PLANT RESPONSES TO A CHANGING ATMOSPHERE: THE INFLUENCE OF EXPOSURE TO MULTIPLE GASES

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PLANT RESPONSES TO A CHANGING ATMOSPHERE: THE INFLUENCE OF EXPOSURE TO MULTIPLE GASES

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The overarching goal of the research presented here was to explore how the composition of the future atmosphere will affect the growth and performance of plants. Pursuant to this goal, I determined the single and combined effects of elevated carbon dioxide (CO_2), nitrogen dioxide (NO_2), ozone (O_3), and soil deposited nitrate (NO_3^-) on seedlings of sugar maple (*Acer saccharum*), eastern hemlock (*Tsuga canadensis*), trembling aspen (*Populus tremuloides*), red oak (*Quercus rubra*), and the model annual, *Arabidopsis thaliana*. The chemistry of Earth's atmosphere is changing, largely due to human activities and these changes include rising concentrations of CO_2, NO_2, and O_3. In order to determine how plants will perform under these likely future atmospheric conditions, we need to understand the mechanisms controlling the entry and elimination of these gases in plant leaves and determine how these gases alter growth, chemistry, and phenology of plants when applied alone and in combinations. I determined the relative importance of physical and chemical processes in controlling the leaf-level fluxes of NO_2 and O_3 by pairing leaf-level flux measurements with measurements of stomatal conductance, ascorbate concentration, and nitrate reductase activity and using these measurement to build multiple regression models. These models determined that stomatal conductance was the dominant controller of both NO_2 and O_3 fluxes, explaining 84 and 56 % of the variance in NO_2 and O_3 fluxes, respectively. The addition of ascorbate concentration was particularly useful in the model for O_3 fluxes where it explained an additional 10 % of the variance. Based on the findings of the modeling study, I predicted that any

treatment causing a change in stomatal conductance would likely impact the magnitude of the plant responses to NO_2 and O_3. From 2004-2007, I conducted field experiments using open-top chambers to expose plants to combinations of elevated CO_2, NO_2, O_3, and soil NO_3^-. The three most important findings from these experiments were: 1) The combination of elevated CO_2, NO_2, and O_3 rarely resulted in a change in plant biomass even if the treatments individually did alter biomass, 2) Even when elevated CO_2 did not increase overall biomass, it did alter leaf chemistry and structure by decreasing specific leaf area and % leaf nitrogen while increasing leaf C:N, and 3) Under elevated CO_2, elevated O_3 significantly delayed the production of flowers and pods in addition to decreasing the overall reproductive output of the model annual, *Arabidopsis thaliana*. These findings suggest that current models predicting an increase in tree seedling growth under elevated CO_2 may be overestimating the potential biomass production because they do not account for the effects of elevated NO_2 and O_3. These results also suggest that changes in phenology and leaf structure and chemistry may be greater than the changes in overall plant biomass and deserve greater attention from the scientific community.

BIOGRAPHICAL SKETCH

Allyson Eller grew up in Manchester, Maine with her parents Stanley Eller and Margaret Duston and her sister Emily Eller. Her parents were firm believers in the value of time spent outdoors and she spent much of her childhood hiking, canoeing, and swimming in the woods and waters of Maine. As a teenager, she worked as a camp counselor where she was a lifeguard and taught natural history classes. Sharing her love of the local ecosystems was a source of joy and helped her develop an enjoyment of teaching that she still carries. She graduated from Kents Hill School, Kents Hill, Maine in 1998 knowing that she wanted to pursue a career in science.

Allyson began her undergraduate studies at Smith College in Northampton, MA in 1998 where she majored in biological sciences. She was awarded two summer fellowships from the B. Elizabeth Horner Fund and the Howard Hughes Medical Institute and conducted field work on Mt. Mansfield, Vermont and around the Northampton, MA area with Drs. Robert McMaster and C. John Burk. This research led to an honors thesis project examining the population dynamics of *Diapensia lapponica*, an endangered plant on Mt. Mansfield. The decline of this species in Vermont is largely due to rising temperatures and reduction of habitat and this project was the beginning of Allyson's interest in studying the effects of global change on plants. In 2002 she completed her Bachelor of Arts degree and graduated cum laude with high honors in Biological Sciences.

Through a study abroad program with the School for Field Studies, Allyson spent a semester in the rainforest in Queensland, Australia. This was a wonderful opportunity to explore new ecosystems and think about the problems that arise in places where there is strong conflict between the welfare of the people and the welfare of the environment. She worked on a team project to investigate the effects of an invasive species, *Solanum mauritianum*, on the local birds using it as a food source

and the native plant species competing with it for space to grow. Allyson's experiences with field work in both Australia and Massachusetts helped her decide to pursue a career in research and in 2002 she became a graduate student at Cornell University in the lab of Jed P. Sparks.

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I have greatly appreciated the patience and helpfulness of the staff in the Ecology and Evolutionary Biology department. In particular I would like to thank Rosie Brainard, Linda Harrington, Carol Damm, Alberta Jackson, Deedee Albertsman, Patty Jordan and Janeen Orr for helping with the enormous amount of paperwork generated throughout my disseration, Gary Oltz and RonWolverton for the incredibly useful things they were able to make for me in their shops, and Brian Mlodzinski for saving my computer on so many occasions.

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Evanhoe, Jill Anderson, Sarah Reilly, and many, many others. I am grateful to Carrie for her helping hand in the field, her companionship in the lab, and most importantly, her friendship. I cannot include enough thanks to Kimberlee Sparks for the many, many hours of help in the lab and field, the last minute mailing of forgotten field equipment, her unbelievable ability to salvage situations that seemed completely hopeless, and the constancy of her friendship.

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Finally, I would like to thank my parents Margaret A. Duston and Stanley W. Eller and my sister Emily Eller. Their love and unwavering support has always been a source of my strength and perseverance and I could not have survived my dissertation without them.

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Chapter 1

Introduction

Human activities, particularly the burning of fossil fuels and biomass, have changed the composition of the Earth's atmosphere. These activities have directly increased the concentrations of carbon dioxide (CO_2) and reactive nitrogen (e.g. NO_2 and other oxidized forms of N); and indirectly increased the photochemical production of ozone (O_3) in the troposphere through the release of reactive nitrogen and hydrocarbons. Models that predict future atmospheric conditions suggest that without changes in human behavior, concentrations of CO_2, NO_2, and O_3 will continue to rise (IPCC 2007).

Monitoring and modeling of atmospheric chemistry suggests the atmosphere has already changed, and how it is likely to change in the future. CO_2 emissions have increased 80% since 1970, and the global CO_2 concentration is increasing by 1.9 ppm per year (IPCC 2007). Between 1860 and 2000 the total amount of reactive nitrogen produced by human processes increased from 15 Tg N yr^{-1} to 165 Tg N yr^{-1} with reactive nitrogen from fossil fuel burning increasing from 1 Tg N yr^{-1} to 25 Tg N yr^{-1} (Galloway *et al.* 2003). In urban areas in the United States, the concentration of NO_2 is typically 10-45 ppb (NASA Visible Earth http://visibleearth.nasa.gov/view_rec.php?id=15000) and 22-45% of Europeans living in urban environments now experience background NO_2 levels above 20 ppb (© EEA, Copenhagen, 2008). In addition to rising NO_2 (and in part because of it), O_3 is increasing in both rural and urban areas; many cities now routinely exceed the EPA recommended limit of 80 ppb (EPA, AIRNOW).

Many studies have looked at the influence of elevated CO_2 on gas exchange, growth, reproduction, and phenology of plants. Several review papers have reported that the majority of elevated CO_2 studies find increased photosynthesis (at least

initially), higher total biomass (Ainsworth and Long 2005, Norby *et al.*. 1999, Curtis and Wang 1998), greater reproductive output (Jablonski *et al.* 2002), altered phenology (Springer and Ward 2007) and decreased stomatal conductance (Ainsworth and Long 2005) in response to elevated CO_2. Despite the general trends, there is significant variation in biomass changes (Ainsworth and Long 2005) and the direction and magnitude of phenological shifts (Springer and Ward 2007) depending on the functional group of the plants and the growing conditions in the study.

Elevated O_3 is a strong oxidant and can be severely detrimental to plants. It has been shown to cause visible leaf damage (e.g. Greitner *et al.* 1994, Coleman *et al.* 1995), decrease photosynthesis (*e.g.* reviews by Chappelka and Samuelson 1998, Skärby *et al.* 1998), decrease growth (see review by Fuhrer 2009), decrease reproductive output, and delay flowering (see review by Black *et al.* 2000). The effects of elevated O_3 on growth can be tempered by the addition of elevated CO_2 (*e.g.* Isebrands *et al.* 2001, Karnosky *et al.* 2003, King *et al.* 2005), at least in part because of decreased stomatal conductance under elevated CO_2.

When NO_2 enters plant leaves it disproportionates into nitrate and nitrite and can potentially act as a source of nitrogen and have a beneficial effect on plants (see review by Sparks 2009), but NO_2 has also been found to decrease or have no effect on growth (*e.g.* Zeevart 1976, Rowland *et al.* 1985, Vallano and Sparks 2007). Although little is known about the effects of NO_2 on plant phenology, it is likely the effects will be largely dependent on whether it increases or decreases total plant biomass. When coupled with elevated O_3, the combined oxidative damage caused by the two gases may result in greater damage than seen with either gas singly. Conversely, if elevated NO_2 provided an addition source of nitrogen, it may help sustain the enhancement of biomass caused by CO_2 and contribute to the production of larger plants. Like O_3, the

effect of NO_2 (whether positive or negative) is likely to be decreased when stomatal conductance is low, as may happen under elevated CO_2.

The goals of this thesis were: 1) determine the relative controls of physical and biochemical processes on the entry of pollutant gases into leaves, and 2) determine how growth, biomass production, leaf chemistry, reproduction, and phenology are affected by these pollutants under current and future CO_2 conditions. The following dissertation is divided into four chapters that describe one experiment used to address the first goal and three experiments used to address the second.

To determine the controls on leaf-level fluxes of NO_2 and O_3 we measured the physical resistance to gases entering the leaves (stomatal conductance), the ability of plants to remove the gases from the apoplast (inferred from the concentration of the antioxidant, ascorbate), and the ability of the plants to remove the products of the chemical reactions that eliminated the gases from the apoplast (for NO_2 only, determined by nitrate reductase activity). These parameters were measured in tandem with instantaneous leaf-level fluxes of O_3 or NO_2 to the leaves of *Catharanthus roseus*. I combined these measurements to create multiple regression models and determine which variable combinations provided the best explanation of the variation in O_3 and NO_2 fluxes. The best models and the overall predictive abilities of those models are laid out in chapter 1.

Chapter two outlines the open-top chamber experiment that I conducted to determine the effects of elevated CO_2, NO_2, and soil NO_3^- on sugar maple (*Acer saccharum*) and eastern hemlock (*Tsuga Canadensis*) seedlings. I used a factorial design to compare the single and combined effects of the treatments and identify non-additive effects that are crucial for making accurate predictions of future plant performance, but are difficult to determine from single-treatment studies. I measured the treatment effects on instantaneous photosynthesis, stomatal conductance, biomass,

allocation, and leaf chemistry in order to make predictions about future growth, carbon storage potential, and leaf quality of tree seedlings.

After completing the experiment discussed in chapter two, I expanded the open-top chamber system to include an O_3 treatment in addition to the CO_2 and NO_2 treatments and added seedlings of red oak (*Quercus rubra*) and two clones of trembling aspen (*Populus tremuloides*) to the sugar maple and eastern hemlock seedlings (detailed in chapter three). As in the earlier experiment, I measured photosynthesis, stomatal conductance, biomass production, and biomass allocation. In this analysis, I focused on the differences between species and the cases where our current predictions of seedling growth and carbon storage potential may be inaccurate because they do not include the likely effects of rising NO_2 and O_3.

