glutathione Protocol

The extraction buffer contained 0.2 mM DTPA with 6% (w/v) meta-phosphoric acid. Tissue was incubated in extraction buffer for 10 minutes and then centrifuged for 10 minutes at 12,000 rpm at 4°C. Then the supernatant was removed and centrifuged for an additional five minutes and the clearer supernatant removed for analysis. A new standard curve was created every morning and if fresh reagents were made during the day.

Spike Procedure

In preliminary experiments, we tested the stability of reduced GSH in the tissue by comparing a spiked extract solution, a spiked buffer solution, and a control extract solution. The spiked solution contained 300 nmol GSH, tissue, and extraction buffer. The spiked buffer solution contained only GSH and extraction buffer, while the control contained only tissue and extraction buffer.

Standard Curve Assay

Reduced glutathione (GSH) and oxidized glutathione (GSSG) content of extracts were measured according to the procedure of Griffith (1980) based on the glutathione

reductase recycling reaction described by Tietze (1969). All assays contained 790 µl 100 mM KPi buffer pH 7.5 (3.4 g KH_2PO_4, 186 mg $Na_2EDTA \cdot 2H_2O$ dissolved in 250 ml ddH_2O and adjusted to pH 7.5 with KOH), 100 µl of 6 mM 5,5'-dithio-bis(2-nitrobenzoic acid), (DTNB) (1.2 mg in 5 ml of 100 mM KPi buffer, pH 7.5), 50 µl of 4 mM NADPH (10 mg in 3 ml 100 mM KPi buffer, pH 7.5), 10 µl tissue extract or standard, and 50 µl of glutathione reductase (5 units glutathione reductase per 100 µl 100 mM KPi buffer, pH 7.5). DTNB is a reporter reagent that changes color in the presence of GSH. Absorbance was read at 412 nm at 25°C for 65 seconds to determine the DTNB reaction rate. The standard curve was constructed by plotting reaction rate versus GSH (0 to 1.2 nmol) for comparison with unknowns.

The standards and total glutathione unknowns were run without the triethanolamine (TEA) treatment described by Griffith (1980) because GSH was observed to be unstable in the presence of TEA possibly due to the neutral or slightly basic pH conditions established by TEA. To obtain GSSG values, aliquots of the tissue extract were pretreated with 2-vinyl pyridine (2-VP) using TEA to establish the pH conditions required by the reaction. In solution, 2-VP reacted with GSH allowing GSSG to be measured alone in the DTNB assay.

Guaiacol Peroxidase Protocols

Polyvinyl-polypyrrolidone (insoluble PVPP) was added to ~200 mg frozen ground leaf tissue in a 1:1 mass ratio. Solid AA (0.0881 g) was added to 50 ml of extraction buffer (66 mM KPi at pH 7.0 and 100 mM KCl) prior to tissue extraction in order to prevent phenolic reactions from darkening the tissue extract to an orange brown by the time the extract was removed from the centrifuge. The final AA concentration in the extract was 10 mM AA. The extract solution was centrifuged for 10 minutes at 28,000 rpm at 4° C and

the supernatant filtered through a 5 ml plastic syringe barrel connected by a Luer lock to a filter holder containing a Mira cloth disk to remove suspended tissue and remaining PVPP. The filtered supernatant was centrifuged again at 12,000 rpm for 1 minute and the clearer supernatant removed for analysis.

Gel Columns Procedure

Two ml Sephadex G-25-50 gel filtration columns (0.75 x 4.5 cm) were used to remove AA and endogenous metabolites from the extract. In the days between experiments, the gel columns were treated with 0.1 % (w/v) sodium azide in column buffer (66 mM KPi at pH 7.0, 100 mM KCl) to prevent biological contamination and were washed with 6 ml column buffer at room temperature immediately before assays began.

Two hundred µl of tissue extract was gently placed atop the column media and, after the tissue extract had entered the column, 2.5 ml of column buffer was gently added in 0.5 ml installments. Four fractions were collected in 0.5 ml units with the 3rd fraction usually containing the most enzyme. A fresh column was used for each sample. Enzyme activity was assayed on the day of extraction and then placed in a freezer prior to the BioRad protein assay conducted the following week.

Activity assay

Assay reagents were made fresh daily and consisted of 400 µl assay buffer (100 mM KPi at pH 6.3), 500 µl 80 mM guaiacol (180 µl guaiacol in 19.8 ml assay buffer), 50 µl 200 mM H_2O_2 (50 µl 30.4% (w/w) H_2O_2 in 2.45 ml assay buffer), and 50 µl of desalted crude extract from the Sephadex column. The change in absorbance was read at 430 nm at 25°C in the 50-100 second window in the kinetic mode of the spectrophotometer. The specific

protein activity was acquired from the slope of the absorbance curve (mAbs/min) using an extinction coefficient of 25 $mM^{-1}cm^{-1}$ and the average protein µg/50 µl.

Protein assay

The BioRad Protein Assay used bovine serum albumin as a standard. The reagents were 25 µl protein standard, 775 µl column buffer, and 200 µl of the BioRad reagent. The standard protein concentrations were: 0, 2, 4, 8, 12, 16, and 20 µg. After incubating the solutions for 5 minutes, absorbance was read at 595 nm and a standard curve established. The sample reactions contained 775 µl assay buffer (100 mM KPi at pH 6.3), 25 µl plant extract, and 200 µl Bio-Rad reagent. Protein concentration was determined by comparison with the standard curve.

Detection of Reactive Oxygen Species

Hydrogen Peroxide

For *in situ* detection of possible O_3-induced accumulation of hydrogen peroxide (H_2O_2), leaf sections were stained with the 3,3'-diaminobenzidine (DAB) based on a protocol by Thordal-Christensen et al. (1997) and modified by Kirk Overmyer (personal communication, 2005). The DAB solution consisted of 50 mg DAB dissolved in 50 ml H_2O and 40 µl concentrated HCL. The final product, a clear yellow solution, was achieved after placing the solution in a shaker for 8 hrs at room temperature. The DAB solution was made fresh 8 to 10 hrs ahead of the staining treatment and kept wrapped in aluminum foil to prevent degradation by light.

Superoxide Anion

For *in situ* detection of possible O_3-induced accumulation of superoxide anion, ($\cdot O_2^-$), leaf sections were stained with nitroblue tetrazolium (NBT) based on a protocol from

Hüeckelhoven et al. (2000). Two stocks were made; the first stock contained 10% (w/v) NaN_3 and the second stock contained 100 mg/ml NBT dissolved in a solution of 70% DMF and 30% H_2O. The second stock was then dissolved into a buffer solution of 10 mM KH_2PO_4 at pH 7.8. The final product, a clear yellow solution, was complete when 650 µl of 10% (w/v) NaN_3 from stock one was stirred into 1 ml of stock two containing the NBT solution and buffer. NBT solution was made fresh for the first rest day, and kept refrigerated for use in the 2nd rest day and for the following acclimation day that started the next repetition.

Leaf samples were stained with DAB and NBT on the acclimation days and the 1st and 2nd rest days of each replicated treatment. A potato borer, with 2 cm diameter cutting end (3.14 cm^2 area) was used to remove circular leaf sections from one mature leaf per plant. The leaf discs were placed in a 60 cc plastic syringe containing either the DAB or NBT solution and a vacuum was drawn until the discs were infiltrated (discs visibly changed from light to dark green). After infiltration, the leaf discs were wrapped in moist paper towels, placed in an aluminum foil packet and transported to the lab where, after 4 hours, they were placed in a clearing solution containing a 3:1:1 solution of 95% ETOH: 85% lactate: glycerol. The clearing solution was changed every day until the leaf discs turned visibly translucent.

STATISTICAL ANALYSES

This experiment was a split-plot design with repeated measures, where the O_3 treatment was the whole plot factor and time was the split plot factor. Data were analyzed using a mixed model ANOVA that included leaf age as a block to reduce error variability. The PROC MIXED procedure in SAS 9.1 (SAS Inst., 2002) was used for all analyses. Statistical significance was assumed if $p \leq 0.05$.

RESULTS

In 2005, a preliminary study discovered that fungicide soil soaks were inadequate to either prevent tulip poplar from powdery mildew infection or in killing off extant infections. In the early summer of 2006, fungicides Rhapsody, MilStop, and Cleary's were applied to the leaves in order to prevent leaf necrosis due to powdery mildew. Initial leaf applications involved spraying the surface but the intensely hydrophobic leaf surface confounded light spraying and heavy spraying induced damage. Subsequent applications involved blotting fungicide directly on the infection zones in order to minimize adverse affects. Prior to acclimation day, the leaves were gently sluiced with water to remove fungicide residue and measurements carefully were made on dry, clean green surfaces. Occasionally, additional blotting with fungicide was required during the treatment, but only in the evening, in order to curtail spreading infection

Ozone concentrations

Table 1 shows the mean O_3 concentration per O_3 treatment for the six treatment days across all repetitions. The SUM00 O_3 concentration for acute and chronic O_3 was similar, 2347 ± 25 ppb*hrs and 2342 ± 28 ppb*hrs, respectively. The average concentration for acute O_3 treatments was 192 ± 2 ppb and the average chronic O_3 concentration was 65 ± 0 ppb.

Table 1. Mean ± SE O_3 concentration (ppb*hr) for each O_3 treatment except for acute O_3 treatment on rep 1, rep 2, rep 3 where mean ± STDEV O_3 concentration (ppb*hr).

O_3 Treatment	Hourly [O_3] average	SUM00	SUM60
Acute off			
rep 1	8±2	206	0
rep 2	20±0	473	0
rep 3	14±1	325	0
Total rep	14±3	340±133	0
Acute on			
rep 1	192±22	2298	2298
rep 2	197±22	2363	2356
rep 3	198±22	2380	2380
Total rep	192 ±2	2347±25	2345±24
Chronic			
rep 1	64±1	2286	2066
rep 2	66±1	2374	2252
rep 3	66±1	2365	2276
Total rep	65±0	2342 ±28	2198±66
Control			
rep1	10±2	355	0
rep 2	17±1	625	0
rep 3	14±1	512	0
Total rep	14±2	497±78	0

Table 2 shows the measurements of light levels of photosynthetically active radiation (PAR), air temperature (°Celsius), and % relative humidity (%RH) for each repetition and across all repetitions. Air temperature and %RH held steady throughout the treatment with PAR fluctuating due to variability of cloud cover and light moving across the greenhouse walls and ceiling.

Table 2. Mean ± SE air temperature and relative humidity (RH), and the sum of photosynthetically active radiation (PAR) for each of the 3 O_3 treatment chambers.

O_3 Treatment	Air Temperature	RH	PAR (sum)
Acute			
rep 1	27±1	54±3	13149
rep 2	30±1	41±2	13188
rep 3	27±1	47±2	16488
Total	28±1	47±4	42825
Chronic			
rep 1	27±1	54±3	8957
rep 2	30±0	42±2	21321
rep 3	28±1	47±2	13613
Total	28±1	48±3	30278
Control			
rep 1	26±1	54±3	11733
rep 2	30±0	42±2	19289
rep 3	27±1	47±2	9348
Total	28±1	48±3	40370

Stomatal conductance (g_s)

Repetition 2 produced irregular results for g_s with a significant interaction between treatment and chamber ($p < 0.0001$), so g_s was analyzed both with (Figures 1 and 3) and without (Figures 2 and 4) this repetition. In the analyses of all three repetitions and of only the 1st and 3rd repetitions together, morning g_s was significantly higher than afternoon ($p < 0.0001$) (Figures 1 and 2, respectively) and was significantly higher in young than old leaves ($p < 0.0001$) (Figures 3 and 4, respectively). No treatment by time interaction was found ($p = 0.5002$). Stomatal conductance did show a treatment effect ($p < 0.0001$) that was driven by the anomalous data in repetition 2, but as neither repetition 1 nor repetition 3 showed similar treatment effects, the overall lack of effect of O_3 exposure on g_s is concluded. Repetition 2 may have had higher temperature and lower relative humidity than repetition 1 and 3, and level of PAR was higher in chronic and control O_3 treatments but not acute (Table 2).