Finally, I expanded the ability to make predictions about future plant performance by adding the model annual *Arabidopsis thaliana* to the open-top chamber experiment with factorial combinations of CO_2, NO_2, and O_3. This experiment, discussed in chapter four, provided an opportunity to investigate the responses of plant reproduction and phenology, in addition to those of biomass and leaf chemistry. The timing of reproduction is particularly important in annual plants, where there is only one opportunity to leave offspring, and the findings of this project provide valuable insight about how reproduction and phenology of annual species is likely to be altered by the future chemical composition of the atmosphere.

Together these four chapters provide explanations for the differences in leaf-level fluxes of NO_2 and O_3 and uses these findings help explain the effects of these gases on growth and carbon storage potential of tree seedlings and reproduction and phenology of annuals. By looking at the effects of NO_2 and O_3 under current and predicted future concentrations of CO_2, we are able to make predictions about how plant responses will be different in the future and how local air quality will affect

future plant performance. The findings presented here also remind us of the importance of species-specific responses and provide a warning about using the effects of single-treatment studies to make predictions about plant responses to multiple, simultaneous atmospheric changes.

REFERENCES

Ainsworth E.A. and S.P. Long. (2005). What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO_2. *New phytologist*, 165: 351–372

Black V.J., C.R. Black, J.A. Roberts, and C.A. Stewart. (2000). Impact of ozone on the reproductive development of plants. *New Phytologist*, 147:421-447

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

Coleman M.D., R.E. Dickerson, J.G. Isebrands, and D.F. Karnosky. (1995). Photosynthetic productivity of aspen clones varying in sensitivity to tropospheric ozone. *Tree Physiology* 15:585-592

Curtis P.S., and X. Wang. (1998). A meta-analysis of elevated CO_2 effects on woody plant mass, form, and physiology. *Oecologia* 113:299-313

EEA, Copenhagen, 2008 http://themes.eea.europa.eu/IMS/IMS/ISpecs/I Specification 20080701123452/IAssessment1219309276318/view_content (5/17/2009)

Fuhrer J. (2009). Ozone risk for crops and pastures in present and future climates. *Naturwissenschaften*, 96:173-194

Galloway J.N., J.D. Aber, J.W. Erisman, S.P. Seitzinger, R.W. Howarth, E.B. Cowling, and B.J. Cosby. (2003). The nitrogen cascade. *Bioscience* 53(4): 341-356

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

IPCC (2007). Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change [Solomon, S., D. Qin, M. Manning, Z. Chen, M. Marquis, K.B. Averyt, M.Tignor and H.L. Miller (eds.)]. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Isebrands JG, McDonald EP, Kruger E, Hendrey G, Pregitzer K, Percy K, Sôber J, Karnosky DF. 2001. Growth responses of *Populus tremuloides* clones to interacting carbon dioxide and tropospheric ozone. *Environmental Pollution* 115: 359–371

Jablonski L.M., X. Wang, P.S. Curtis. 2002. Plant reproduction under elevated CO_2 conditions: a meta-analysis of reports on 79 crop and wild species. *New Phytologist,*156: 9-26

Karnosky DF, Zak DR, Pregitzer KS, Awmack CS, Bockheim JG, Dickson RE, Hendrey GR, Host GE, King JS, Kopper BJ, Kruger EL, Kubiske ME, Lindroth RL, Mattson WJ, McDonald EP, Noormets A, Oksanen E, Parsons WFJ, Percy KE, Podila GK, Riemenschneider DE, Sharma P, Thakur R, Sôber J, Jones WS, Anttonen S, Vapaavuori E, Mankovska B, Heilman W, Isebrands JG. (2003). Tropospheric O3

moderates responses of temperate hardwood forests to elevated CO2: a synthesis of molecular to ecosystem results from the Aspen FACE project. *Functional Ecology* 17:289–304.

King, J.S., K.S. Pregitzer, D.R. Zak, M.E. Kubiske, and W.E. Holmes (2001). Correlation of foliage and litter chemistry of sugar maple, *Acer saccharum*, as affected by elevated CO2 and varying N availability, and effects on decomposition. *Oikos* 94:403-416.

NASA Visible Earth http://visibleearth. nasa.gov/view_rec.php?id=15000 (05/17/2009)

Norby R.J., S.D. Wullschleger, C.A. Gunderson, D.W. Johnson, and R. Ceulemans. (1999). Tree responses to rising CO_2 in field experiments: implications for the future forest. *Plant, Cell and Environment* 22:683-714

Rowland, A. J., M. C. Drew and A. R. Wellburn (1987). Foliar Entry and Incorporation of Atmospheric Nitrogen-Dioxide into Barley Plants of Different Nitrogen Status. *New Phytologist* 107(2): 357-371.

Skärby L., H. Ro-Poulsen, F.A.M. Wellburn, and L.J. Sheppard. (1998). Impacts of ozone on forests: a European perspective. *New Phytologist*, 139:109-122

Springer C.J. and J.K. Ward (2007). Flowering time and elevated atmospheric CO_2. *New Phytologist,* 176: 243–255

Sparks J. P. (2009). Ecological ramifications of the direct foliar uptake of nitrogen. *Oecologia* 159:1-13

Vallano D.M. and J.P Sparks. (2007). Quantifying foliar uptake of gaseous nitrogen dioxide using enriched foliar $\delta^{15}N$ values. *New Phytologist*, 177:946-955

Zeevaart, A. J. (1976). Some Effects of Fumigating Plants for Short Periods with No2. *Environmental Pollution* 11(2): 97-108.

Chapter 2

Predicting leaf-level fluxes of O_3 and NO_2: the relative roles of diffusion and biochemical processes

Abstract:

Pollutants like O_3 and NO_2 enter leaves through the stomata and cause damage during reactions with components of biological cell membranes. The steady-state flux rates of these gases into the leaf are determined by a series of physical and biochemical resistances including stomatal aperture, reactions occurring within the cell wall and the ability of the leaf to remove the products of apoplastic reactions. In the present study, multiple regression models incorporating stomatal conductance, apoplastic and symplastic ascorbate concentrations, and nitrate reductase (NR) activities were generated to explain the observed variations in leaf-level flux rates of O_3 and NO_2. These measurements were made on the plant *Catharanthus roseus* (Madagascar periwinkle). The best-fit model explaining NO_2 flux included stomatal conductance, apoplastic ascorbate and NR activity. This model explained 89% of the variation in observed leaf fluxes and suggested physical resistances, reaction between NO_2 and apoplastic ascorbate, and the removal rate of nitrate (generated by reactions of NO_2 and water) from the apoplast all play controlling roles in NO_2 flux to leaves. O_3 flux was best explained by stomatal conductance and symplastic ascorbate explaining 66% of the total variation in leaf flux. Both models demonstrate the importance of measuring processes other than stomatal conductance to explain steady-state leaflevel fluxes of pollutant gases.

Introduction:

Many pollutants, including ozone (O_3) and nitrogen dioxide (NO_2) have significantly increased in the Earth's atmosphere and are expected to continue rising

(IPCC 2001). This increase of reactive compounds in the atmosphere has the potential to significantly alter plant performance. Ozone and nitrogen dioxide are both reactive oxidants that can react with components of biological systems disrupting function and thereby decreasing growth (Greitner *et al.* 1994). Further, in the case of NO_2, the endpoint of the chemistry between the pollutant and the plant is itself a nutrient that under certain circumstances can be utilized by the plant (Segschneider *et al.* 1995).

Plants are directly affected by pollutants in the atmosphere, but because they remove certain compounds from the air, plants also influence the atmospheric concentrations of many gases. The plant uptake of gases such as NO_2 and O_3 can reduce the overall concentration in the atmosphere (Hill 1971; Fowler *et al.* 1998) and potentially alter local air quality. The flux of NO_2 (Morikawa *et al.* 1998) and O_3 (Fiscus *et al.* 2005) into plant leaves varies greatly among species, but the basis of this variation has not been fully resolved.

The sensitivity of plant leaves to damage from atmospheric pollution is a function of both the rate at which pollutant compounds diffuse through the stomata and the rate they are eliminated from the sub-stomatal cavity (Laisk, Kull, and Moldau 1989; Ramge *et al.* 1993). Both O_3 and NO_2 enter leaves primarily by passing through stomata, allowing plants to exert some level of control over the amount of O_3 and NO_2 entering the leaf by altering the diffusional resistance of entry (Fowler *et al.* 1998). After entry into the leaf, O_3 and NO_2 react either with organic material (cell wall components, plasma membranes, etc.) or undergo secondary chemistry with antioxidant compounds within the cell wall (Chameides 1989; Laisk, Kull, and Moldau 1989; Moldau 1998). One strong, ubiquitous antioxidant often associated with oxidant tolerance in plants is ascorbate (vitamin C; Chameides 1989; Cross *et al.* 1998; Lyons, Ollerenshaw, and Barnes 1999a). The apoplastic concentration of ascorbate has been shown to increase under O_3 exposure (Castillo and Greppin 1988;

Ranieri *et al.* 1996) and higher concentrations of ascorbate have been correlated with decreased O_3 sensitivity in several species including, snap bean (Burkey 1999; Burkey *et al.* 2000; Burkey, Eason, and Fiscus 2003), common bean (Moldau, Bichele, and Huve 1998), broad bean (Turcsanyi *et al.* 2000), *Plantago major* (Zheng *et al.* 2000), spinach (Luwe, Takahama, and Heber 1993), and soybean (Lee *et al.* 1984; Robinson and Britz 2000). Also, the *Arabidopsis* mutant *vtc1*, an ascorbate under-producer, exhibits greater O_3 sensitivity compared to wildtype (Conklin, Williams, and Last 1996; Conklin *et al.* 1997). Ascorbate is a general antioxidant and is likely to react strongly with both O_3 and NO_2. Although a number of studies have examined the relationship between ascorbate and O_3 sensitivity, few have looked at the direct relationship between ascorbate and leaf O_3 flux rate.

To effectively protect living plant cell components from the damage caused by O_3 and NO_2, the ascorbate-oxidant reaction would necessarily occur in the cell wall (i.e., the apoplastic space) prior to the reactant reaching the living membrane. Therefore, resistance to damage caused by gaseous oxidants is dependent upon both the size of the ascorbate pool within the apoplastic space and the rate at which that pool can be replenished (Plöchl *et al.* 2000). When ascorbate in the apoplast reacts with an oxidant, it is oxidized and must be transported back into the plant cell to be reduced before it can return to the apoplast to again react with oxidants (Smirnoff 1996; Horemans, Foyer, and Asard 2000; Smirnoff and Wheeler 2000; Heber *et al.* 2003). Therefore, the concentration of ascorbate within the apoplast, where the direct reaction occurs, or within the symplast, where the ascorbate is reduced, could be related to the steady-state flux of oxidant gases into the leaf.

The apoplastic reactions involving NO_2 and O_3 are very different. In the case of NO_2, it is known to react with water or ascorbate yielding nitrate or nitrite, respectively (Zeevaart 1976; Yoneyama, Hashimoto, and Totsuka 1980; Lee and

Schwartz 1981; Rowland, Drew, and Wellburn 1987; Ramge *et al.* 1993). Both reactions are irreversible and dependent upon the concentration of NO_2^-/NO_3^- in solution (Remmler and Campbell 1986; Stulen *et al.* 1998). Therefore, the rate of transport of the products from the cell wall solution to the interior of the cell could influence the total flux rate of NO_2 at steady state (Ramge *et al.* 1993). Many studies have found that plants grown under elevated NO_2 increase their nitrate reductase activity (Zeevaart 1976; Murray and Wellburn 1985; Rowland *et al.* 1987; Bender, Weigel, and Jager 1991; Thoene *et al.* 1991; Hur and Wellburn 1994; Hufton, Besford, and Wellburn 1996) which can be an indication of increased ability to remove nitrate from the apoplast. Measuring the rate of this transport directly is difficult, but the activity of the enzyme nitrate reductase is often an indication of this transport capacity (Thoene *et al.* 1991; Hereid and Monson 2001) and is used in this study as a proxy for NO_3^- cellular uptake. Unlike the apoplastic reactions of NO_2, the reactions of O_3 produce no nutritive products. O_3 can react directly with apoplastic ascorbate producing dehydroascorbate, water, and oxygen (Chameides 1989) or O_3 can react with other apoplastic constituents generating other reactive oxygen species (ROS; Grimes *et al.* 1993). Subsequent ROS products are then destroyed by reactions with antioxidants including ascorbate or damage to the plasmamembrane (Lyons *et al.* 1999b).

The focus of this study was to use empirical measurements to develop correlative models to describe the flux of O_3 and NO_2 into plant leaves. We based our empirical measurements on the conceptual model that three basic processes control the leaf fluxes of NO_2 and O_3: 1) diffusional resistances of gas entry into the leaf, 2) reactions with chemical components within the leaf cell walls, and 3) the removal of the chemical end products within the leaf (Fig. 2.1). Undoubtedly, some part of the leaf flux is defined by the reaction between oxidants and cellular structures within the

leaf, but these processes are small, likely not sustainable over longer timescales, and are not considered in this study.

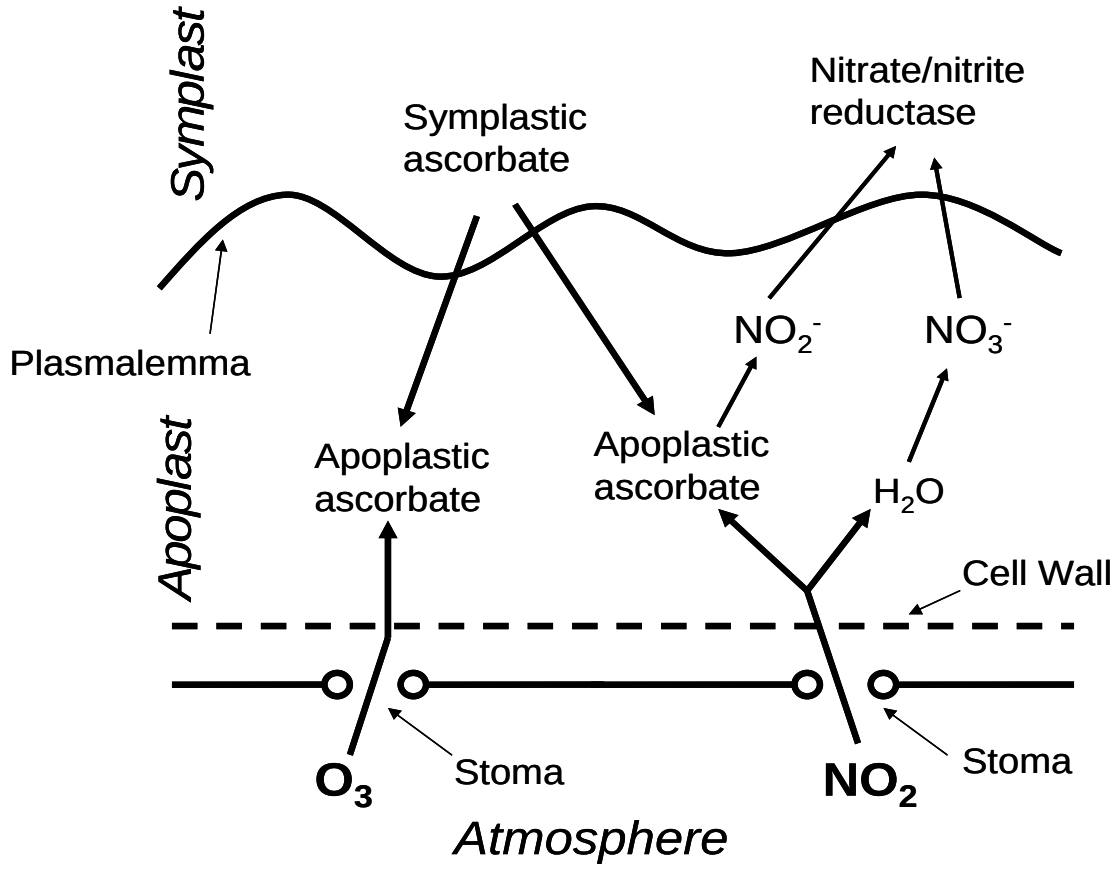

Figure 2.1: Conceptual model describing the processes associated with O_3 and NO_2 flux to plant leaves that are included in the correlative model.

For both O_3 and NO_2, we measured stomatal conductance as an estimate of the diffusional resistances to the gases entering the leaf, and the concentrations of apoplastic and symplastic ascorbate to infer the reaction rates between the gases and chemicals within the leaf cell wall. For the case of NO_2 flux, nitrate reductase activity was also measured to estimate the leaf capacity to remove the endpoint products of the NO_2+water and NO_2+ascorbate reactions from the cell wall.

The explicit goals of this research were to investigate the relative importance of stomatal control and leaf chemistry to the flux of pollutant gases to leaves and then to use the results of these investigations to generate correlative leaf uptake models. These models provide an empirical test of the importance of ascorbate in the flux of O_3 and NO_2 and help to validate the theoretical models proposed by Chameides (1989) and Plöchl *et al.* (2000).

Methods:

Plant Material and Growth Conditions

Catharanthus roseus (Madagascar Periwinkle) was used in this study because it had been observed to express leaf ascorbate contents dependent upon ambient light conditions during growth. It exhibits higher ascorbate levels when grown under higher light (Eller and Sparks unpublished data). Twenty plants (original seed stock from Trobilabs Inc., Largo, FL, USA) were propagated from cuttings and grown under greenhouse conditions with a 12-hour daylength and temperatures of 21/29^{o}C (night/day), watered daily, and fertilized with 21-5-20 N-P-K once per week. The plants were grown under these conditions for 4 months before the start of the experiment. Half of the plants were grown under a 75% shade cloth to induce differences in ascorbate concentration across individuals.

Leaf O_3 and NO_2 flux

A portable leaf gas exchange system (Model 6400, LiCor, Lincoln, NE) was used for all gas exchange measurements. The system was modified such that trace gases could be added to the sample flow before the leaf cuvette. Sources of O_3 or NO_2 were added to the empty cuvette with target concentrations of 65 ppb and 4 ppb for O_3

and NO_2, respectively. Ozone was measured using a Thermo Environmental (Franklin, MA) model 49 U.V. Photometric O_3 Analyzer and NO_2 was measured using an ECO PHYSICS AG (Duernten, Switzerland) CLD 770 AL ppt with a PLC 760 photolytic converter. Fluxes were calculated using the difference in partial pressure between an empty (C_a) and a leaf-filled cuvette (C_l). Stomatal conductance was recorded when C_l was stable (coefficient of variation < 5%). Positive fluxes were defined as fluxes into the leaf; negative fluxes as emissions from the leaf.

Leaf fluxes (J) of NO_2 and O_3 were calculated as:

$$J = \frac{f * (C_a - C_i)}{A} \quad (1)$$

where f is the flow rate through the cuvette (μmol s^{-1}) and A is the leaf area enclosed within the cuvette (m^2). $C_a - C_l$ is the difference in gas partial pressure between the empty and leaf-filled cuvette, expressed in nmol mol^{-1}. After measurement, the leaf was removed from the cuvette, excised, and immediately used in the apoplastic ascorbate extraction, nitrate reductase assay, or frozen at -80 ^oC.

Extraction of Apoplastic Fluid

The method of extracting the apoplastic fluid was based on a modified version of the method described by Luwe and Heber (1995). Immediately following the gas exchange measurement, the leaf was cut along the midrib with a razor blade, separated into two halves, and the cut surfaces rinsed with deionized water (this procedure was tested against uncut leaves and no significant differences in apoplastic ascorbate concentration or nitrate reductase content were found). One half of the leaf was used for the apoplastic fluid extraction and the other half was immediately used in the

nitrate reductase assay. The leaf was weighed, then placed in a 15 mL test tube filled with 2% metaphosphoric acid 2mM EDTA buffer and vacuum infiltrated. The leaf and buffer were placed under 80 kPa of vacuum pressure for 30 seconds, then released from the vacuum for 30 seconds. The cycle was repeated 3 times or until the leaf was fully infiltrated with buffer. As leaves became infiltrated, there was a distinct change in color as the leaf became dark green and translucent. The infiltration process was repeated until the entire leaf had undergone this change in color. The infiltrated leaf was then placed into a small funnel attached to an eppendorf tube containing 50 µl of EDTA buffer. The combination of the leaf funnel and eppendorf tube were then placed in a conical tube and centrifuged at 4000 RPM for 10 minutes. This process forced the apoplastic fluid out of the leaf and into the eppendorf tube where it was collected. The apoplastic fluid and the leaf tissue were then separately frozen in liquid nitrogen and stored at -80 ^{o}C until assayed for ascorbate content.

Ascorbate Assay

Leaf tissue (post leaf apoplastic fluid extraction) was ground in 1 mL of 2% metaphosphoric acid 2mM EDTA buffer and centrifuged at 13,000 RPM for 10 minutes. The supernatant (symplastic extract) was collected and frozen at -80 ˚C.

The assay of ascorbate in both apoplastic and symplastic fractions followed the procedure of Rao and Ormrod 1995 and Conklin *et al.* 1996 with the modifications outlined below. A room-temperature reaction mixture of 500 µL 0.2 M Sodium Phosphate buffer (pH 5.6), 270 µL sterile H_2O, 20 µL 0.5M HEPES-KOH (pH 7.5), and 20 µL 200mM DTT were mixed for each sample. The symplastic extract was neutralized by adding 60 µL of 10% Sodium Citrate to 90 µL of extract and the apoplastic fluid was neutralized by adding 60 µL of 10% Sodium Citrate to 40 µL of apoplastic fluid. One hundred µL of each neutralized sample was then placed on ice.

Absorbance of each sample was measured at 265 nm using a spectrophotometer (Beckman, DU 640). Immediately before measuring absorbance, the neutralized sample was mixed with the reaction mixture to reduce all of the ascorbate in the sample. The absorbance was read at 265 nm, then 10 µL of ascorbate oxidase (0.4/U µl) was added to oxidize all the ascorbate and the sample absorbance was read a second time. The difference in absorbance between the reduced and oxidized samples was compared to a standard curve to determine the concentration of ascorbate in the sample expressed in µmol g^{-1} fresh weight. The standard curve was prepared using purified ascorbic acid (Sigma, St. Louis, MO, USA).

Nitrate Reductase Assay

Nitrate reductase activity was measured using a procedure modified from Jones, Tucker, and Ort 1998. One half of each leaf exposed to NO_2 was vacuum infiltrated in 10 mL of incubation buffer [0.1M potassium phosphate (pH 7.4), 0.05M KNO_3, 1% v/v propanol] then incubated for 30 minutes in a dark shaking water bath at 30 ^oC. Following incubation, 100 µL of incubation buffer was diluted with 400 µL H_2O, then mixed with 250 µL 1% sulfanilic acid in 1.5M HCl followed by 250 µl of color development agent [N-(1-naphthyl)ethylenediame-HCl (200mg/L)]. At least 20 minutes were allowed for the color development reaction, then absorbance of the sample was measured at 540 nm using a spectrophotomer (Beckman DU 640). The samples were compared to a standard curve generated using a stock solution of KNO_3.

Statistical Analysis

The SAS/STAT software (version 8 for Windows, copyright SAS Institute Inc. Cary, NC, USA) was used in all statistical analyses. Multiple linear regression models were constructed using stomatal conductance, apoplastic ascorbate concentration,

symplastic ascorbate concentration, and nitrate reductase activity (for NO_2 only) to explain variation in leaf O_3 and NO_2 fluxes. The r^2 selection method was used to choose the combination of predictors that resulted in the best multiple regression models explaining O_3 and NO_2 flux.

Results:

NO_2

Single linear regressions were used to test stomatal conductance, apoplastic ascorbate concentration, symplastic ascorbate concentration, and nitrate reductase activity as potential predictors of NO_2 flux to the leaves of *C. roseus*. Each of the predictors had a significant ($p < 0.05$) positive relationship with NO_2 flux. When the strength of each predictor was tested singly, stomatal conductance explained 84% of the variation in NO_2 flux, while nitrate reductase activity, apoplastic ascorbate, and symplastic ascorbate explained 37, 56, and 43% respectively (Fig. 2.2).