Fig. 1. Mean stomatal conductance (g_s) for all repetitions for four measurement times in a treatment day. Values represent mean ± se (n = 3). The unit represents one plant for each of the three O_3 treatments. Each of the three repetitions employed two plants per O_3 treatment with measurements made on one young and one old leaf on each plant. Results were averaged across leaves, plants, and repetitions for each of the three O_3 treatments.

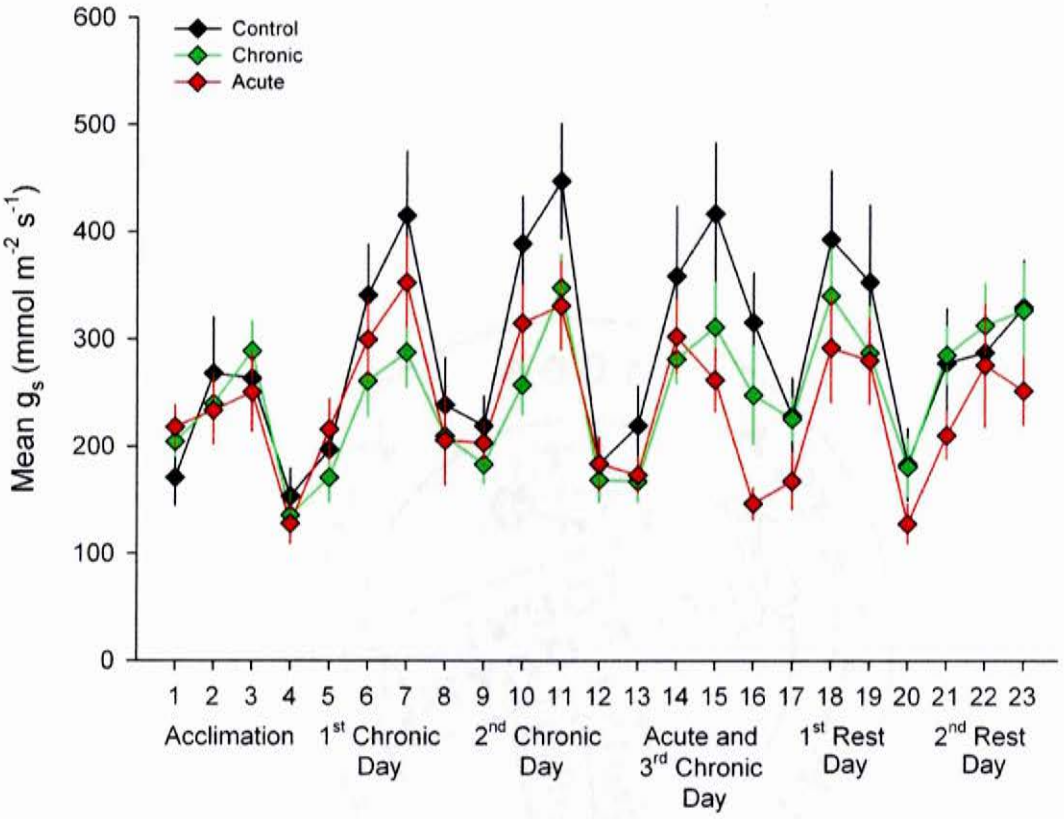


Fig. 2. Mean stomatal conductance (g_s) minus repetition 2 for four measurement times in a treatment day. Values represent mean ±se (n = 3). The unit represents one plant for each of the three O_3 treatments. Each of the three repetitions employed two plants per O_3 treatment with measurements made on one young and one old leaf on each plant. Results were averaged across leaves, plants, and repetitions for each of the three O_3 treatments.

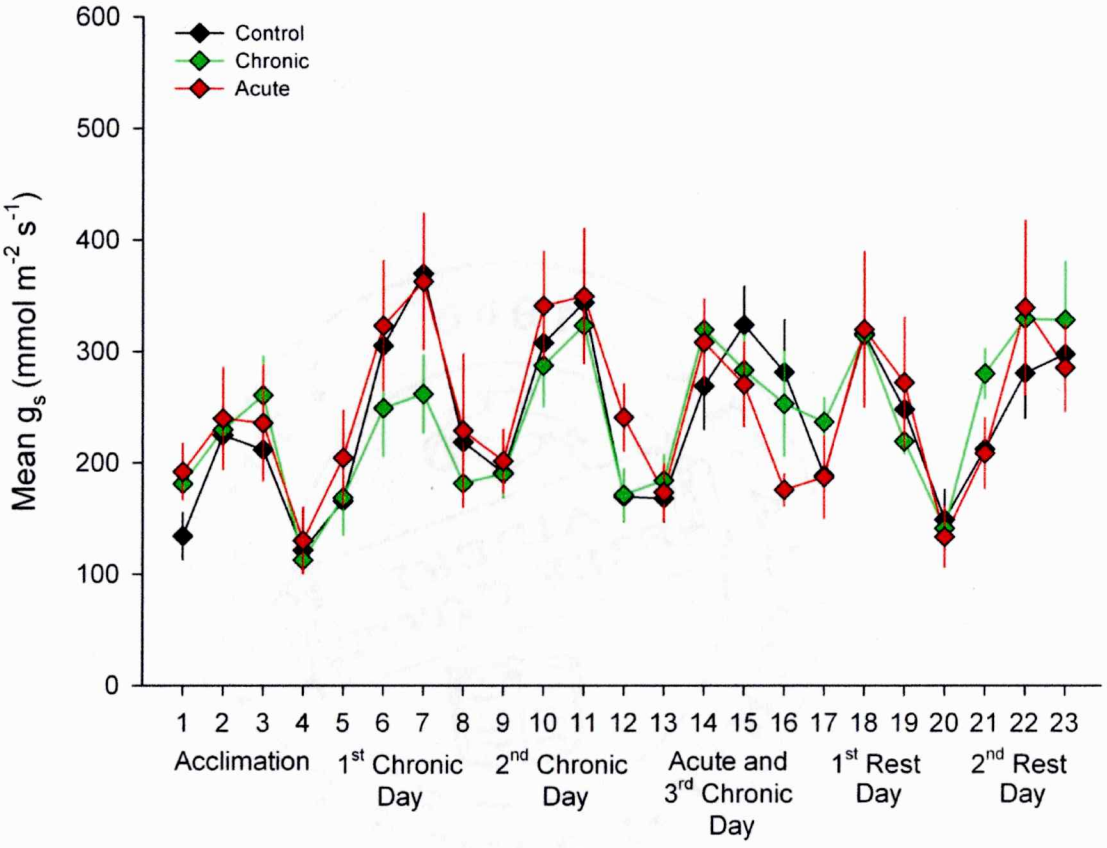


Fig. 3. Maximum mean stomatal conductance for all reps for young and old leaves across treatment days. Values represent mean ± se (n = 6). The unit represents two leaves for each of the three O_3 treatments. Each of the three repetitions employed two plants per O_3 treatment with measurements made across time on one young and one old leaf on each plant. Results for each leaf were averaged across plants and repetitions. Dashed lines indicate young leaves and solid lines indicate old leaves.

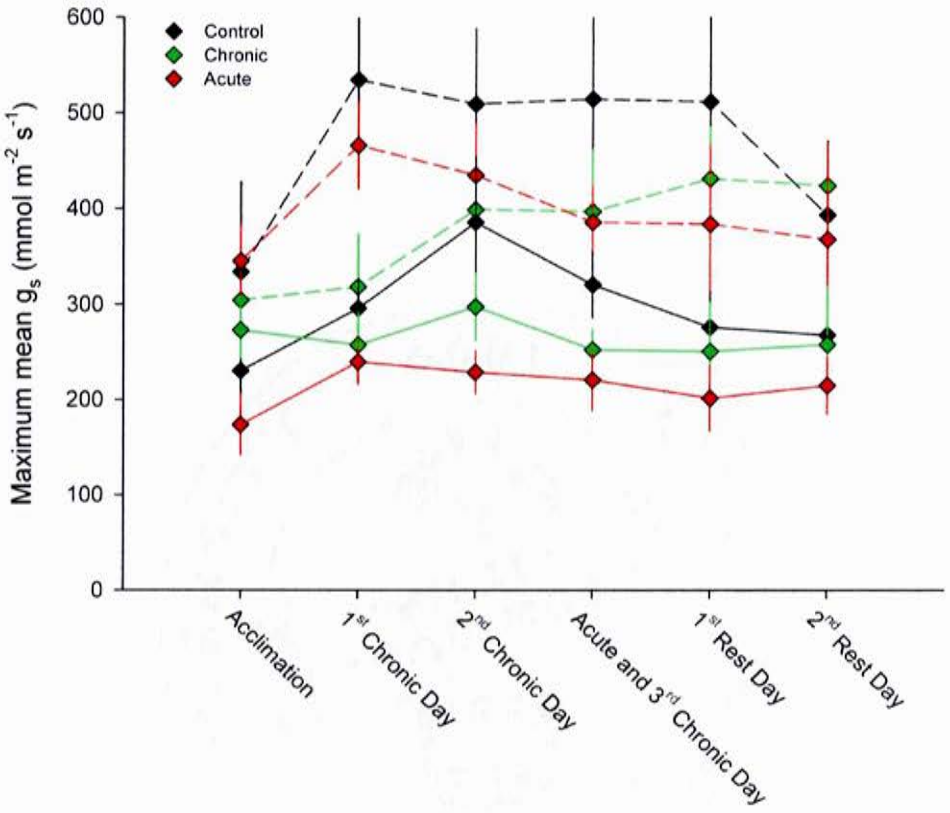
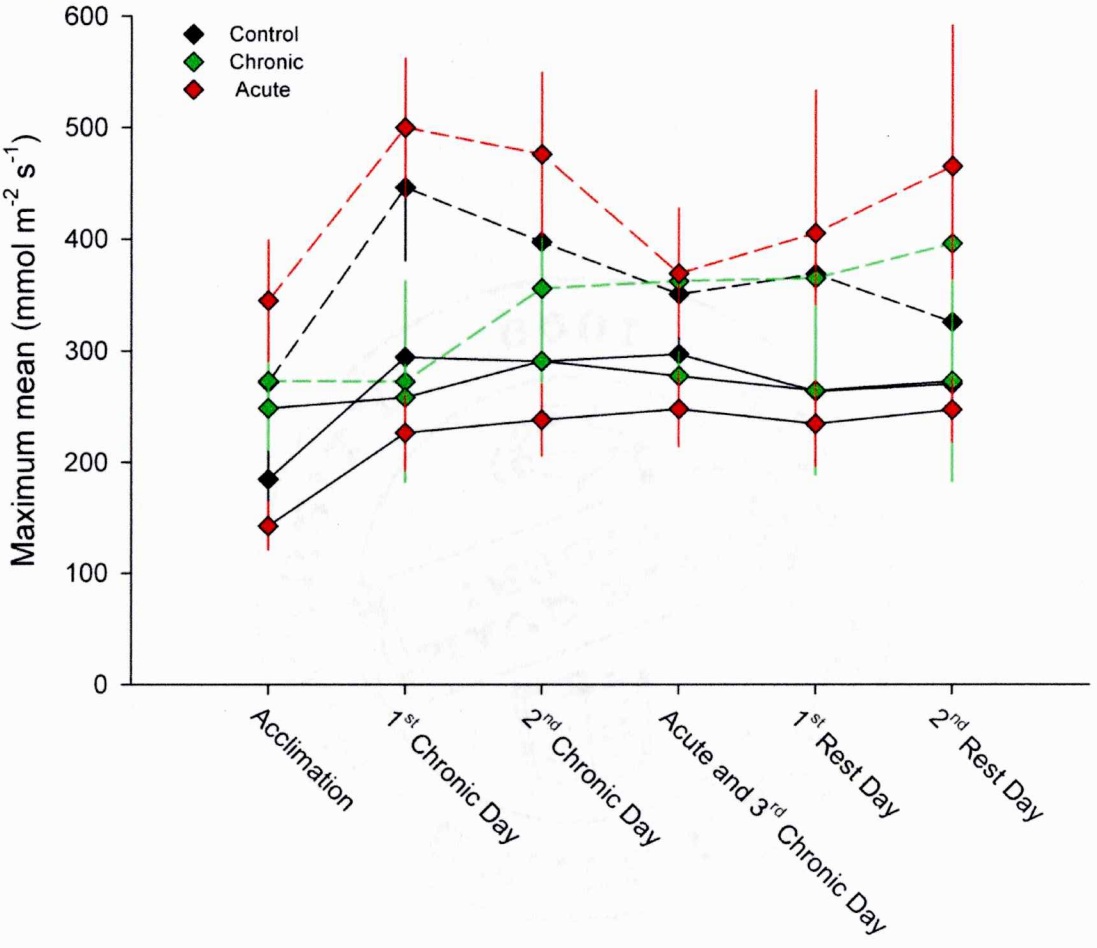