Using the R-squared model selection technique it was found that the flux rate of NO_2 was best explained by a multiple regression model containing stomatal conductance, nitrate reductase activity and apoplastic ascorbate concentration (Table 2.1). Symplastic ascorbate did not explain any additional variation in the model. The multiple linear regression model containing these three predictors explained 89% of the variation in NO_2 flux. All three predictors were positively correlated with flux and the y-intercepts were negative.

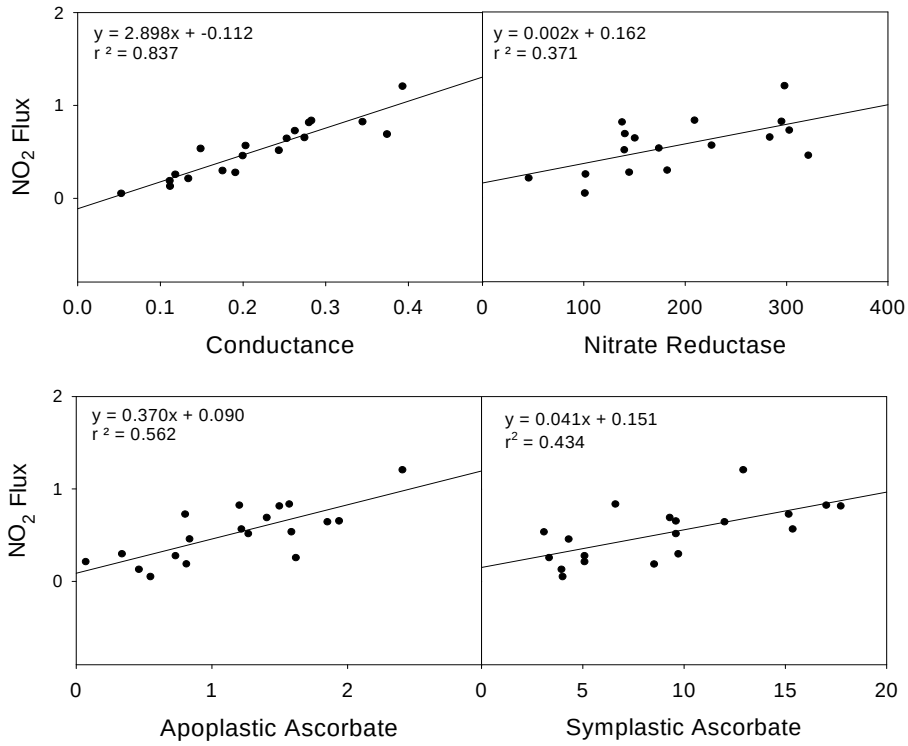

Figure 2.2: Relationships between leaf NO_2 flux and conductance (mol H_2O m^{-2} s^{-1}) ($p < 0.0001$), nitrate reductase activity (µmol KNO_2^- g FW^{-1} h^1) ($p = 0.0095$), apoplastic ascorbate concentration (µmol g FW^{-1}) ($p = 0.0002$), and symplastic ascorbate concentration (µmol g FW^{-1}) ($p = 0.0022$).

Table 2.1: Results of the best fit multiple regression model for NO_2 flux into the leaf. Symplastic ascorbate concentration did not explain additional variance and was not included in the final model formulation. The variables included in the model are stomatal conductance (mol H_2O m^{-2} s^{-1}) nitrate reductase activity (µmol KNO_2^- g FW^{-1} h^1), and apoplastic ascorbate concentration (µmol g FW^{-1}).

Multiple Regression Model for NO_2 Flux: **Flux = $1.982x_1 + 0.00060x_2 + 0.134x_3 + -0.182$**			
	F-value	P-value	**R^2**
Overall Model	35.28	< 0.0001	**0.89**
Model Components			
x_1 = Conductance	95.99	< 0.0001	
x_2 = Nitrate Reductase Activity	2.85	0.1151	
x_3 = Apoplastic Ascorbate	7.00	0.0202	

Partial residual plots were generated to determine how much of the variation in NO_2 flux was explained by each predictor after accounting for the effects of the other two predictors. All three predictors had a strong relationship with the residuals of the partial models, indicating that they each add explanatory power to the overall model. Stomatal conductance explained 34% of the variation that remained after accounting for leaf chemistry (apoplastic ascorbate and nitrate reductase activity). Ten percent of the variation in NO_2 flux that remained after accounting for stomatal conductance and apoplastic ascorbate was explained by nitrate reductase activity. Apoplastic ascorbate concentration explained 22% of the residual variation remaining after considering stomatal conductance and nitrate reductase (Fig. 2.3).

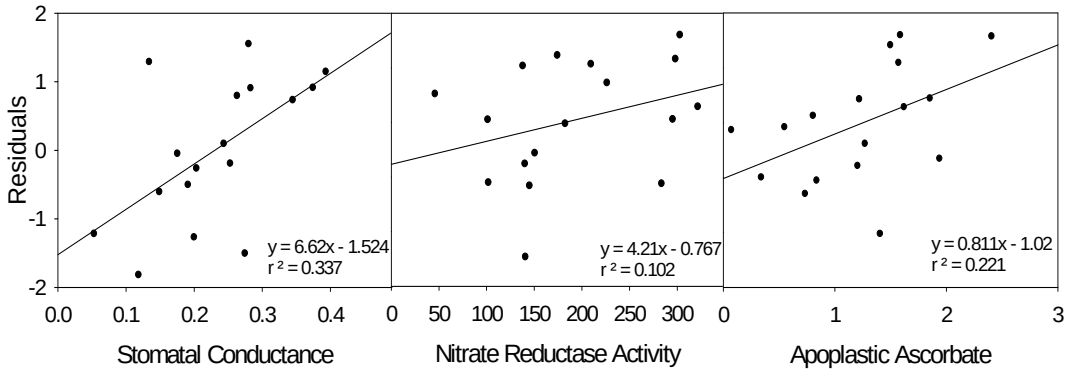

Figure 2.3: Partial residual plots for the predictor variables included in the multiple regression model explaining NO_2 flux. (A) the correlation of conductance (mol H_2O m^{-2} s^{-1}) ($p = 0.015$) and the residuals of the model describing NO_2 flux with nitrate reductase activity and apoplastic ascorbate concentration. (B) the correlation of nitrate reductase activity (µmol KNO_2^- g FW^{-1} h^1) ($p = 0.210$) and the residuals of the model describing NO_2 flux with conductance and apoplastic ascorbate concentration. (C) correlation between apoplastic ascorbate concentration (µmol g FW^{-1}) ($p = 0.057$) and the residuals of the model describing NO_2 flux with conductance and nitrate reductase activity.

O_3

The examined predictors of O_3 flux were stomatal conductance, apoplastic ascorbate, and symplastic ascorbate. Singly, stomatal conductance, symplastic ascorbate, and apoplastic ascorbate were each significantly ($p < 0.05$) and positively correlated with O_3 flux and explained 56, 39, and 25%, respectively, of the variation in leaf O_3 flux (Fig. 2.4).

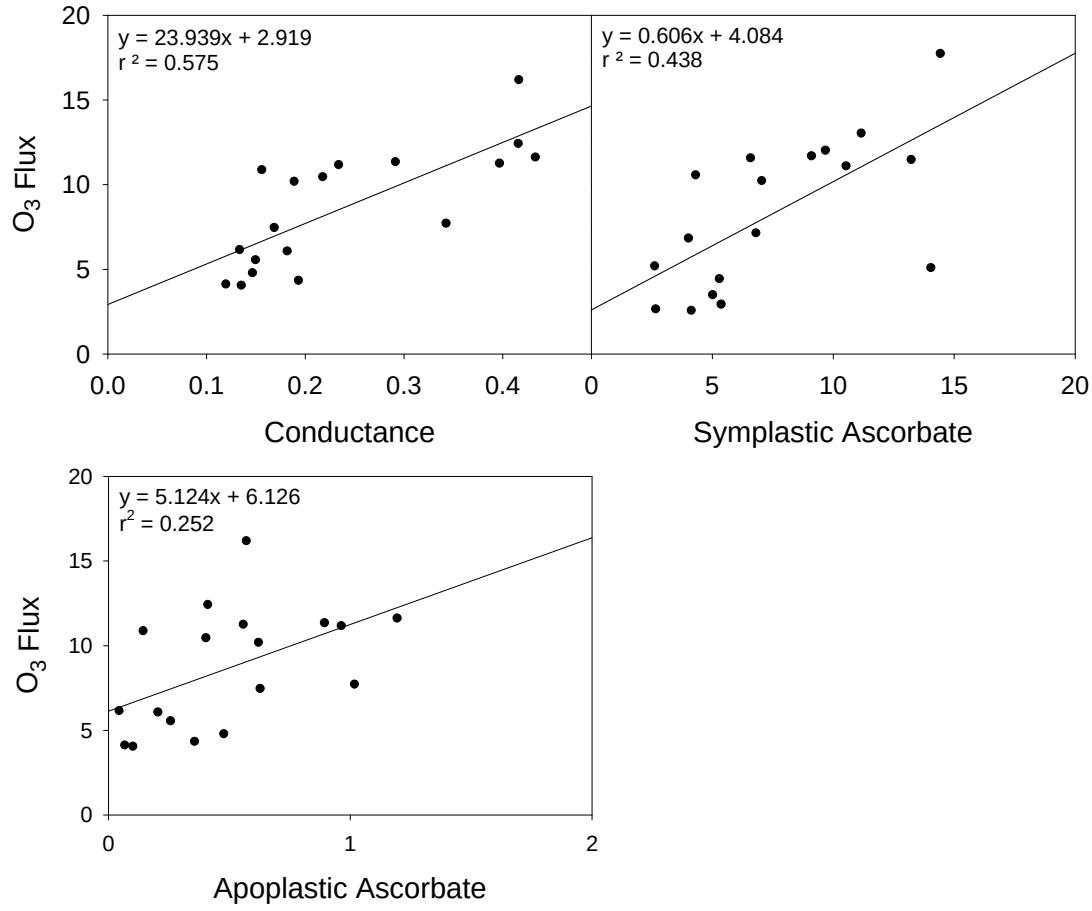

Figure 2.4: Relationship between the flux of O_3 and conductance (mol H_2O m^{-2} s^{-1}) ($p < 0.0001$), symplastic ascorbate concentration (µmol g FW^{-1}) ($p = 0.0088$), and apoplastic ascorbate concentration (µmol g FW^{-1}) ($p = 0.0338$).

Similar to the NO_2 analysis, the R-squared selection method was used to determine the best fit multiple-linear regression model. The best overall model explained 66% of the variation in O_3 flux and included stomatal conductance and symplastic ascorbate concentration. As others have found (Noctor and Foyer, 1998), apoplastic ascorbate accounted for ~10% of the total ascorbate in the leaf, and did not explain any additional variation in the combined model. Both stomatal conductance and symplastic ascorbate were positively correlated with O_3 flux and the y-intercept was positive (Table 2.2).

Table 2.2: Results of the best fit multiple regression model for the flux of O_3 into the leaf. Apoplastic ascorbate concentration did not explain any additional variance and was not included in the model. The variables included in the model are stomatal conductance (mol H_2O m^{-2} s^{-1}) and symplastic ascorbate concentration (μmol g FW^{-1})

Best Multiple Regression Model for O_3 Flux: **Flux = $18.846x_1 + 0.272x_2 + 1.933$**			
	F-value	P-value	**R^2**
Overall Model	14.74	0.0003	**0.66**
Model Components			
x_1 = Stomatal Conductance	25.59	< 0.0001	
x_2 = Symplastic Ascorbate	3.89	0.0674	

Partial residual plots show that both stomatal conductance and symplastic ascorbate explain a significant portion of the flux of O_3 into leaves even after accounting for the effect of the other predictor. Stomatal conductance explained 34% of the variation remaining after symplastic ascorbate was included in the model. Symplastic ascorbate explained 16% of the variation that was not explained by stomatal conductance (Fig. 2.5).