Fig. 4. Maximum mean stomatal conductance for young and old leaves across treatment days without repetition 2. Values represent mean ± se (n = 6). The unit represents two leaves for each of the three O_3 treatments. Each of the three repetitions employed two plants per O_3 treatment with measurements made across time on one young and one old leaf on each plant. Results for each leaf were averaged across plants and repetitions. Dashed lines indicate young leaves and solid lines indicate old leaves.

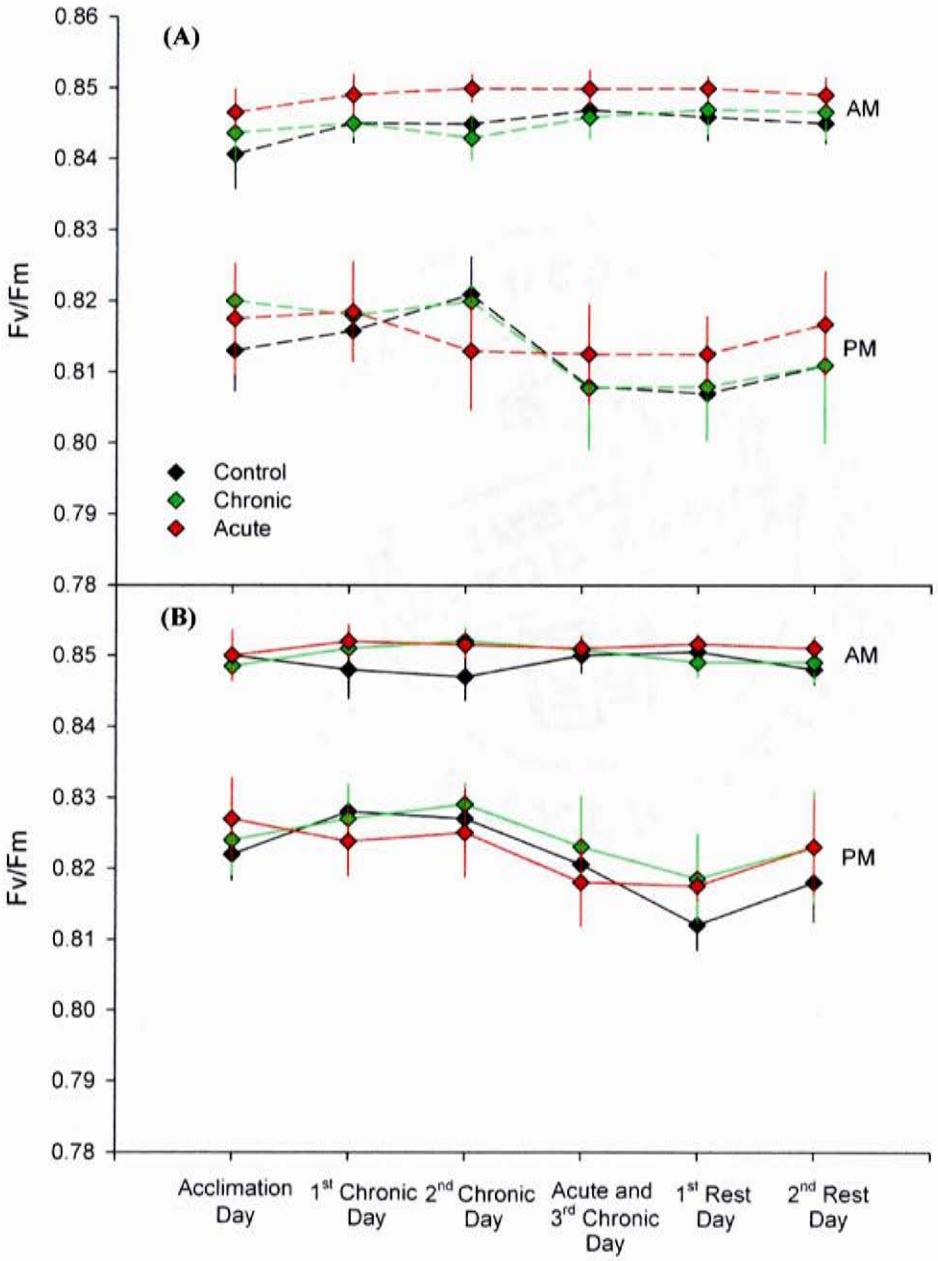


Chlorophyll fluorescence

Graphs of Fv/Fm and Area contain all 3 repetitions. Fv/Fm remained high throughout the treatment and followed the expected diurnal pattern of higher values in the morning followed by afternoon depressions (Figure 5). Fv/Fm in the morning was significantly higher than in the afternoon ($p < 0.0001$) and older leaves had significantly higher Fv/Fm than younger

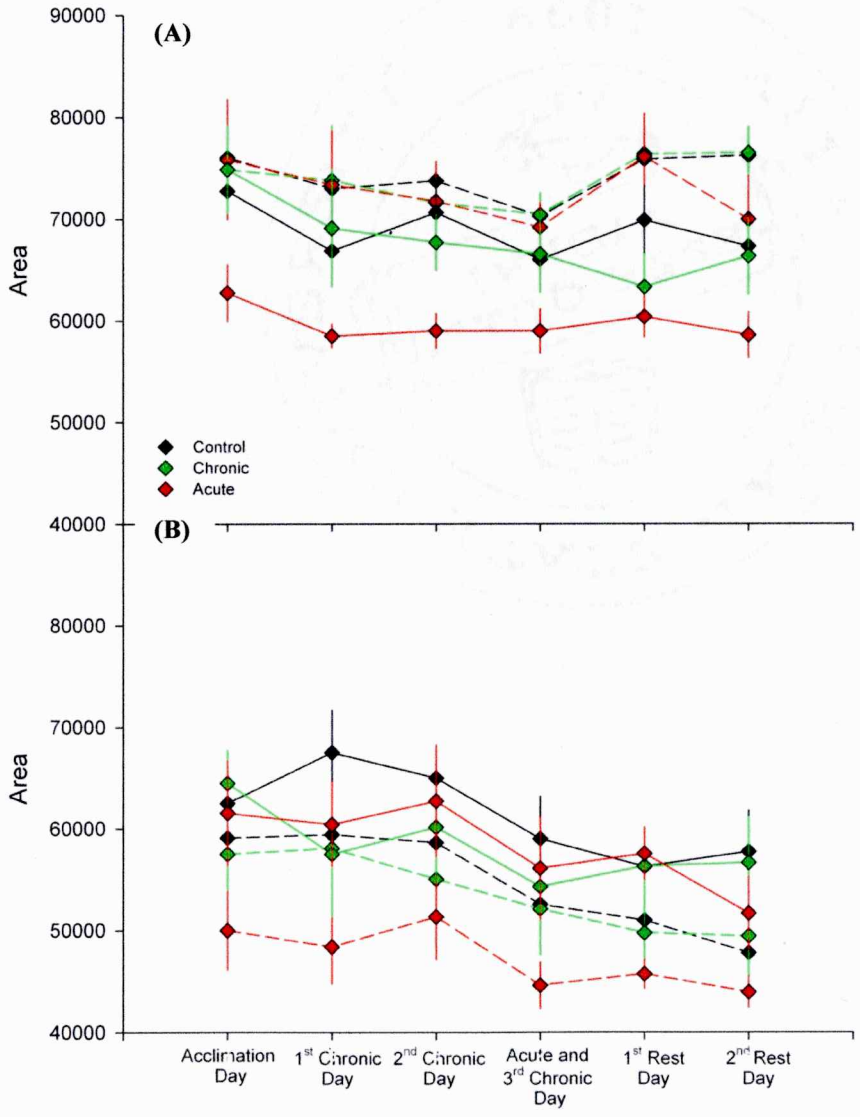
leaves ($p < 0.0001$). However, Fv/Fm in young and old leaves was not affected by O_3 treatment ($p = 0.1694$).

Fig. 5. Fv/Fm values for (A) young leaves for morning and afternoon and (B) old leaves for morning and afternoon of each treatment day. Values represent mean ± se (n = 6). The unit represents two leaves for each of the three O_3 treatments. Each of the three repetitions employed two plants per O_3 treatment with measurements made across time on one young and one old leaf on each plant. Results for each leaf were averaged across plants and repetitions. Dashed lines indicate young leaves and solid lines indicate old leaves.



Area also followed a diurnal pattern with a morning high followed by an afternoon low (Figure 6). Younger leaves had a significantly higher F_a than older leaves ($p < 0.0001$) in the morning and older leaves had significantly higher F_a than younger leaves in the afternoon. Morning F_a measurements were significantly higher than afternoon measurements ($p < 0.0001$). No significant relationship was found between F_a and O_3 treatment.

Fig. 6. Area values for (A) young and old leaves in the morning and (B) young and old leaves in the afternoon for each treatment day. Values represent mean ± se (n = 6). The unit represents two leaves for each of the three O_3 treatments. Each of the three repetitions employed two plants per O_3 treatment with measurements made across time on one young and one old leaf on each plant. Results for each leaf were averaged across plants and repetitions. Dashed lines indicate young leaves and solid lines indicate old leaves.



Antioxidants

There were no effects of O_3 on either (1) total ascorbic acid content (AA or DHA) ($p = 0.3659$) or reduced AA content ($p = 0.5688$; Figure 7), (2) total glutathione content (GSH + GSSG) ($p = 0.4757$) or GSH content ($p = 0.4169$; Figure 8) or (3) GPX activity, ($p = 0.0813$; Figure 9). However, a possible trend can be seen for an increase in GPX specific activity over time in the acute O_3 treatment relative to the chronic and control treatments.

Fig. 7. Total ascorbic acid (AA + DHA), AA, DHA, and redox status (AA/(DHA + AA)). Values represent mean ± se (n=3). The unit represents one plant for each of the three O_3 treatments. Each of the three repetitions employed two plants per O_3 treatment with measurements made on one leaf per plant. Results for each leaf were averaged across plants and repetitions.