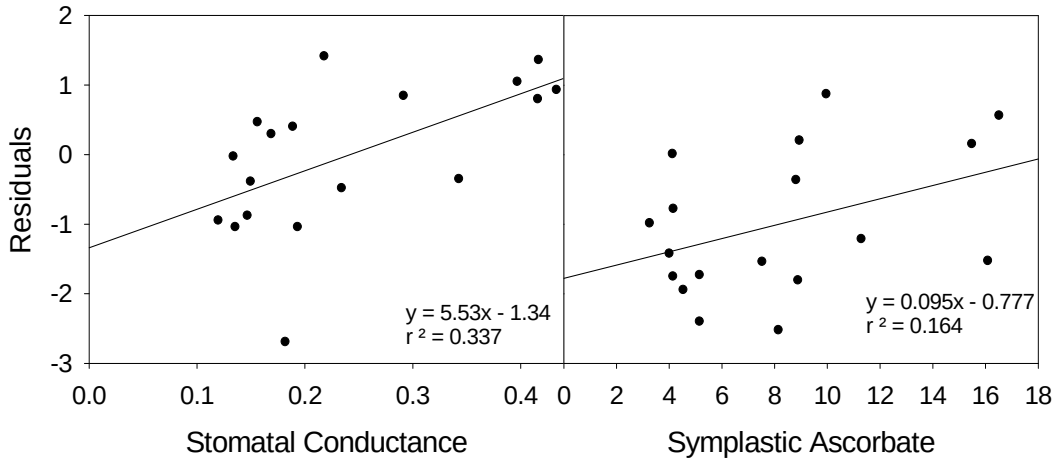

Figure 2.5: Partial residual plots for the predictor variables included in the multiple regression model explaining O_3 flux. (A) the correlation between conductance (mol H_2O m^{-2} s^{-1}) ($p = 0.012$) and the residuals of the model describing O_3 flux with symplastic ascorbate concentration. (B) the correlation of symplastic ascorbate concentration (µmol g FW^{-1}) ($p = 0.096$) and the residuals of the model describing O_3 flux with conductance.

Discussion

The regression model including stomatal conductance, apoplastic ascorbate, and nitrate reductase activity explained 89% of the variation in NO_2 flux, making it a robust predictive model. This supports the initial conceptual model (Fig. 2.1) and suggests that three resistances (i.e., stomatal aperture, apoplastic antioxidant reactions, and removal of end products from the apoplast) largely control the influx of NO_2 into the leaf.

Our findings agree with others that stomatal conductance is the dominant controller of NO_2 flux (Hanson and Lindberg 1991; Hargreaves *et al.* 1992; Duyzer and Fowler 1994), but not that it is the only predictor. Others have suggested that there is a mesophyllic resistance to NO_2 flux (Thoene *et al.* 1991; Thoene, Rennenberg, and Weber 1996; Hereid and Monson 2001; Sparks *et al.* 2001; Gut *et al.* 2002; Teklemariam and Sparks 2006), but have not identified that resistance. In the

mathematical model by Ramge *et al.* (1993), apoplastic ascorbate concentration was proposed as the primary component of the mesophyllic resistance, and this study provides empirical data supporting that model. While many have found a relationship between elevated NO_2 and increased nitrate reductase activity (Zeevaart 1976; Murray and Wellburn 1985; Rowland *et al.* 1987; Bender *et al.* 1991; Thoene *et al.* 1991; Hur and Wellburn 1994; Hufton *et al.* 1996), this is the first to demonstrate that nitrate reductase activity is directly correlated with the flux rate of NO_2 into leaves.

The relatively simple statistical model produced in this study robustly describes the variation of NO_2 leaf fluxes observed in *C. roseus*. Also, there is evidence that this model can reasonably extrapolate beyond the range of data observed in this study. For example, other studies have reported an emission of NO_2 that is only detectable when the atmospheric NO_2 concentration is below the compensation point (Rondon and Granat 1994; Weber and Rennenberg 1996; Hereid and Monson 2001; Sparks *et al.* 2001). The negative y-intercept of the model generated here suggests an emission of NO_2 of a similar magnitude to that observed in other studies (Table 2.1).

Antioxidant compounds such as ascorbate, if located in the plant cell wall, may act as the first line of defense protecting cell membranes from oxidants that have entered leaves (Moldau 1998). As mentioned earlier, after apoplastic ascorbate is oxidized by NO_2, it is no longer able to react with oxidants and must be transported back into the plant cell where it can be reduced and returned to the cell wall to participate in further NO_2 reactions (Smirnoff 1996; Smirnoff and Wheeler 2000). Therefore, depending upon the ambient oxidant concentrations and time of exposure, the standing pool of apoplastic ascorbate or the ability of local cells to replenish this pool may both be correlated to leaf flux. Ultimately, the rate of apoplastic pool replenishment may control how much of the steady-state flux into the leaf is related to ascorbate concentration. During the present study, we observed for *C. roseus* under

relatively short exposures (< 10 min.), apoplastic ascorbate concentration was a stronger predictor of NO_2 flux than symplastic ascorbate (Fig. 2.2) suggesting that the pool of ascorbate in the apoplast was not completely oxidized at ambient NO_2 concentrations of 4 ppb.

Nitrate reductase (NR) reduces nitrate within the cytosol and may be a good indicator of the rate of removal of nitrate from the cell wall (Srivastava and Ormrod 1989). Its significance in the model suggests that, under some circumstance, the rate of NO_3^- removal from the cell wall influences the steady-state leaf NO_2 flux. This observation is in general agreement with prior measurements that found gross correlations between NO_2 fumigation and leaf NR activity or leaf NO_2 flux and bulk leaf nitrogen content where the authors suggested a controlling role of NR in steady-state leaf NO_2 flux (Thoene *et al.* 1991; Ramge *et al.* 1993; Hufton *et al.* 1996; Weber and Rennenberg 1996; Hereid and Monson 2001). Nitrate reductase activity is variable across plant species and these differences may help to explain the wide variation in uptake rates of NO_2 observed in other studies (Morikawa *et al.* 1997; Morikawa *et al.* 1998). Plants with higher nitrate reductase activities may express higher uptake rates especially at higher NO_2 concentrations where the cell wall may become saturated with NO_3^-/NO_2^-.

Assuming this model is representative of species beyond *C. roseus*, it could be used to improve NO_2 uptake models based solely on stomatal conductance. Several studies (Saxe 1986; Thoene *et al.* 1991; Rondon, Johansson, and Granat 1993; Weber and Rennenberg 1996; Hereid and Monson 2001) have shown a linear relationship between stomatal conductance and NO_2 uptake within single plant species. However, the magnitude of NO_2 uptake at a particular conductance varies widely between species (Morikawa *et al.* 1997; Morikawa *et al.* 1998). The differences in uptake independent of stomatal conductance are likely associated with mesophyllic processes

like those examined in this study. Therefore, using data like those presented here will allow us to modify existing stomatal algorithms using species specific physiology data (e.g., Ramge *et al.* 1993).

In contrast to NO_2, we found only 66% of O_3 flux was explained by a multiple regression considering stomatal conductance and symplastic ascorbate. Our model predictions disagree with previous studies suggesting stomatal conductance can explain nearly all of the variation observed in O_3 fluxes to plant leaves (Gut *et al.* 2002; Wieser *et al.* 2003; Dittmar *et al.* 2005). When considered alone, we found stomatal conductance explains only 56% of the variation in O_3 leaf flux suggesting other factors play a strong and controlling role.

The model proposed by Chameides (1989) suggested that, at a constant stomatal conductance, apoplastic ascorbate is the dominant control over the magnitude of O_3 reaching the cell membrane. The findings of the present study support the general hypothesis that ascorbate plays an important role in the rate of leaf O_3 flux (Fig. 2.4). However, we found that symplastic ascorbate was a stronger predictor than apoplastic ascorbate. Other studies have suggested that apoplastic ascorbate concentration may be too low to protect leaves from O_3 damage (Luwe and Heber 1995; Kollist *et al.* 2000; Van Hove *et al.* 2001; Sanmartin *et al.* 2003; D'Haese *et al.* 2005). The significance of symplastic ascorbate as a predictor of O_3 flux suggests that the ability of a plant to regenerate apoplastic ascorbate is more important than the absolute concentration of apoplastic ascorbate. As the apoplastic ascorbate reacts with O_3 it becomes oxidized and must be transported to the symplast to be regenerated before it can undergo further reactions with O_3. The concentration of ascorbate in the symplast may indicate the rate at which ascorbate can be returned to the apoplast and exerts significant control over the steady-state fluxes of O_3.

We found that stomatal conductance and ascorbate alone were not enough to adequately predict O_3 flux as had been suggested in previous studies (Plöchl *et al.* 2000; Turcsanyi *et al.* 2000). There are three factors that are not considered in our conceptual model that may play a role in controlling O_3 flux to leaves. The first is the reaction of O_3 directly with the leaf surface. Stomatal conductance explains a large portion of the variation in O_3 flux, but the positive y-intercept of the model (Table 2.2) implies O_3 reacts directly with the leaf surface, a process that has been observed in other studies (Fowler *et al.* 1998; Gut *et al.* 2002). The second factor is the presence of antioxidant chemicals in the cell wall other than ascorbate. It is likely other antioxidants exist in the apoplast that readily react with ozone. For example, glutathione is a compound that others have suggested is related to ozone sensitivity (Guri 1983; Burkey *et al.* 2000; Chernikova *et al.* 2000). Finally, no estimates of leaf internal surface areas were made in this study. This was reasonable because this study addresses uptake in only one plant species and the internal surface area is likely to be similar among leaves of similar age and size. However, variation in O_3 flux across plant species will be influenced by differences in the relationship between leaf internal volume per unit leaf external area (Ranieri *et al.* 1996; Lyons *et al.* 1999b; Ranieri *et al.* 1999; Plöchl *et al.* 2000).

These models serve two important purposes. First, the models presented here show that while stomatal conductance is the dominant controller of O_3 and NO_2 leaf flux, it alone is not sufficient to predict these fluxes. We must also account for apoplastic reactions and the removal of compounds produced in those reactions. Second, these models provide empirical data that can be used to parameterize mathematical models and generate more accurate predictions of leaf-level O_3 and NO_2 fluxes. Future work should focus on modifying existing stomatally-based canopy models to incorporate leaf biochemical information.

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REFERENCES

Bender J., Weigel H. & Jager H. (1991) Response of Nitrogen-Metabolism in Beans (Phaseolus-Vulgaris L) after Exposure to Ozone and Nitrogen-Dioxide, Alone and in Sequence. New Phytologist 119, 261-267.

Burkey K.O. (1999) Effects of ozone on apoplast/cytoplasm partitioning of ascorbic acid in snap bean. Physiologia Plantarum 107, 188-193.

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

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

Castillo F.J. & 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.

Chameides W.L. (1989) The Chemistry of Ozone Deposition to Plant-Leaves - Role of Ascorbic-Acid. Environmental Science & Technology 23, 595-600.

Chernikova T., Robinson J., Lee E. & Mulchi C. (2000) Ozone tolerance and antioxidant enzyme activity in soybean cultivars. Photosynthesis Research 64, 15-26.

Conklin P.L., Pallanca J., Last R. & Smirnoff N. (1997) L-Ascorbic Acid Metabolism in the Ascorbate-Deficient Arabidopsis Mutant vtc1. Plant Physiology 115, 1277-1285.

Conklin P.L., Williams E. & Last R.(1996). Environmental stress sensitivity of an ascorbic acid-deficient Arabidopsis mutant. PNAS 93, 9970-9974.

Cross C.E., van der Vliet A., Louie S., Thiele J. & Halliwell B. (1998) Oxidative stress and antioxidants at biosurfaces: Plants, skin, and respiratory tract surfaces. Environmental Health Perspectives 106, 1241-1251.

D'Haese D., Vandermeiren K., Asard H. & 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.

Dittmar C., Pfaffelmoser K., Rotzer T. & Elling W. (2005) Quantifying ozone uptake and its effects on the stand level of common beech (Fagus sylvatica L.) in Southern Germany. Environmental Pollution 134. 1-4.

Duyzer J. & Fowler D. (1994) Modeling Land Atmosphere Exchange of Gaseous Oxides of Nitrogen in Europe. Tellus Series B-Chemical and Physical Meteorology 46, 353-372.

Fiscus E.L., Booker F. & Burkey K. (2005) Crop responses to ozone: uptake, modes of action, carbon assimilation and partitioning. Plant, Cell and Environment 28, 997-1011.

Fowler D., Flechard C., Skiba U., Coyle M. & Cape J. (1998) The atmospheric budget of oxidized nitrogen and its role in ozone formation and deposition. New Phytologist 139, 11-23.

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

Grimes H.D., Perkins K. & Boss W. (1983) Ozone degrades into hydroxyl radicals under physiological conditions. Plant Physiology 72, 1016-1020.

Guri A. (1983) Variation in Glutathione and Ascorbic-Acid Content among Selected Cultivars of Phaseolus-Vulgaris Prior to and after Exposure to Ozone. Canadian Journal of Plant Science 63, 733-737.

Gut A., Scheibe M., Rottenberger S., Rummel U., Welling M., Ammann C., Kirkman G., Kuhn U., Meixner F., Kesselmeier J., Lehmann B., Schmidt W., Muller E. & Piedade M. (2002) Exchange fluxes of NO2 and O-3 at soil and leaf surfaces in an Amazonian rain forest. Journal of Geophysical Research-Atmospheres 107(D20).

Hanson P.J. & Lindberg S. (1991) Dry Deposition of Reactive Nitrogen-Compounds - a Review of Leaf, Canopy and Non-Foliar Measurements. Atmospheric Environment Part a-General Topics 25, 1615-1634.

Hargreaves K.J., Fowler D., Storetonwest R. & Duyzer J. (1992) The Exchange of Nitric-Oxide, Nitrogen-Dioxide and Ozone between Pasture and the Atmosphere. Environmental Pollution 75, 53-59.

Heber U., Bukhov N., Wiese C. & Hedrich R. (2003) Energized uptake of ascorbate and dehydroascorbate from the apoplast of intact leaves in relation to apoplastic steady state concentrations of ascorbate. Plant Biology 5, 151-158.

Hereid D.P. & Monson R. (2001) Nitrogen oxide fluxes between corn (Zea mays L.) leaves and the atmosphere. Atmospheric Environment 35, 975-983.

Hill A.C. (1971) Vegetation - Sink for Atmospheric Pollutants. Journal of the Air Pollution Control Association 21, 341-346.

Horemans N., Foyer C. & Asard H. (2000) Transport and action of ascorbate at the plant plasma membrane. Trends in Plant Science 5, 263-267.

Hufton C.A., Besford R. & Wellburn A. (1996) Effects of NO (+NO2) pollution on growth, nitrate reductase activities and associated protein contents in glasshouse lettuce grown hydroponically in winter with CO2 enrichment. New Phytologist 133, 495-501.

Hur J.S., & Wellburn A. (1994) Effects of Atmospheric No2 on Azolla-Anabaena Symbiosis. Annals of Botany 73, 137-141.

IPCC (2001) A report of working group I of the intergovernmental panel on climate change.

Jones T.L., Tucker D. & Ort D. (1998) Chilling Delays Circadian Pattern of Sucrose Phosphate Synthase and Nitrate Reductase Activity in Tomato. Plant Physiologist 118, 149-158.

Kollist H., Moldau H., Mortensen L., Rasmussen S. & Jorgensen L. (2000) Ozone flux to plasmalemma in barley and wheat is controlled by stomata rather than by direct reaction of ozone with cell wall ascorbate. Journal of Plant Physiology 156, 645-651.

Laisk A., Kull O. & Moldau H. (1989) Ozone Concentration in Leaf Intercellular Air Spaces Is Close to Zero. Plant Physiology 90, 1163-1167.

Lee E.H., Jersey J., Gifford C. & Bennett J. (1984) Differential Ozone Tolerance in Soybean and Snapbeans - Analysis of Ascorbic-Acid in O-3-Susceptible and O-3-Resistant Cultivars by High-Performance Liquid-Chromatography. Environmental and Experimental Botany 24, 331-341.

Lee Y.N. & Schwartz S. (1981) Reaction-Kinetics of Nitrogen-Dioxide with Liquid Water at Low Partial-Pressure. Journal of Physical Chemistry 85, 840-848.

Luwe M. & Heber U. (1995) Ozone Detoxification in the Apoplasm and Symplasm of Spinach, Broad Bean and Beech Leaves at Ambient and Elevated Concentrations of Ozone in Air. Planta 197, 448-455.

Luwe M., Takahama U. & Heber U. (1993) Role of Ascorbate in Detoxifying Ozone in the Apoplast of Spinach (Spinacia-Oleracea L) Leaves. Plant Physiology 101, 969-976.

Lyons T., Ollerenshaw J. & Barnes J. (1999a) Impacts of ozone on Plantago major: apoplastic and symplastic antioxidant status. New Phytologist 141, 253-263.

Lyons T., Plochl M., Turcsanyi E. & Barnes J. (1999b) Extracellular antioxidants: a protective screen against ozone? in Environmental Pollution and Plant Responses. (eds S. Agrawal, M. Agrawal and D. Krizek). CRC Press/Lewis, New York.

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

Moldau H. (1998) Hierarchy of ozone scavenging reactions in the plant cell wall. Physiologia Plantarum 104, 617-622.

Moldau H., Bichele I. & Huve K. (1998) Dark-induced ascorbate deficiency in leaf cell walls increases plasmalemma injury under ozone. Planta 207, 60-66.

Morikawa H., Higaki A., Nohno M., Takahashi M., Kamada M., Nakata M., Toyohara G., Okamura Y., Matsui K., Kitani S., Fujita K., Irifune K. & Goshima N. (1997) NO2 assimilation ability of plants varies more than 600-fold among 217 taxa. Plant Physiology 114, 506-506.

Morikawa H., Higaki A., Nohno M., Takahasi M., Kamada M., Nakata M., Toyohara G., Okamura Y., Matsui K., Kitani S., Fujita K., Irifune K. & Goshima N. (1998) More than a 600-fold variation in nitrogen dioxide assimilation among 217 plant taxa. Plant Cell and Environment 21, 180-190.

Murray A. & Wellburn A. (1985) Differences in Nitrogen-Metabolism between Cultivars of Tomato and Pepper During Exposure to Glasshouse Atmospheres Containing Oxides of Nitrogen. Environmental Pollution Series a-Ecological and Biological 39, 303-316.

Plöchl M., Lyons T., Ollerenshaw J. & Barnes J. (2000) Simulating ozone detoxification in the leaf apoplast through the direct reaction with ascorbate. Planta 210, 454-467.

Ramge P., Badeck F., Plochl M. & Kohlmaier G. (1993) Apoplastic Antioxidants as Decisive Elimination Factors within the Uptake Process of Nitrogen-Dioxide into Leaf Tissues. New Phytologist 125, 771-785.

Ranieri A., Castagna A., Padu E., Moldau H., Rahi M. & Soldatini G. (1999) The decay of O-3 through direct reaction with cell wall ascorbate is not sufficient to explain the different degrees of O-3-sensitivity in two poplar clones. Journal of Plant Physiology 154, 250-255.

Ranieri A., Durso G., Nali C., Lorenzini G. & Soldatini G. (1996) Ozone stimulates apoplastic antioxidant systems in pumpkin leaves. Physiologia Plantarum 97, 381-387.

Rao, M. & Ormrod D. (1995) Ozone exposure decrease UVB sensitivity in a UVB-sensitive flavonoid mutant of arabidopsis. Photochemistry and photobiology 61, 71-78.

Remmler J.L. & Campbell W. (1986) Regulation of Corn Leaf Nitrate Reductase .2. Synthesis and Turnover of the Enzymes Activity and Protein. Plant Physiology 80, 442-447.

Robinson J.M. & Britz S. (2000) Tolerance of a field grown soybean cultivar to elevated ozone level is concurrent with higher leaflet ascorbic acid level, higher ascorbate-dehydroascorbate redox status, and long term photosynthetic productivity. Photosynthesis Research 64, 77-87.

Rondon A. & Granat L. (1994) Studies on the dry deposition of NO2 to coniferous species at low NO2 concentrations. Tellus 46B, 339-352.

Rondon A., Johansson C. & Granat L. (1993) Dry Deposition of Nitrogen-Dioxide and Ozone to Coniferous Forests. Journal of Geophysical Research-Atmospheres 98(D3), 5159-5172.

Rowland A.J., Drew M. & Wellburn A. (1987) Foliar Entry and Incorporation of Atmospheric Nitrogen-Dioxide into Barley Plants of Different Nitrogen Status. New Phytologist 107, 357-371.

Sanmartin M., Drogoudi P., Lyons T., Pateraki I., Barnes J. & Kanellis A. (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, 918-928.

Saxe H. (1986) Stomatal-dependent and stomatal-independent uptake of NOx. New Phytologist 103, 199-205.

Segschneider H., Wildt J. & Forstel H. (1995) Uptake of $^{15}NO_2$ by sunflower (*Helianthus annuus*) during exposures in light and darkness: quantities, relationship to stomatal aperture and incorporation into different nitrogen pools within the plant. New Phytologist 131, 109-119.

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

Smirnoff N. & Wheeler G. (2000) Ascorbic acid in plants: Biosynthesis and function. Critical Reviews in Biochemistry and Molecular Biology 35, 291-314.

Sparks J.P., Monson R., Sparks K. & Lerdau M. (2001) Leaf uptake of nitrogen dioxide (NO2) in a tropical wet forest: implications for tropospheric chemistry. Oecologia 127, 214-221.

Srivastava H.S. & Ormrod D. (1989) Nitrogen-Dioxide and Nitrate Nutrition Effects on Nitrate Reductase-Activity and Nitrate Content of Bean-Leaves. Environmental and Experimental Botany 29, 433-438.

Stulen I., Perez-Soba M., De Kok L. & Van der Eerden L. (1998) Impact of gaseous nitrogen deposition on plant functioning. New Phytologist 139, 61-70.

Teklemariam T.A., & Sparks J. (2006) Leaf fluxes of NO and NO2 in four herbaceous plant species: The role of ascorbic acid. Atmospheric Environment 40, 2235-2244.

Thoene B., Rennenberg H. & Weber P. (1996) Absorption of atmospheric NO2 by spruce (Picea abies) trees .2. Parameterization of NO2 fluxes by controlled dynamic chamber experiments. New Phytologist 134, 257-266.

Thoene B., Schroder P., Papen H., Egger A. & Rennenberg H. (1991) Absorption of Atmospheric No2 by Spruce (Picea-Abies L Karst) Trees .1. No2 Influx and Its Correlation with Nitrate Reduction. New Phytologist 117, 575-585.

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

Van Hove L., Bossen M., San Gabino B. & Sgreva C. (2001) The ability of apoplastic ascorbate to protect poplar leaves against ambient ozone concentrations: a quantitative approach. Environmental Pollution 114, 371-382.

Weber P., & Rennenberg H. (1996) Dependency of nitrogen dioxide (NO2) fluxes to wheat (Triticum aestivum L) leaves from NO2 concentration, light intensity, temperature and relative humidity determined from controlled dynamic chamber experiments. Atmospheric Environment 30, 3001-3009.

Wieser G., Matyssek R., Kostner B. & Oberhuber W. (2003) Quantifying ozone uptake at the canopy level of spruce, pine and larch trees at the alpine timberline: an approach based on sap flow measurement. Environmental Pollution 126, 5-8.

Yoneyama T., Hashimoto A. & Totsuka T. (1980) Absorption of Atmospheric No2 by Plants and Soils .4. 2 Routes of Nitrogen Uptake by Plants from Atmospheric No2 - Direct Incorporation into Aerial Plant-Parts and Uptake by Roots after Absorption into the Soil. Soil Science and Plant Nutrition 26, 1-7.

Zeevaart A.J. (1976) Some Effects of Fumigating Plants for Short Periods with No2. Environmental Pollution 11, 97-108.

Zheng Y.B., Lyons T., Ollerenshaw J. & Barnes J. (2000) Ascorbate in the leaf apoplast is a factor mediating ozone resistance in Plantago major. Plant Physiology and Biochemistry 38, 403-411.