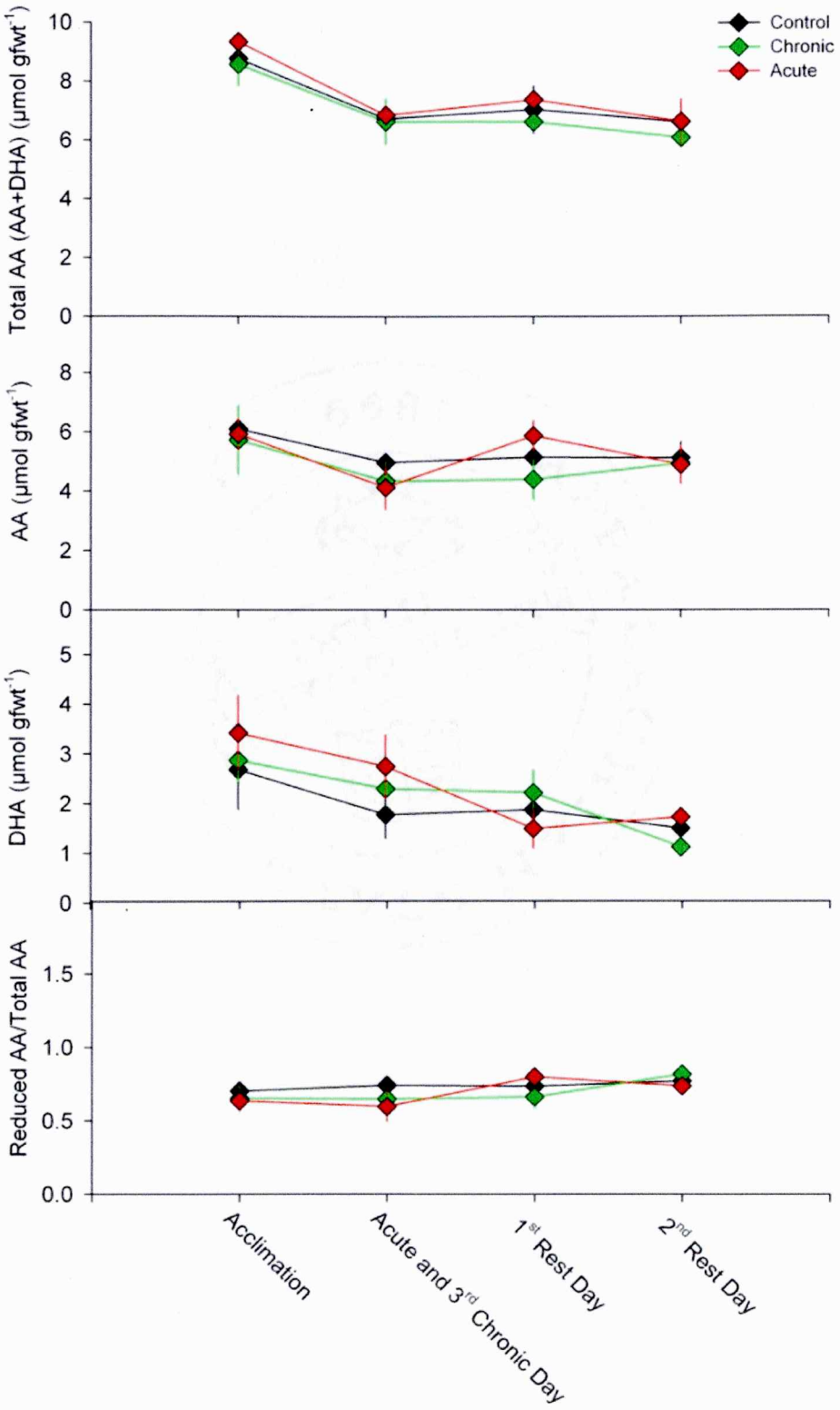


Fig. 8. Total glutathione (GSH + GSSG), GSH, GSSG, and redox status (GSH/(GSH + GSSG)). Values represent mean ± se (n =3). The unit represents one plant for each of the three O_3 treatments. Each of the three repetitions employed two plants per O_3 treatment with measurements made on one leaf per plant. Results for each leaf were averaged across plants and repetitions.

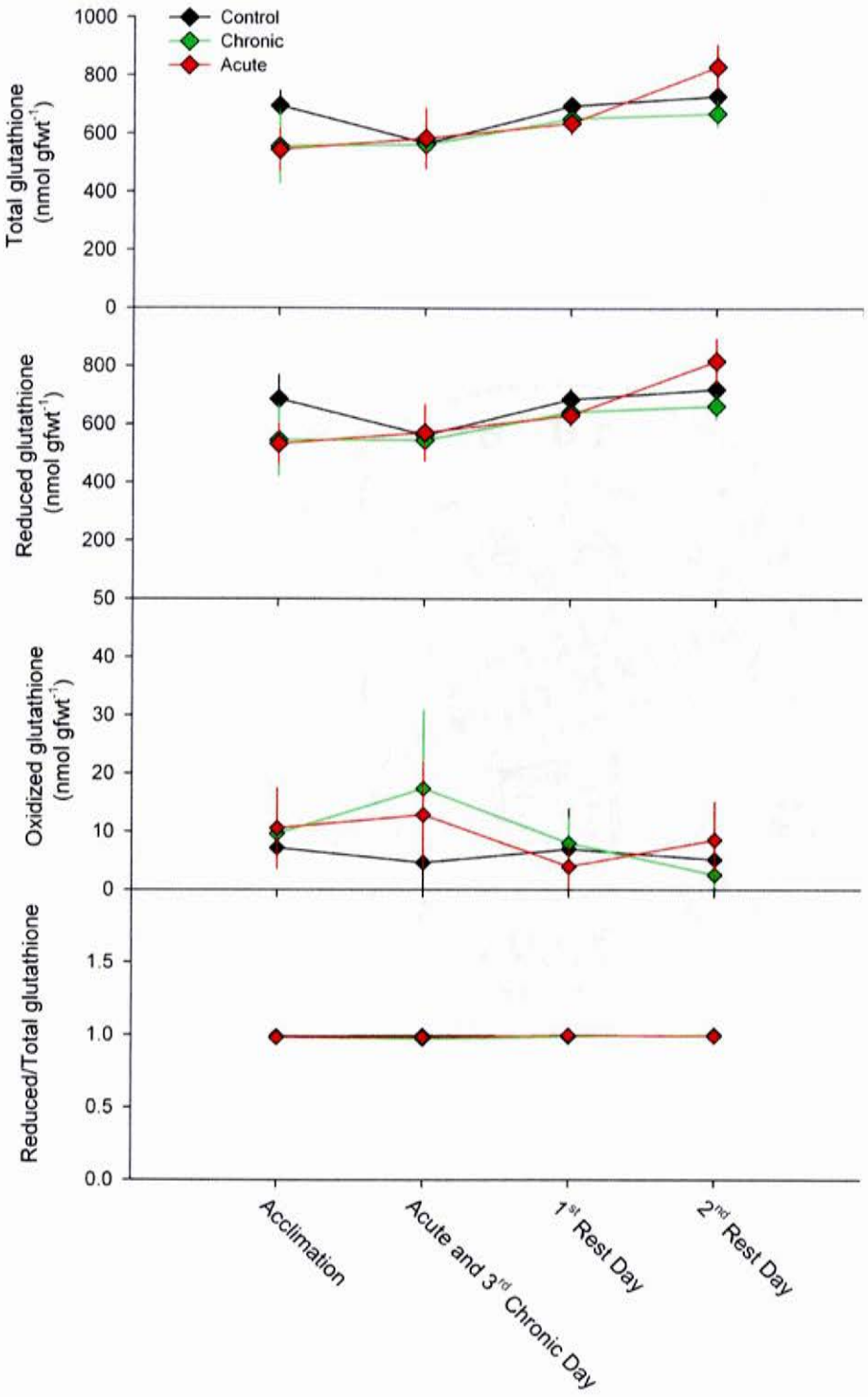
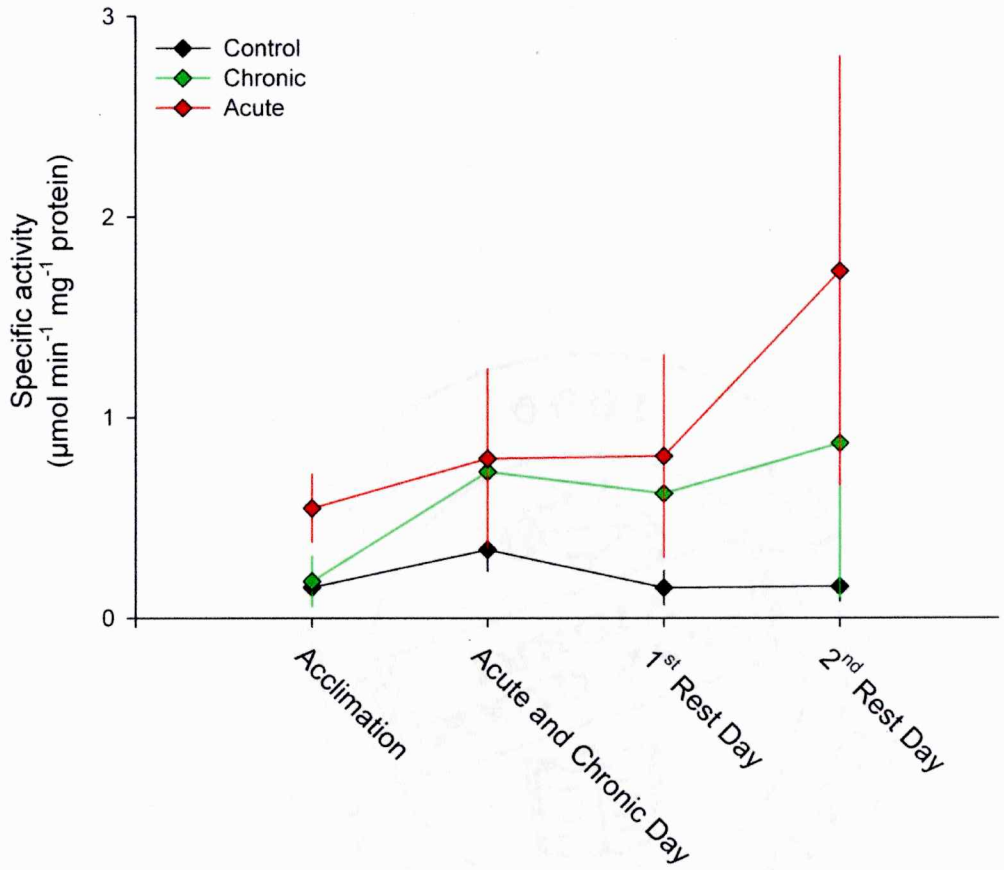


Fig. 9. Guaiacol peroxidase activity (µmol min^{-1} mg^{-1} protein) across treatment days. Values represent mean ± se (n = 3). The unit represents one plant for each of the three O_3 treatments. Each of the three repetitions employed two plants per O_3 treatment with measurements made on one leaf per plant. Results for each leaf were averaged across plants and repetitions.

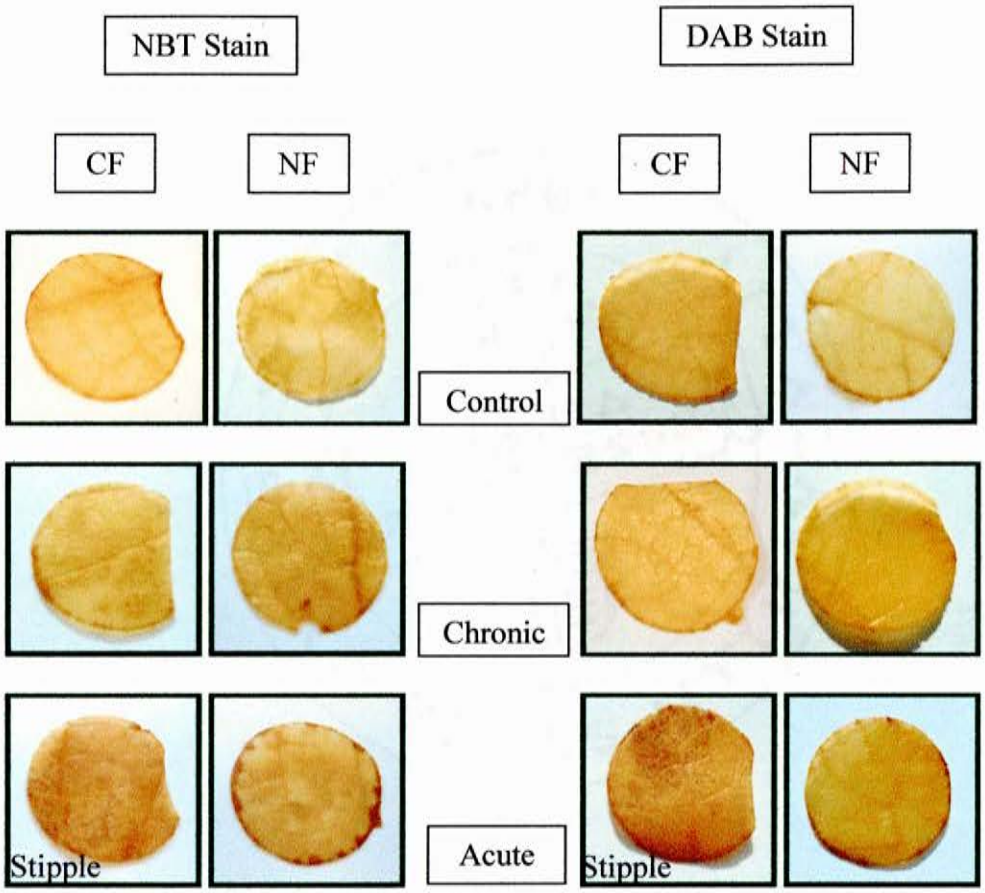


Observation of ROS Accumulation

The results of the DAB and NBT staining procedure were inconclusive. The leaf discs in NBT developed a uniform purpling, which may have been due to incomplete infiltration (Wohlgemuth et al. 2002) resulting possibly from late morning stomatal closure (Rebbeck et al. 2004), or from normal staining of superoxide produced not from O_3, but from other physiological processes, such as mitochondrial functioning (Turrens 1997). A preliminary staining study with DAB recorded development of brown stipple on the small older leaves of two year old saplings exposed to ~200 ppb O_3 for 12 hours, but only on

saplings that had been raised in charcoal filtered air, not on saplings raised in ambient air (Figure 10). In this study, faint stippling could be seen on some DAB stained leaf discs, but given the prevalence of powdery mildew and fungicide related damage, relating the staining unequivocally to O_3 treatment was not possible.

Fig. 10. Leaf discs stained with either (A) nitroblue tetrazolium (NBT) or (B) 3,3'- diaminobenzidine (DAB) from saplings grown in either charcoal filtered air (CF) or non-filtered air (NF) and subsequently exposed to chronic O_3 (~65 ppb x 36 hrs, 12 hrs/day) or acute O_3 (~165 ppb x 12 hrs, 12 hrs/day) or control with NF air.



DISCUSSION

In this study, exposure to short term episodes of acute and chronic O_3 did not significantly affect leaf physiological properties such as g_s or Fv/Fm, or biochemical responses such as the content and redox values of AA or GSH or the activity of GPX. Only F_a was significantly lower by the acute O_3 treatment in comparison to both the chronic and control O_3 treatments.

A major complication of this study was the presence of powdery mildew on the leaves that were used for measurements. Consequently, the results of this study may have been confounded by this infection. As this study was not designed to separate the two stressors, I cannot conclusively state that powdery mildew did not influence any physiological or biochemical parameters, and for that reason, the results must be viewed with some caution. However, powdery mildew is a normal component of the environment where the experiment was performed and two powdery mildew species are known to commonly infect tulip poplar (Hepting 1971), and, consequently, my results reflect the natural situation of exposure to O_3 and powdery mildew simultaneously.

Leaves infected by powdery mildew infection can exhibit impaired stomatal regulation (Gordon and Duniway 1982) and reduced rates of photosynthesis (Rabbinge et al. 1985, Scholes et al. 1994, Nail and Stanley 2004, Moriondo et al. 2005, Swarbrick et al. 2006). Moriondo et al. (2005) state that reductions in CO_2 assimilation in powdery mildew infected leaf tissue and in neighboring non-infected tissue resulted from mildew-induced

stomatal closure. Rabbinge et al. (1985) reported that while the CO_2 assimilation rate was sharply reduced by mildew, the cause of the decline was due to enhanced carboxylation resistance, and that reductions in g_s were the consequence of, but not the cause of, the reduced assimilation rate. Other research has shown that reductions in photosynthesis seen in powdery mildew reactions are a consequence of decreased Rubisco activity resulting from a fungal-induced increase in carbohydrate levels (Scholes et al. 1994, Swarbrick et al. 2006).

In this study, no significant differences were found between g_s values for control, chronic and acute O_3 treatments. Including all repetitions and leaf age, and going from 8 am to 6 pm over the course of a day, g_s in the acute, chronic, and control O_3 treatment ranged from 60 – 500 mmol m^{-2} s^{-1}, 104 – 396 mmol m^{-2} s^{-1}, and 100 – 447 mmol m^{-2} s^{-1}, respectively. These ranges of g_s values are similar to those for uninfected tulip poplar saplings as found by Huang (1992), who reported mean g_s values of old and young leaves exposed to CF air of 0.24 mol m^{-2} s^{-1} and 0.33 mol m^{-2} s^{-1}, respectively. With 2x O_3 exposure, the mean g_s of old leaves was 0.16 mol m^{-2} s^{-1}, and for young leaves it was 0.26 mol m^{-2} s^{-1}, still not significantly different from control leaves. Rebbeck and Loats (1997) recorded similar seasonal mean g_s of tulip poplar saplings of 0.20 mol m^{-2} s^{-1} in CF air, 0.190 mol m^{-2} s^{-1} in 1x O_3, and 0.168 mol m^{-2} s^{-1} in 2x O_3). The strong diurnal pattern that I found, which indicates relatively unimpaired stomatal regulation (Grulke et al. 2007), coupled with g_s values that are similar to those for trees without powdery mildew or O_3 stress, suggest that the powdery mildew infection in my study did not significantly affect g_s. This means that uptake of ozone was not compromised by either treatment-induced reductions in g_s, nor by infection with powdery mildew.

Fv/Fm values were also comparable between the three O_3 treatments, remaining in the unstressed zone between 0.75-0.85 (Bolhàr-Nordenkampf et al. 1989), even during afternoon depressions. However, F_a significantly declined in the acute O_3 treatment compared to both the chronic and control treatments. Area values are thought to represent the activity or size of the plastoquinone pool (Kurreck et al.2000), which is composed of soluble electron carriers that ferry electrons from PSII to the cytochrome b/f complex in the chloroplast electron transport chain. The disturbance of the plastoquinone pool did not significantly influence quantum efficiency (i.e., no changes in Fv/Fm), possibly due to effective quenching mechanisms or antioxidant activities present to absorb excess electrons or minimize ROS accumulation.

Along those same lines, the content and redox values of the antioxidants AA and GSH, along with the activity of GPX, were unresponsive to O_3 treatment, suggesting that either their pool sizes were sufficient to detoxify the O_3-induced ROS or that other detoxifying systems were primarily responsible for the O_3 tolerance of the saplings. The mean level of total AA in the acute O_3 treatment was highest during the acclimation day at 9.4 μmol $gfwt^{-1}$, and then continuously declined to the 2^{nd} rest day at 6.6 μmol $gfwt^{-1}$. Similarly, mean total AA content for the chronic and control O_3 treatment was highest prior to O_3 exposure (8.6 μmol $gfwt^{-1}$, 8.8 μmol $gfwt^{-1}$, respectively) and lowest after two rest days (6.1 μmol $gfwt^{-1}$, 6.6 μmol $gfwt^{-1}$, respectively).

Over the four measurement days, the total AA content of the tulip poplar saplings in this study remained lower than that found for adult field grown Scots pine, whose current-year needle total AA levels spanned ~7 – 23 μmol $gfwt^{-1}$ over one growing season (Haberer et al. 2006). However, my values were much lower compared to tolerant and sensitive

genotypes of quaking aspen, both of which ranged from 10 – 30 μmol gfwt^{-1} when exposed to either 1.7x or 3x O_3 for one growing season (Yun and Laurence 1999). In this study, redox values ranged between 60 – 80% for all treatments, often with the lowest values on acclimation day, indicating no connection of AA content with O_3 exposure. The redox value for total glutathione across the four measurement days stayed very near to 1.0, indicating that the accumulation of reduced AA was not limited by the regeneration of symplastic GSH.

The total glutathione content was higher than that reported by Luwe (1996) for 30 year old field grown beech (*Fagus sylvatica* L.) exposed to ambient O_3, which ranged between 200 – 350 nmol gfwt^{-1} in July, while the redox state held steady between 80 – 90%. With a slight trend in the opposite direction, the mean total glutathione content for the acute O_3 treatment was lowest on acclimation day at 543 nmol gfwt^{-1} and highest on the 2nd rest day at 827 nmol gfwt^{-1}. Likewise, mean total glutathione content for the chronic O_3 treatment was lowest prior to O_3 exposure at 554 nmol gfwt^{-1}, and highest on the 2nd rest day at 667 nmol gfwt^{-1}. Given that no stipple was observed in these experiments, which indicates that ROS were being efficiently scavenged before they could initiate these symptoms, this data indicated that antioxidant activity would have been high in these leaves, but, antioxidant levels remained the same between control and O_3 treatments. Possibly, neither AA nor GSH may be the primary defense mechanisms against O_3 damage in tulip poplar leaves, or background levels of AA and GSH were sufficient to prevent stippling and the impairment of stomatal conductance and photosynthetic efficiency.

GPX activity has been used as a general indicator of oxidative stress in plants (Burkey et al. 2000) but in my study GPX activity was not statistically linked to O_3 concentrations. For both the acute and chronic O_3 treatment, GPX activity was lowest prior

to the onset of O_3 fumigation at 0.55 μmol mg^{-1} protein min^{-1} and 0.18 μmol mg^{-1} protein min^{-1}, respectively, with activity levels rising to peak values on the 2nd rest day to 1.7 μmol mg^{-1} protein min^{-1} and 0.87 μmol mg^{-1} protein min^{-1}, respectively. This GPX activity is similar to that reported by Burkey et al. (2000) who fumigated seven varieties of snap bean with O_3. After a seven day O_3 exposure period, they found GPX activities between 1.2 – 2.6 μmol mg^{-1} protein min^{-1}, which were higher than for non-fumigated plants (0.59 – 1.6 μmol mg^{-1} protein min^{-1}). However, they did not find a significant correlation between O_3 sensitivity and GPX activity.