Chapter 3

Responses of sugar maple and eastern hemlock seedlings to increasing carbon dioxide, nitrogen dioxide, and nitrate

Abstract

Various human-induced changes to the atmosphere have caused carbon dioxide (CO_2), nitrogen dioxide (NO_2), and nitrate deposition (NO_3^-) to increase in many regions of the world. The goal of this study was to examine the simultaneous influence of these three factors on tree seedlings. We used open-top chambers to fumigate sugar maple (*Acer saccharum)* and eastern hemlock (*Tsuga canadensis*) with ambient or elevated CO_2 and NO_2 (760 ppm and 40 ppb, respectively). In addition, we applied an artificial wet deposition of 30 kg ha^{-1} yr^{-1} NO_3^- to half of the open-top chambers. After two growing seasons, hemlocks showed a stimulation of growth under elevated CO_2, but the addition of either elevated NO_2 or NO_3^- eliminated this effect. In contrast, sugar maple seedlings exhibited no growth enhancement under elevated CO_2 alone, decreased growth in the presence of either NO_2 and/or NO_3^-, and the combined treatments of elevated CO_2 and increased NO_2 and/or NO_3^- were similar to control plants. Elevated CO_2 induced changes to the leaves of both species including, decrease specific leaf area, decreased % N, and increased C:N. The effects of elevated CO_2, NO_2, and NO_3^- on growth were not additive and treatments that singly had no effect often modified the effects of other treatments. The growth of both maple and hemlock seedlings under the full combination of treatments (CO_2 + NO_2 + NO_3^-) was similar to that of seedlings grown under control conditions, suggesting that models predicting increased seedling growth under future atmospheric conditions may be overestimating the growth and carbon storage potential of young trees. This study highlights the importance of using the simultaneous application of multiple treatments rather than relying on additive models using single treatment responses.

Introduction

To predict the effects of future atmospheric change on plants, most studies examine only single factors. This is a logical way to begin, but once single treatment responses have been established, it is important to conduct multi-factor experiments. When plants are exposed to simultaneous treatments, the responses are not always what would have been predicted from single-treatment studies. Here we used a factorial design to examine the single and combined effects of elevated carbon dioxide (CO_2), gaseous nitrogen dioxide (NO_2) and the soil deposition of nitrate (NO_3^-). We used this approach to explore instances where the combination of treatments may not be simply additive and to better predict how plants are likely to respond to changes in future atmospheric composition.

There is general scientific concensus that human activities, particularly the burning of fossil fuels, are changing the chemistry of Earth's atmosphere and increasing the global emissions of CO_2 and reactive nitrogen. CO_2 emissions have increased 80% since 1970, and the global CO_2 concentration is increasing by 1.9 ppm per year (IPCC 2007). Between 1860 and 2000, the total amount of reactive nitrogen produced by human processes increased from 15 to 165 Tg N yr^{-1} with reactive nitrogen from fossil fuel burning increasing from 1 Tg N yr^{-1} to 25 Tg N yr^{-1} (Galloway et al. 2003).

Reactive nitrogen in the atmosphere can be deposited to the biosphere via either wet or dry deposition. In the focus region of this study, northern lower Michigan, about 60% of the total wet deposition of nitrogen is in the form of NO_3^- (Pregitzer et al. 2008). In 2004 and 2005, NO_3^- deposition throughout the Midwestern United States ranged from 11-18 kg ha^{-1} yr^{-1} (National Atmospheric Deposition Program, NADP http://nadp.sws.uiuc.edu), but some of the highest nitrogen

deposition sites in Europe receive as much at 60 kg N ha^{-1} yr^{-1} (MacDonald et al. 2002).

Nitrogen dioxide is one component of dry deposition that is increasing locally along roadways and around point sources. In the Northeastern United States, about 50% of the NO_x (NO_2 + NO) emissions come from vehicle emissions and another 25% come from production of electricity. In urban areas in the United States, the concentration of NO_2 is typically 10-45 ppb (NASA Visible Earth http://visibleearth.nasa.gov) and 22-45% of Europeans living in urban environments now experience background NO_2 levels above 20 ppb (© EEA, Copenhagen, 2008).

Many studies have looked at the influence of elevated CO_2 on plant gas exchange and growth of plants. Several review papers report that the majority of elevated CO_2 studies find an increase in photosynthesis (at least initially) and a decrease in stomatal conductance in response to elevated CO_2 (Ainsworth and Long 2005, Norby et al. 1999, Curtis and Wang 1998), although the magnitude of the variation in percent change was dependent on the functional group of the plants and growing conditions in the study (Ainsworth and Long 2005). The observed increase in photosynthetic rates typically leads to an increase in above-ground biomass (Ainsworth and Long 2005, Norby et al. 1999, Curtis and Wang 1998) with the exception of plants whose growth is limited by nutrients (Curtis and Wang 1998, Oren et al. 2001) or water (Housman et al. 2006).

In addition to alterations in gas exchange and growth, CO_2 has been shown to alter the elemental ratios of tissues and the allocation of biomass within the plant. In particular, most studies find that elevated CO_2 decreases the %N of leaf material (Curtis and Wang 1998, Norby et al. 1999, Ainsworth and Long 2005) leading to an increase in the ratio of carbon to nitrogen in plant biomass (C:N). Because elevated CO_2 provides more carbon substrate for photosynthesis and increases the C:N of

leaves, many researchers have hypothesized plants should increase allocation to roots because the extra carbon will increase the limitation by other nutrients. However, few studies have actually seen this effect (Curtis and Wang 1998, Norby et al. 1999).

Wet deposition of NO_3^- can affect photosynthesis and growth in two opposing ways. Since nitrogen is limiting in many forested ecosystems (Vitousek and Howarth 1991), wet deposition of NO_3^- can alleviate nitrogen limitation causing an increase in foliar N content (e.g. Fenn 1998, Magill et al. 2000, Boggs et al. 2005) and overall tree growth (Pregitzer et al. 2008). However, long-term high deposition of N can lead to nitrogen saturation. This occurs when there is an excess of nitrogen in the ecosystem such that it affects the balance of soil processes and leads to depletion of base cations (particularly calcium and magnesium), and acidification of the soil (Fenn 1998). This process leads to a decline in plant growth and can increase tree mortality (e.g. McNulty et al. 1996).

Increased gaseous NO_2 in the atmosphere can be directly incorporated through the foliage and can theoretically increase or decrease photosynthesis and growth (Sparks 2009). When NO_2 enters plant leaves it disproportionates into nitrate and nitrite (Rennenberg and Geβler 1999, Siegwolf et al. 2001) and has been shown to contribute nitrogen to the formation of plant tissue, suggesting that plants can use it as a source of nitrogen (Vallano and Sparks 2007). Siegwolf et al. 2001 found a significant increase in biomass under 100 ppb NO_2 and as much as 15% of a plant's nitrogen can come from NO_2 (Vallano and Sparks 2007). Like increasing NO_3^- deposition, it is expected that in a nitrogen limited system, elevated NO_2 could stimulate plant growth. However, NO_2 is also an oxidant and when it enters plant leaves it has the potential to react with cell membranes and damage internal cellular structures. At very high concentrations of NO_2, investigators have found reduced

plant growth (Rowland et al. 1985) and increased mortality (Strivastava et al. 1992, Qiao and Murray 1997) although the responses tend to be species-specific.

When elevated CO_2 is combined with nitrogen, the increase in photosynthesis and total biomass can be greater than the sum of the two single treatments (Oren et al. 2001). Increased nitrogen is likely to increase the %N of the leaf tissue, which may counteract the effect of CO_2. Increased nitrogen may also decrease the allocation of biomass to the roots if the plant can get the same nutrients from a smaller soil volume (Zak et al. 2000). If NO_2 doesn't cause oxidative damage, then it may provide an additional source of nitrogen and the plant responses may be similar to the responses to increased NO_3^-.

In this study, we examined the single and combined effects of elevated CO_2, NO_2, and increased wet deposition of NO_3^- and predicted the following combinatorial responses:

1) Additional N as either NO_2 or NO_3^- will increase growth under elevated CO_2 by alleviating N limitation.

2) Elevated CO_2 will decrease the effects of NO_2 as a result of decreased stomatal conductance limiting NO_2 entry into leaves.

3) The effects of NO_2 will be less pronounced under increased NO_3^- because the magnitude of additional N from NO_2 will be small compared with that from NO_3^-.

Methods

Site description:

This study was conducted in an open field at the University of Michigan Biological Station near Pellston, MI USA (45°33′14″N 84°47′4″W). Over the two year study period, the average high and low June-August temperatures at the site were 23 and 13^{o} C, respectively. In order to reduce the ambient light level and approximate the understory environment, a shade cloth was erected over the entire site, reducing the incoming light by 50%. The shade cloth was porous and allowed at least some natural precipitation to pass. The total summer precipitation in 2004 and 2005 was 20.7 and 27 cm, respectively, and experimental plants were watered every three days (if there was no natural precipitation) to avoid drought stress.

Plant material and chambers:

Bare-rooted seedlings of hemlock (*Tsuga canadensis*) and sugar maple (*Acer saccharum*) seedlings were purchased from Pikes Peak Nursery (Penn Run, PA). The seedlings were 3-5 years old and 45-60 cm tall when planted into the experiment in May 2004.

Rootboxes were prepared by drilling approximately 30 2.5 cm holes into the bottoms of 160 plastic storage tubs (volume = 0.16 m^3). The boxes were buried so that the top of each box was level with the surrounding soil surface and the boxes were filled with sand and covered with 10 cm of topsoil (both from local sources). This sand/soil layering mimicked the soil structure in the surrounding forests.

Two seedlings of each species were randomly assigned to each rootbox and inoculated with 50 cm^3 of forest topsoil removed from a predominantly sugar maple or hemlock stand to establish a microbial community similar to that of the local forests. In May 2004, two rootboxes were placed under each chamber and in September one

box from each chamber was harvested. In May 2005, the harvested rootboxes were replanted with new seedlings resulting in cohorts of single-season and two-season seedlings harvested in 2005.

One flow-through open-top chamber (0.8m x 0.8m x 1m) was placed over each pair of rootboxes. The chamber frames were made of 1/2 inch PVC pipe and the frames were wrapped with transparent 0.8 mm PVC film. Fans encased in metal blower boxes were connected to a perforated ring of PVC that was placed at the bottom of the chamber. The bulk flow from the blower box through the chamber was 600-700 lpm resulting in a turnover time of less than two minutes. A smoke test showed that the chambers became fully mixed in less than 10 seconds.

The chambers were assigned to a block (ten total blocks) based on their location in the field in order to minimize the effects of possible light, wind, and moisture differences across the field. Within each block, each chamber was randomly assigned one of the eight possible treatment combinations, for a total of 80 chambers.

Treatments:

Carbon dioxide was purchased from Airgas (Charlevoix, MI) as a liquid and NO_2 was purchased in 10,000 ppm tanks from Scott-Marin Specialty Gas (Riverside, CA). The gas from each tank was delivered to a manifold block where it was split into 40 lines and the flow of each line was controlled by a needle valve and flowmeter (Aalborg, Orangeburg, NY). Each needle valve controlled the flow of either CO_2 or NO_2 to a single chamber. Opaque black Teflon (PTFE) tubing was used for all NO_2 lines and Poly Flo tubing (J.F. Good Company, OH) was used for CO_2 lines. Return lines placed in each chamber were used to bring air from the chamber to a solenoid system that automatically sampled each chamber every 4 hours. An infrared gas analyzer (LiCor 6252, Lincoln, NE, USA) was used for analysis of CO_2 concentration

and a chemiluminescence analyzer (EcoPhysics CLD 760, Switzerland) fitted with a NO_2 converter (Ecophysics, PLC 660, Switzerland) was used for measurement of NO_2 and NO concentrations. Both analyzers were calibrated weekly using sequential dilution of certified calibration tanks (Scott-Marrin Specialty Gas, Riverside, CA).