The lack of any significant GPX activity in tulip poplars in response to the O_3 treatments may indicate an absence of detectable oxidative stress, or that oxidative stress responses might have developed more than 48 hours since the end of the exposure event. Diop et al. (1997) fumigated two cultivars of snap bean for four hours with 120 ppb O_3 and found that the GPX activity in the O_3-tolerant line Groffy peaked 96 hours after fumigation began, rising to ~2 U mg^{-1} protein. In contrast, the GPX activity in the O_3-sensitive line Pinto rose immediately after onset of fumigation and peaked 48 hrs later at ~4 U mg^{-1} protein. If the tulip poplar saplings in my study were moderately O_3 tolerant, it is possible that GPX activity did not respond until after the 48 hour measurement period. Therefore, future studies should follow responses for at least four days after the onset of exposures in order to capture the full response.

The complete absence of stipple formation and DAB staining suggests that defense systems must have prevented accumulation of O_3-induced ROS and PCD. In a prior preliminary study, stipple only formed on leaves of tulip poplar saplings raised in CF air and which were then exposed to ~200 ppb O_3 for 12 hours, while similarly exposed plants that

were raised in ambient air did not develop stipple. This suggests acclimation may be a possible key to O_3 tolerance (Held et al. 1991) although stipple primarily occurred on the older mature leaves, indicating a diminished detoxification capacity with leaf age and/or with increased time of O_3 exposure. Possibly, the indeterminate habit of tulip poplar elevates the susceptibility of aging leaves to adverse environmental impacts through the continuous redistribution of leaf resources into new leaf development (Greitner et al. 1994).

As the saplings in this current study were grown in ambient air (to minimize powdery mildew infection, which seemed to be exacerbated by the open-top chamber environment), those chronic O_3 levels may have been sufficient to induce defense related mechanisms and provide increased O_3 tolerance to high O_3 concentrations (Conklin and Barth 2004). Pea seedlings grown in chronic O_3 showed increased tolerance to high O_3 concentrations (Dickinson et al. 1991). Chronic exposure to O_3 is reported to minimize the production of ethylene that is typically stimulated by high O_3 concentrations, and this would limit stipple development (Dickinson et al. 1991).

Whether induced by pathogens or O_3, the HR stimulates the production of secondary signaling molecules, such as salicylic acid (SA), jasmonic acid, and ethylene, that are required for the development of PCD and SAR (Rao et al. 2000). The progress of SAR is dependent upon SA which produces ROS microbursts that induce microscopic cell death (Kangasjärvi et al. 2005) in distant non-inoculated tissue (Lee and Hwang 2005). It is by this process that viruses, bacteria, and fungal pathogens can elicit increased O_3 tolerance in non-inoculated tissues of a variety of species of plants (Violini 1995).

Yarwood and Middleton (1954) discovered O_3 resistance in Pinto bean leaf tissue inoculated with *Uromyces phaseoli* (Pers.) and also in neighboring non-inoculated tissue.

Davis and Smith (1976) and Vargo et al. (1978) reported an increase in O_3 protection in non-inoculated primary leaves that were opposite those of inoculated leaves on pinto bean and soybean (*Glycine max* L.), respectively. Inoculating lower pepper leaves with the compatible strain Bv5-4a of *Xanthomonas. campestris* pv. *vesicatoria*, created disease resistance to the incompatible strain Ds1 in the upper leaves of pepper that were not inoculated (Lee and Hwang 2005). Both powdery mildew and prior exposure to chronic O_3 may have stimulated the defensive systems of the tulip poplar saplings and increased the level of O_3 tolerance to the short term episodes of high O_3 concentrations used in this study.

The broad range of O_3 response in tulip poplar has been well noted over the years (Rebbeck and Loats 1997). Tulip poplar was labeled O_3-tolerant by Jensen (1973) who found no growth responses after one year old potted seedlings were fumigated with 250-350 ppb O_3 for five months. Davis and Skelly (1992) reported that fumigation with 150 ppb O_3 for eight weeks was needed to elicit foliar injury on potted two year old tulip poplar saplings, whereas 75 ppb O_3 for 12 weeks failed to elicit any injury. In addition, Rebbeck and Loats (1997) found negligible foliar injury on potted tulip poplar saplings exposed to 1.7x O_3 for two seasons. Huang (1992) only found foliar injury on one and two year old tulip poplar saplings in the 1.5x and 2.0x O_3 treatments in open-top chambers in the Smokies.

In contrast, both Duchelle et al. (1982) and Simini et al. (1992), who studied field planted tulip poplar saplings, reported foliar injury occurring in ambient O_3. Hildebrand et al. (1996) and Chappelka et al. (1999) supported the classification of tulip poplars as O_3-sensitive due to the induction of foliar injury on mature field grown tulip poplars exposed to ambient O_3. The inconsistent responses of tulip poplars to O_3 may be, at least, dependent upon differences in methodology, e.g. whether the plants are potted or field planted and

whether the plants are saplings or mature trees (Rebbeck 1996, Loats and Rebbeck 1999). However, methodology cannot explain the different sensitivities of tulip poplar to O_3, as other experiments using potted tulip poplar saplings have recorded development of severe foliar injury (Tjoelker and Luxmoore 1991)

Rebbeck (1996) proposed that the contradictory responses of tulip poplars to O_3 could result from different sensitivities of populations across its distributional range. Tulip poplar may consist of populations of O_3-sensitive and O_3-tolerant trees, as has been found for aspen and white pine (Karnosky et al. 2007). Somers et al. (1998) found mixed populations of mature O_3-sensitive and O_3-tolerant tulip poplars in Great Smoky Mountains National Park that consistently differed in the levels of foliar injury induced by ambient O_3. Trees that reliably developed foliar injury showed significant growth declines over a five year period when compared to trees that were asymptomatic. This shows that tulip poplar may contain considerable genetic variability among individuals in the sensitivity to this pollutant, even within a single geographic area. Additionally, O_3 concentrations may be a determinant of the levels of O_3 sensitivity found in certain tree species. In support of this, Berrang et al. (1991) claim that ambient O_3 concentrations are positively correlated with aspen O_3 resistance; areas with high O_3 are dominated by O_3 tolerant aspen, while areas without high O_3 contain both sensitive and tolerant clones. Additional research has reported similar results in areas of high O_3 for populations of red maple (Dickinson et al. 1991) white pine (*Pinus strobus* L.) (Armentano and Menges 1987, Bennett et al. 1994), quaking aspen, (Dickinson et al. 1991, Heagle et al. 1991, Chappelka and Samuelson 1998) green ash (*Fraxinus pennsylvanica* Marsh.) and white ash (*F. americana* L.) (Chappelka and Samuelson 1998).

However, anecdotal evidence suggests that the source population for the saplings in my study was not completely from O_3-tolerant stock. Trees from my study that were planted outside after the experiments in the Boone, NC area have shown foliar stipple during the summer of 2007, which suggests that my trees may have been a mix of O_3-sensitive and O_3-tolerant saplings. Their mixed responses to the O_3 treatments may have inflated the error variances such that no statistically detectable differences could be found, especially given the small sample sizes (a function of the small number of fumigation chambers and time constraints in which to conduct the study). The error variance idea is partially supported by the occurrence of the abnormal data for repetition 2, which may have contained trees that differed genetically from those in the other two repetitions.

CONCLUSION

Research using short term hourly O_3 exposures is important in order to discern the immediate response to O_3 by plants and how those initial reactions lead to the late stage responses found after extended exposures. This study suggests that tulip poplar saplings are relatively insensitive to short-term O_3 episodes, at least when infected by powdery mildew, even when the hourly concentrations are high, and that multiple episodes appear to be necessary to induce visible and adverse physiological and biochemical effects. Future research should extend the post-exposure measurement period to at least 96 hours, in case responses take a long time to develop, and to investigate additional anti-oxidants to determine their importance to the ability of this species to tolerate high concentrations of O_3.

GLOSSARY

AA	Ascorbic acid
APX	Ascorbate peroxidase
CAT	Catalase
DAB	3,3'-diaminobenzidine
DHA	Dehydroascorbic acid
F_a	Area over fluorescence induction curve reflective of plastoquinone pool size
GPX	Guaiacol peroxidase
g_s	Stomatal conductance
GSH	Reduced glutathione
GSSG	Oxidized glutathione, glutathione disulfide
H_2O_2	Hydrogen peroxide
HR	Hypersensitive response
NBT	Nitroblue tetrazolium
$\cdot O_2^-$	Superoxide anion
$\cdot O_2H$	Hydroperoxide
O_3	Ozone
$\cdot OH$	Hydroxyl radical
PAR	Photosynthetically active radiation
PCD	Programmed cell death
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	Systemic acquired resistance
SUM00	Sum of O_3 concentrations > 0 ppb
SUM60	Sum of O_3 concentrations > 60 ppb

REFERENCES

Armentano, T.V., and Menges, E.S. 1987. Air-pollution-induced foliar injury to natural populations of jack and white pine in a chronically polluted environment. Water, Air, and Soil Pollution 33: 395-409.

Beck, D.E. 1990. *Liriodendron tulipifera* L. yellow poplar. *In* Silvics of North America Volume 2, Hardwoods. Agricultural Handbook 654. *Edited by* R.M Burns. and B.H. Honkala. U.S.D.A. Forest Service. Washington, D.C. pp. 406-416.

Bennett, J.P., Anderson, R.L., Mielke, M.L., and Ebersole J.J. 1994. Foliar injury air pollution surveys of eastern white pine (*Pinus strobus* L.). Environmental Monitoring and Assessment 30: 247-274.

Berrang, P., Karnosky, D.F., and Bennett, J.P. 1991. Natural selection for ozone tolerance in *Populus tremuloides*: an evaluation of nationwide trends. Canadian Journal of Forest Research 21: 1091-1097.

Bolhàr-Nordenkampf, H.R., Long, S.P., Baker, N.R., Öquist, G., Schreiber, U., and Lechner, E.G. 1989. Chlorophyll fluorescence as a probe of the photosynthetic competence of leaves in the field: a review of current instrumentation. Functional Ecology 3: 497-514.

Brace S., Peterson, D.L., and Bowers, D. 1999. A guide to ozone injury in vascular plants of the Pacific Northwest. General Technical Report PNW-GTR-446. U.S.D.A. Forest Service. Portland, Oregon. pp. 1-9.

Burkey, K.O., Wei, C., Eason, G., Ghosh, P., and Fenner, G. 2000. Antioxidant metabolite levels in ozone-sensitive and tolerant genotypes of snap bean. Physiologia Plantarum 110: 195-200.

Burkey, K.O., Eason, G., and Fiscus, E.L. 2003. Factors that affect leaf extracellular ascorbic acid content and redox status. Physiologia Plantarum 117: 51-57.

Burkey, K.O., Neufeld, H.S., Souza, L., Chappelka, A.H., and Davison, A.W. 2006. Seasonal profiles of leaf ascorbic acid content and redox state in ozone-sensitive wildflowers. Environmental Pollution 143: 427-434.

Castillo, F.J., and Greppin, H. 1988. Extracellular ascorbic acid and enzyme activities related to ascorbic acid metabolism in *Sedum album* L. leaves after ozone exposure. Environmental and Experimental Botany 28: 231-238.

Chappelka, A.H., Chevone, B.I., and Seiler, J.R. 1988. Growth and physiological responses of yellow-poplar seedlings exposed to ozone and simulated acidic rain. Environmental Pollution 49: 1-8.

Chappelka, A.H., and Samuelson, L.J. 1998. Ambient ozone effects on forest trees of the eastern United States: a review. New Phytologist 139: 91-108.

Chappelka, A., Somers, G., and Renfro, J. 1999. Visible ozone injury on forest trees in Great Smoky Mountains National Park. Water, Air, and Soil Pollution 116: 255-260.

Chen, A., and Gallie, D.R. 2005. Increasing tolerance to ozone by elevating foliar ascorbic acid confers greater protection against ozone than increasing avoidance. Plant Physiology 138: 1673-1689.

Chernikova, T., Robinson, J.M., Lee, E.H., and Mulchi, C.L. 2000. Ozone tolerance and antioxidant enzyme activity in soybean cultivars. Photosynthesis Research 64: 15-26.

Conklin, P.L., Williams, E.H., and Last, R.L. 1996. Environmental stress sensitivity of an ascorbic acid-deficient Arabidopsis mutant. Proceedings of the National Academy of Sciences of the United States of America 93: 9970-9974.

Conklin, P.L., and Barth, C. 2004. Ascorbic acid, a familiar small molecule intertwined in the response of plants to ozone, pathogens, and the onset of senescence. Plant, Cell and Environment 27: 959-970.

Davis, D.D., and Smith, S.H. 1976. Reduction of ozone sensitivity of pinto bean by virus-induced local lesions. Plant Disease Reporter 60: 31-34.

Davis, D.D., and Skelly, J.M. 1992. Foliar sensitivity of eight eastern hardwood tree species to ozone. Water, Air, and Soil Pollution 62: 269-277.

Degl'Innocenti, E., Guidi, L., and Soldatini, G.F. 2007. Effects of elevated ozone on chlorophyll *a* fluorescence in symptomatic and asymptomatic leaves of two tomato genotypes. Biologia Plantarum 51: 313-321.

D'Haese, D., Vandermeiren, K., Asard, H., and Horemans, N. 2005. Other factors than apoplastic ascorbate contribute to the differential ozone tolerance of two clones of *Trifolium repens* L. Plant, Cell and Environment 28: 623-632.

Dickinson, N.M., Turner, A.P., and Lepp, N.W. 1991. How do trees and other long-lived plants survive in polluted environments? Functional Ecology 4: 5-11.

Diop, C.T., Nali, C., Messsori, L., Heimler, D., and Lorenzini, G. 1997. Peroxidase and superoxide dismutase activities in bean in response to ozone. Journal of Plant Pathology 79: 107-113.

Duchelle, S.F., Skelly, J.M., and Chevone, B.I. 1982. Oxidant effects on forest tree seedling growth in the Appalachian Mountains. Water, Air, and Soil Pollution 18: 363-373.

Edreva, A. 2005. Generation and scavenging of reactive oxygen species in chloroplast: a submolecular approach. Agriculture, Ecosystems and Environment 106: 119-133.

Foyer, C.H. 1993. Ascorbic acid. *In* Antioxidants in Higher Plants. *Edited by* R.G. Alscher and J.L. Hess CRC Press. Boca Raton, FL. pp 31-58.

Foyer, C.H., and Noctor, G. 2005. Oxidant and antioxidant signaling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. Plant, Cell and Environment 28: 1056-1071.

Francini, A., Nali, C., Picchi, V., and Lorenzini, G. 2007. Metabolic changes in white clover clones exposed to ozone. Environmental and Experimental Botany 60: 11-19.

Fuhrer, J., and Booker, F. 2003. Ecological issues related to ozone: agricultural issues. Environment International 29: 141-154.

Gordon, T.R., and Duniway, J.M. 1982 Stomatal behavior and water relations in sugar beet *Beta vulgaris* leaves infected by *Erysiphe polygoni*. Phytopathology 72: 723-726.

Greitner, C.S., Pell, E.J., and Winner, W.E. 1994. Analysis of aspen foliage exposed to multiple stresses: ozone, nitrogen deficiency and drought. New Phytologist 127: 579-589.

Griffith, O.W. 1980. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Analytical Biochemistry 106: 207-212.

Grulke, N.E., Neufeld, H.S., Davison, A.W., Roberts, M., and Chappelka, A.H. 2007. Stomatal behavior of ozone-sensitive and –insensitive coneflowers (*Rudbeckia laciniata* var. *digitata*) in Great Smoky Mountains National Park. New Phytologist 173: 100-109.

Guéra, A., Calatayud, A., Sabater, B., and Barreno, E. 2005. Involvement of the thylakoidal NADH-plastoquinone-oxidoreductase complex in the early responses to ozone exposure of barely (*Hordeum vulgare* L.) seedlings. Journal of Experimental Botany 56: 205-218.

Guidi, L., Degl'Innocenti, E., and Soldatini, G.F. 2002. Assimilation of CO_2, enzyme activation and photosynthetic electron transport in bean leaves, as affected by high light and ozone. New Phytologist 156: 377-388.

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.

Guzy, M.R., and Heath, R.L. 1993. Responses to ozone of varieties of common bean (*Phaseolus vulgaris* L.). New Phytologist 124: 617-625.

Haberer, K., Jaeger, L., and Rennenberg, H. 2006. Seasonal patterns of ascorbate in the needles of Scots Pine (*Pinus sylvestris* L.) trees: correlation analyses with atmospheric O_3 and NO_2 gas mixing ratios and meteorological parameters. Environmental Pollution 139: 224-231.

Harkov, R.S., and Brennan, E. 1981. An ecophysiological analysis of the response of woody and herbaceous plants to oxidant injury. Journal of Environmental Management 15: 251-261.

Heagle, A.S., McLaughlin, M.R., Miller, J.E., Joyner, R.L., and Spruill, S.E. 1991. Adaptation of a white clover population to ozone stress. New Phytologist 119: 61-68.

Held, A.A., Mooney, H.A., and Gorham, J.N. 1991. Acclimation to ozone stress in radish: leaf demography and photosynthesis. New Phytologist 118: 417-423.

Hepting, G.H. 1971. Diseases of forest and shade trees of the United States. U.S.D.A. Forest Service, Agriculture Handbook Number 386. Washington, D.C. pp. 658.

Herbinger, K., Tausz, M., Wonisch, A., Soja, G., Sorger, A., and Grill, D. 2002. Complex interactive effects of drought and ozone stress on the antioxidant defense systems of two wheat cultivars. Plant Physiology and Biochemistry 40: 691-696.

Herbinger, K., Then, C., Löw, M., Haberer, K., Alexous, M., Koch, N., Remele, K., Heerdt, C., Grill, D., Rennenber, H., Häberle, K.H., Matyssek, R., Tausz, M., and Wieser G. 2005. Tree age dependence and within-canopy variation of leaf gas exchange and antioxidant defence in *Fagus sylvatica* under experimental free-air ozone exposure. Environmental Pollution 137: 476-482.

Hildebrand, E., Skelly, J.M., and Fredericksen, T.S. 1996. Foliar response of ozone-sensitive hardwood tree species from 1991 to 1993 in the Shenandoah National Park, Virginia. Canadian Journal of Forest Research 26: 658-669.

Huang, S. 1992. Response of growth and gas exchange of tulip poplar (*Liriodendron tulipifera* L.) seedlings to ozone exposure. Masters Thesis. pp. 117.

Hückelhoven, R., Fodor, J., Trujillo, M., and Kogel, K.H. 2000. Barely *Mla* and *Rar* mutants compromised in the hypersensitive cell death response against *Blumeria graminis* f.sp. *hordei* are modified in their ability to accumulate reactive oxygen intermediates at sites of fungal invasion. Planta 212: 16-24.

Jabs, T., Deitrich, R.A., and Dangl, J.L. 1996. Initiation of runaway cell death in an *Arabidopsis* mutant by extracellular superoxide. Science 273: 1853-1856.

Jensen, K.F. 1973. Response of nine forest tree species to chronic ozone fumigation. Plant Disease Reporter 57: 914-916.

Kangasjärvi, J., Jaspers, P., and Kollist, H. 2005. Signalling and cell death in ozone-exposed plants. Plant, Cell and Environment 28: 1021-1036.

Karnosky, DF., Skelly, J.M., Percy, K.E., and Chappelka, A.H. 2007. Perspectives regarding 50 years of research on effects of tropospheric ozone air pollution on US forests. Environmental Pollution 147: 489-506.

Kronfuss, G., Wieser, G., Havranek, W.M., and Polle, A. 1996. Effects of ozone and mild drought stress on total and apoplastic guaiacol peroxidase and lipid peroxidation in current-year needles of young Norway spruce (*Picea abies* L., Karst.). Journal of Plant Physiology 148: 203-206.

Krupa, S., McGrath, M.T., Andersen, C.P., Booker, F.L., Burkey, K.O., Chappelka, A.H., Chevone, B.I., Pell, E.J., and Zilinskas, B.A. 2000. Ambient ozone and plant health. Plant Disease 85: 4-12.

Kurreck, J., Schödel, R., and Renger, G. 2000. Investigation of the plastoquinone pool size and fluorescence quenching in thylakoid membranes and photosystem II (PSII) membrane fragments. Photosynthesis Research 63:171-182.

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, G., Wohlgemuth, H., Kschieschan, S., Grün, S., and Sandermann, H. 2002. Oxidative burst and cell death in ozone-exposed plants. Plant Physiology and Biochemistry 40:567-575.

Lee, S.C., and Hwang, B.K. 2005. Induction of some defense-related genes and oxidative burst is required for the establishment of systemic acquired resistance in *Capsicum annuum*. Planta 221: 790-800.

Lefohn, A.S., and Runeckles, V.C. 1987. Establishing standards to protect vegetation-ozone exposure/dose considerations. Atmospheric Environment 21: 561-568.

Loats, K.V., and Rebbeck, J. 1999. Interactive effects of ozone and elevated carbon dioxide on the growth and physiology of black cherry, green ash, and yellow-poplar seedlings. Environmental Pollution 106: 237-248.

Long, S.P., and Naidu, S.L. 2002. Effects of oxidants at the biochemical, cell and physiological levels, with particular reference to ozone. *In* Air Pollution and Plant Life 2nd Edition. *Edited by* J.N.B. Bell and M. Treshow. John Wiley and Sons, Ltd., Chichester. England. pp. 70 – 88.

Luwe, M. 1996. Antioxidants in the apoplast and symplast of beech (*Fagus sylvatica* L.) leaves: seasonal variations and responses to changing ozone concentrations in air. Plant, Cell and environment 19: 321-328.

Mittler, R. 2002. Oxidative stress, antioxidants and stress tolerance. Trends in Plant Science 7: 405-410.

Moraes, R.M., Furlan, C.M., Bulbovas, P., Domingos, M., Meirelles, S.T., Salatino, A., Delitti, W.B.C., and Sanz, M.J. 2004. Photosynthetic responses of tropical trees to short-term exposure to ozone. Photosynthetica 42: 291-293.

Moriondo, M., Orlandini, S., Guintoli, A., and Bindi, M. 2005. The effect of downy and powdery mildew on grapevine (*Vitis vinifera* L.) leaf gas exchange. Journal of Phytopathology 153: 350-357.

Musselman, R.C., Younglove, T., and McCool, P.M. 1994. Response of *Phaseolus vulgaris* L. to differing ozone regimes having identical total exposure. Atmospheric Environment 28: 2727-2731.

Musselman, R.C., Lefohn, A.S., Massman, W.J., and Heath, R.L. 2006. A critical review and analysis of the use of exposure- and flux-based ozone indices for predicting vegetation effects. Atmospheric Environment 40: 1869-1888.

Nail, W.R., and Stanley, H.G. 2004. Effects of powdery mildew of grape on carbon assimilation mechanism of potted 'Chardonnay' grapevines. Hortscience 39: 1670-1673.

Nali, C., Pucciariello, C., Mills, G., and Lorenzini, G. 2005. On the different sensitivity of white clover clones to ozone: physiological and biochemical parameters in a multivariate approach. Water, Air, and Soil Pollution 164: 137-153.

Neufeld, H.S., Renfro, J.R., Huang, S., Hacker, W.D., Mangis, D., Chappelka, A.H., Hogsett, W.E., Herstrom, A.A., Laurence, J.A., Lee, E.H., Weber, J.E., and Tingey, D.T. 1993. Assessing ozone effects on plants native to the southeastern United States. Proceedings of XI International Association of Botanic Gardens Conference. China Agriculture Press. Beijing, China. pp. 105 – 122.

Neufeld, H.S., Lee, E.H., Renfro, J.R., Hacker, W.D., and Yu, B. 1995. Sensitivity of seedlings of black cherry (*Prunus serotina* Ehrh.) to ozone in Great Smoky Mountains National Park. New Phytologist 130: 447-459.

Neufeld, H.S., Lee, E.H., Renfro, J.R., and Hacker, W.D. 2000. Seedling insensitivity to ozone for three conifer species native to Great Smoky Mountains National Park. Environmental Pollution 108: 141-151.

Noctor, G., and Foyer, C.H. 1998. Ascorbate and glutathione: keeping active oxygen under control. Annual Review of Plant Physiology and Molecular Biology 49: 249-279.

Overmyer, K., Brosché, M., and Kangarjärvi, J. 2003. Reactive oxygen species and hormonal control of cell death. Trends in Plant Science 8: 335-342.

Pasqualini, S., Batini, P., Ederli, L., Porceddu, A., Piccioni, C., De Marchis, F., and Antonielli, M. 2001. Effects of short-term ozone fumigation on tobacco plants: response of the scavenging system and expression of the glutathione reductase. Plant, Cell and Environment 24: 245-252.

Pell, E.J., Schlagnhaufer, C.D., and Arteca, R.N. 1997. Ozone-induced oxidative stress: mechanisms of action and reaction. Physiologia Plantarum 100: 264-273.

Pellinen, R., Palva, T., and Kangasjärvi, J. 1999. Subcellular localization of ozone-induced hydrogen peroxide production in birch (*Betula pendula*) leaf cells. The Plant Journal 20: 349-356.

Pino, M.E., Mudd, J.B., and Bailey-Serres, J. 1995. Ozone-induced alterations in the accumulation of newly synthesized proteins in leaves of maize. Plant Physiology 108: 777-785.

Polle, A. 2001. Dissecting the superoxide dismutase-ascorbate-glutathione-pathway in chloroplasts by metabolic modeling. Computer simulations as a step towards flux analysis. Plant Physiology 126: 445-462.

Prasad, T.K., Anderson, M.D., and Sterwart, C.R. 1995. Localization and characterization of peroxidases in the mitochondria of chilling-acclimated maize seedlings. Plant Physiology 108: 1597-1605.

Rabbinege, R., Jorritsma, I.T.M., and Schans, J. 1985. Damage components of powdery mildew in winter wheat. Netherlands Journal of Plant Pathology 91: 235-247.

Ranieri, A., D'Urso, G., Naili, C., Lorenzini, G., and Soladatini, G.F. 1996. Ozone stimulates apoplastic antioxidant systems in pumpkin leaves. Physiologia Plantarum 97: 381-387.

Ranieri, A., Petacco, F., Castagna, A., and Soldatini, G.F. 2000. Redox state and peroxidase system in sunflower plants exposed to ozone. Plant Science 159: 159-167.

Ranieri, A., Castagna, A., Pacini, J., Baldan, B., Sodi A.M., and Soldatini, G.F. 2003. Early production and scavenging of hydrogen peroxide in the apoplast of sunflower plants exposed to ozone. Journal of Experimental Botany 54: 2529-2540.

Rao, M.V., Koch, J.R., and Davis, K.R. 2000. Ozone: a tool for probing programmed cell death in plants. Plant Molecular Biology 44: 345-358.

Rebbeck, J. 1996. Chronic ozone effects on three northeastern hardwood species: growth and biomass. Canadian Journal of Forest Research 26: 1788-1798.

Rebbeck, J., and Loats, K.V. 1997. Ozone effects on seedling sugar maple (*Acer saccharum*) and yellow-poplar (*Liriodendron tulipifera*): gas exchange. Canadian Journal of Forest Research 27: 1595-1605.

Rebbeck, J., and Scherzer, A.J. 2002. Growth responses of yellow-poplar (*Liriodendron tulipifera* L.) exposed to 5 years of O_3 alone or combined with elevated CO_2. Plant, Cell and Environment 25: 1527-1537.

Rebbeck, J., Scherzer, A.J., and Loats, K.V. 2004. Foliar physiology of yellow-poplar (*Liriodendron tulipifera* L.) exposed to O_3 and elevated CO_2 over five seasons. Trees 18: 253-263.

Sanmartin, M., Drogoudi, P.D., Lyons, T., Pateraki, I., Barnes, J., and Kanellis, A.K. 2003. Over-expression of ascorbate oxidase in the apoplast of transgenic tobacco results in altered ascorbate and glutathione redox states and increased sensitivity to ozone. Planta 216: 918-929.

Scebba, F., Soldatini, G., and Ranieri, A. 2003. Ozone differentially affects physiological and biochemical responses of two clover species; *Trifolium repens* and *Trifolium pratense*. Environmental Pollution 123: 209-216.

Scholes, J.D., Lee, P.J., Horton, P., and Lewis, D.H. 1994. Invertase: understanding changes in the photosynthetic and carbohydrate metabolism of barley leaves infected with powdery mildew. New Phytologist 126: 213-222.

Schraudner, M., Moeder, W., Wiese, C., Van Camp, W., Inzé, D., Langebartels, C., and Sandermann, Jr. H. 1998. Ozone-induced oxidative burst in the ozone biomonitor plant, tobacco Bel W3. The Plant Journal 16: 235-245.

Sheng, Y., Podila, G.K., and Karnosky, D.F. 1997. Differences in O_3-induced superoxide dismutase and glutathione antioxidant expression in O_3 tolerant and sensitive trembling aspen (*Populus tremuloides* Michx.) clones. Forest Genetics 4: 25-33.

Simini, M., Skelly, J.M., Davis, D.D., and Savage, J.E. 1992. Sensitivity of four hardwood species to ambient ozone in north central Pennsylvania. Canadian Journal of Forest Research 22: 1789-1799.

Smirnoff, N. 1996. The function and metabolism of ascorbic acid in plants. Annals of Botany 78: 661-669.

Smirnoff, N. (2000) Ascorbic acid: metabolism and functions of a multi-facetted molecule. Current Opinion in Plant Biology 3: 229-235.

Somers G.L., Chappelka A.H., Rosseau P., and Renfro J.R. 1998. Empirical evidence of growth decline related to visible ozone injury. Forest Ecology and Management 104: 129-137.

Strohm M., Eiblmeier M., Langebartels C., Jouanin L., Polle, A., Sandermann, H., and Rennenberg, H. 2002. Responses of antioxidative systems to acute ozone stress in transgenic poplar (*Populus tremula* x *P. alba*) over-expressing glutathione synthetase or glutathione reductase. Trees 16: 262-273.

Swarbrick, P.J., Schulze-lefert, P., and Scholes, J.D. 2006. Metabolic consequences of susceptibility and resistance (race-specific and broad-spectrum) in barley leaves challenged with powdery mildew. Plant, Cell and Environment 29: 1061-1076.

Thordal-Christensen, H., Z.G. Zhang, Y.D. Wei and D.B. Collinge. 1997. Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. Plant Journal 11: 1187-1194.

Tietze, F. 1969. Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione: Applications to mammalian blood and other tissues. Analytical Biochemistry 27: 502-522.

Tjoelker, M.G., and Luxmoore, R.J. 1991. Soil nitrogen and chronic ozone stress influence physiology, growth and nutrient status of *Pinus taeda* L. and *Liriodendron tulipifera* L. seedlings. New Phytologist 119: 69-81.

Treshow, M., and Bell, J.N.B. 2002. Historical perspectives. *In* Air Pollution and Plant Life 2nd Edition. *Edited by* J.N.B. Bell and M. Treshow. John Wiley and Sons Ltd. Chichester, England. pp. 465.

Turcsányi, E., Lyons, T., Plöchl, M., and Barnes, J. 2000. Does ascorbate in the mesophyll cell walls form the first line of defense against ozone? Testing the concept using broad bean (*Vicia faba* L.). Journal of Experimental Botany 51: 901-910.

Turrens, J.F. 1997. Superoxide production by the mitochondrial respiratory chain. Bioscience Reports 17: 3-8.

van Hove, L.W.A., Bossen, M.E., San Gabino, B.G., and Sgreva, C. 2001.The ability of apoplastic ascorbate to protect poplar leaves against ambient ozone concentrations: a quantitative approach. Environmental Pollution 114: 371-382.

Vargo, R.H., Pell, E.J., and Smith, S.H. 1978. Induced resistance to ozone injury of soybean by tobacco ringspot virus. Phytopathology 68: 715-719.

Violini, G. 1995. Ozone and plant pathogens: an overview. Rivista di Patologia Vegetale 5: 113-130.

Wang, D., Hinckley, T.M., Cumming, A.B., and Braatne, J. 1995. A comparison of measured and modeled ozone uptake into plant leaves. Environmental Pollution 89: 247-254.

Wieser, G., Havranek, W.M., Loidoldt-Nagele, M., Kronfuss, G., and Polle, A. 1998. Responses of photosynthesis, carbohydrates and antioxidants in needles of Norway spruce to slow and rapid changes in ozone. Botánica Acta 111: 35-41.

Wohlgemuth, H., Mittelstrass, K., Kschieschan, S., Bender, J., Weigel, H.J., Overmyer, K., Kangasjärvi, J., Sandermann, H., and Langebartels, C. 2002. Activation of an oxidative burst is a general feature of sensitive plants exposed to the air pollutant ozone. Plant, Cell and Environment 25: 717-726.

Woo, S.Y., Lee, S.H., Kwon, K.W., Lee, J.C., and Choi, J.H. 2004. Growth, photosynthesis, and ascorbate peroxidase activity of several species to the ozone exposure. Journal of Korean Forestry Society 93: 409-414.

Yarwood, C.E., and Middleton, J.T. 1954. Smog injury and rust infection. Plant Physiology 29: 393–395.

Yun, S.C., and Laurence, J.A. 1999. The response of clones of *Populus tremuloides* differing in sensitivity to ozone in the field. New Phytologist 141: 411-421.

Zheng, Y., Lyons, T., Ollerenshaw, J.H., and Barnes, J.D. 2000. Ascorbate in the leaf apoplast is a factor mediating ozone resistance in *Plantago major*. Plant Physiology and Biochemistry 38: 403-411.

BIOGRAPHICAL INFORMATION

Ann Gretchen Huyler was born on December 27, 1966, in Belleville, O'Fallon, Illinois, making a triplicate from a doublet of adopted fraternal twins, Jennifer and Susan, born almost exactly 3 months prior. Schooling through high school took place in Arden, North Carolina, and college experiences, over the course of 15 years, developed at Warren Wilson College, Asheville Buncombe Technical College, and University of North Carolina at Asheville (twice), with the final capture of an undergraduate degree in Environmental Science at UNCA occurring in 2004. A biology Masters degree was slowly completed at Appalachian State University, Boone, NC, by the end of 2008, during which a doctorate in Forestry was begun at Auburn University, Auburn, Alabama in August, 2007. Presently, Ann is investigating soil carbon pools in residential areas in Auburn, and plans to finish her dissertation prior to entering a nursing home, where she can read and chat with friends at her leisure.