In 2004, the elevated CO_2 treatment began 20-June and the NO_2 treatment began 10-July. Both treatments were ended on 15-September, 2004. In 2005, elevated CO_2 and NO_2 treatments began on 13-June and ended 22-August. Elevated CO_2 chambers were set to 760 ppm CO_2, elevated NO_2 chambers received 40 ppb NO_2, and the treatments were applied between 7 am and 7 pm daily. The concentration of each gas in each chamber was checked daily and adjusted to the target if needed. The NO concentration in elevated NO_2 chambers was typically 3-7ppb. The ambient CO_2 concentration was 365 ppm and ambient NO_2 and NO concentrations were both < 1 ppb. Three times each summer the ozone concentration in each chamber was measured and we found that the addition of NO_2 did not increase ozone levels above ambient.

Half the chambers were given additional soil nitrogen in the form of $NaNO_3$ at a rate of 30 kg N ha^{-1} yr^{-1}. Each year, solid fertilizer was applied and immediately watered in four times at two week intervals beginning 22-June in 2004 and 14-June in 2005.

Photosynthesis measurements:

The Li-Cor 6400 portable gas exchange system (Li-Cor, Lincoln, NE) was used for all gas exchange measurements. Photosynthesis and stomatal conductance were measured in July and August during both years, however, only the maple seedlings that were planted in 2004 were measured. As a result, the gas exchange data for the single season treatment was collected in 2004 on seedlings planted in 2004.

The second season data was collected on the same seedlings in 2005. In all other cases, the data presented for seedlings after one growing season are from seedlings that were planted and harvested in 2005.

Both photosynthesis and stomatal conductance were measured at two CO_2 concentrations for each seedling. First, each seedling was measured at the CO_2 concentration under which it was growing (i.e. seedlings grown under ambient CO_2 were measured at 380 ppm CO_2 and those grown under elevated CO_2 were measured at 760 ppm CO_2) and then each seedling was measured at 760 ppm. We used the first measurements to look for absolute differences in gas exchange. The second measurements were used to look for acclimation of photosynthesis and stomatal conductance to elevated CO_2 by comparing the data collected on plants grown under elevated CO_2 to plants grown at ambient CO_2 and instantaneously exposed to elevated CO_2.

Growth and Allocation Measurements:

At the time of planting, ten seedlings of each species were harvested and the height, stem diameter, and total dry biomass were measured. A multiple linear regression model was developed using height and stem diameter to predict initial dry biomass and create an estimate of the initial dry biomass for each seedling planted in the study. Estimated initial biomass was used as a covariate in all subsequent analyses of biomass.

In September 2005, all seedlings were harvested by removing the rootboxes from the ground and placing the soil and seedlings onto a 2 mm mesh screen to rinse soil from the roots. Once clean, the roots, stems, and leaves were separated, the roots frozen, and the leaves pressed. The hemlock twigs were clipped at the most recent bud scar so that the biomass generated in 2005 was separated from the rest of the

biomass. The separated seedlings were transported to Cornell University in Ithaca, NY where the roots were thawed and separated into three diameter size classes: <1 mm, 1-2 mm, and >2 mm. All plant tissues were dried for three days at 50 ^{o}C and weighed to determine dry mass [the same procedure was used to harvest plants in 2004 (data not shown)].

Total leaf area of maple seedlings was determined using a leaf area meter (LI-3100, Li-Cor Lincoln, NE). Leaf area was divided by total leaf biomass to determine specific leaf area (SLA). To determine hemlock leaf area, a photographic method was used and only leaves produced during the second growing season were measured. A white piece of paper was placed on a black cloth and a subset of hemlock needles was laid out on the paper and a photograph of the paper was taken. Using Adobe Photoshop software (Adobe Systems Inc. San Jose, CA) every pixel was changed to black or white, so that the piece of paper was entirely white pixels and the leaves on the paper were entirely black pixels. By selecting the piece of paper and using the "histogram" function, the program determined the percent of the pixels within the piece of paper that were black; indicating the percent of the area of the paper covered with leaves. The photographed leaves were then weighed to determine specific leaf area.

Dried leaf tissue was ground and percent C and N determined using an Elemental Analyzer (ThermoFinnegan, FlashEA 1112, Pittsburg, PA).

Statistical Analysis:

In this randomized block design there were 10-20 replicates of each species in each treatment depending on seedling mortality. Most seedling mortality occurred shortly after being transplanted to the field and was not related to treatment (data not shown). Treatment means were compared using mixed model ANOVA and

ANCOVA techniques with pairwise comparisons. All of the analyses of mass included estimated initial biomass as a covariate and were transformed by taking the square root in order to eliminate increasing variance in the residuals. All statistical analyses were completed using SAS statistical software (SAS Institute Inc., SAS Version 9.1.3, Cary, NC) and figures were generated in SigmaPlot (SPSS Science, Chicago, IL).

Results

Biomass:

Elevated CO_2 and increased N (as either NO_2 or NO_3^-) tended to have opposing effects on biomass (Fig 3.1). CO_2 either increased total biomass or balanced the effects of NO_2 and/or NO_3^- depending on the species and the duration of treatment. When elevated NO_2 or NO_3^- had an effect on biomass, it was negative; causing a reduction in maple biomass under ambient CO_2 and eliminating the growth stimulation caused by CO_2 in hemlocks. After only one season, maple seedlings had higher leaf, stem, and total biomass under elevated CO_2. Elevated NO_2 eliminated the effects on leaf and total biomass when NO_3^- was low and on stem biomass regardless of NO_3^- status. Root biomass was the least responsive to CO_2 and was only increased when NO_3^- was high and NO_2 was low. After a second season of fumigation, maples exhibited a reduction in root biomass caused by high NO_2 and/or NO_3^- that resulted in lower total biomass (not significant in the $NO_2 + NO_3^-$ treatment, $p = 0.10$). While CO_2 no longer increased maple biomass, it did eliminate the detrimental effects of NO_2 and/or NO_3^-. In hemlocks, elevated CO_2 increased leaf, stem, root, and total biomass, but adding either NO_2 or NO_3^- eliminated this effect. Neither NO_2 nor NO_3^- had an effect when CO_2 was ambient. The counteracting effects of CO_2 and added N (as either NO_2 or NO_3^-) caused both hemlock and maple seedlings grown under

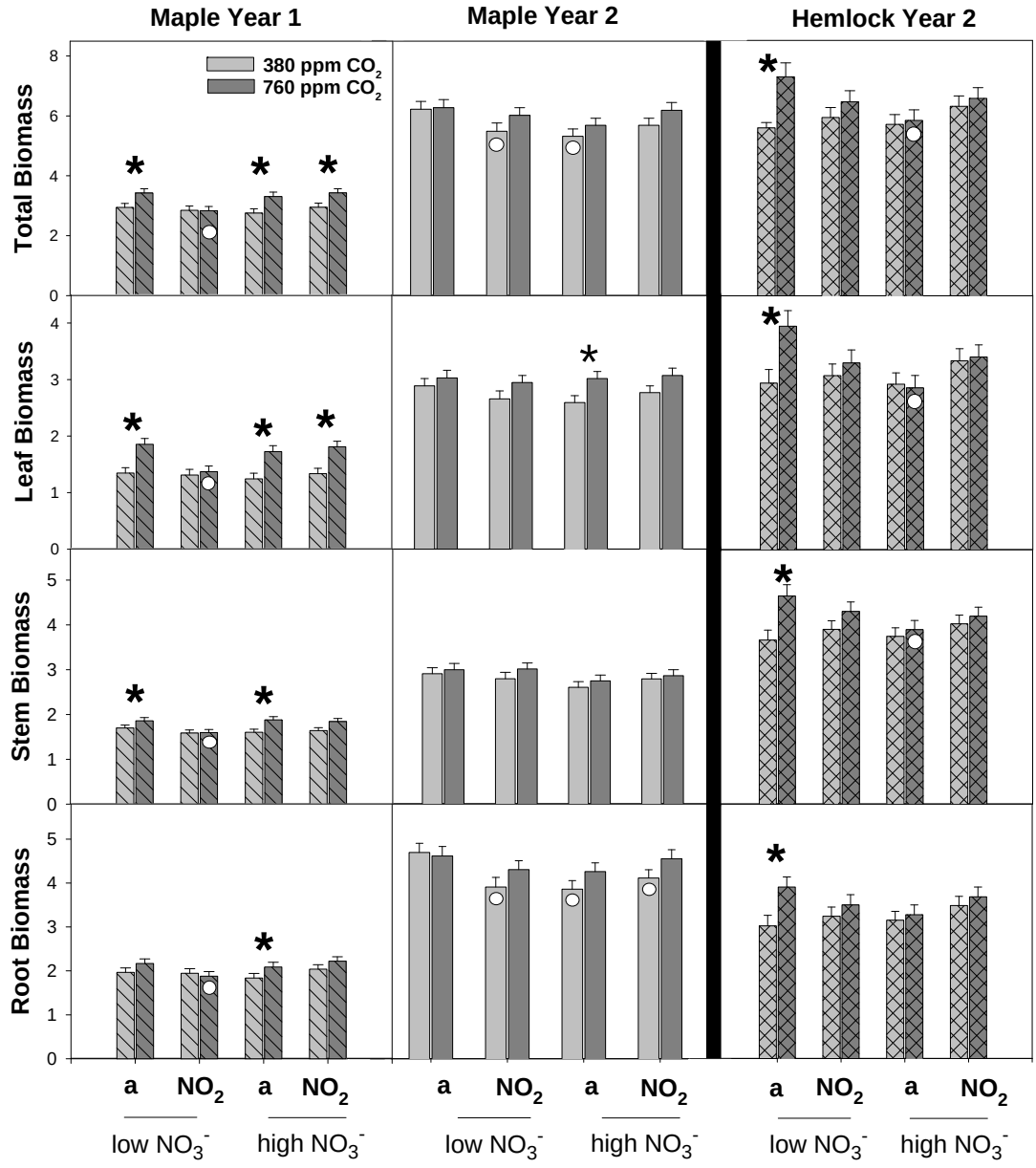

Figure 3.1: Mean total, leaf, stem, and root biomass reported as the square root of dry mass in grams. Light gray bars indicate ambient (380ppm) CO_2 and dark gray bars indicate elevated (760ppm) CO_2. In each panel, the four bars on the left were grown under ambient NO_3^- deposition (low NO_3^-) and the four bars on the right were grown under 30 kg ha^{-1} yr^{-1} NO_3^- deposition (high NO_3^-). On the x-axis, a = ambient NO_2 and NO_2 = 40ppb NO_2. Asterisks indicate a significant difference between the ambient and elevated CO_2 treatments. Open circles indicate a significant difference between that treatment and ambient NO_2 + ambient NO_3^- treatment in the same CO_2 group (e.g. an open circle on the bar of the elevated CO_2 + elevated NO_2 treatment indicates it is significantly different from the CO_2 alone treatment).

combinations of elevated CO_2 and N to exhibit biomass similar to those grown under ambient conditions.

Gas exchange

Gas exchange was only measured on maple seedlings and acclimation of photosynthesis to elevated CO_2 began after two seasons of treatment (Fig 3.2), but reductions in stomatal conductance began in the first year (for some treatments) and became greater after two. At the end of the first season, photosynthesis was higher in all elevated CO_2 treatments (only significant when N was ambient, in elevated NO_2 $p = 0.08$, NO_3^- $p = 0.17$, and $NO_2 + NO_3^-$ $p= 0.08$). When instantaneous photosynthesis was measured at 760 ppm CO_2 on all of the seedlings, there were no differences between those grown under ambient and elevated CO_2 indicating that photosynthesis had not acclimated to the higher concentration of CO_2. Stomatal conductance was decreased by elevated CO_2, but only when NO_2 or NO_3^- were singly elevated. After two seasons of fumigation, there were no differences in photosynthesis when measured under the seedlings' CO_2 growing conditions. However, when all seedlings were compared at 760 ppm CO_2, those from the ambient treatment had higher photosynthetic rates than those from the elevated CO_2 treatment. This acclimation of the photosynthetic process to elevated CO_2 was coupled with a decline in stomatal conductance in every treatment to which CO_2 was added.

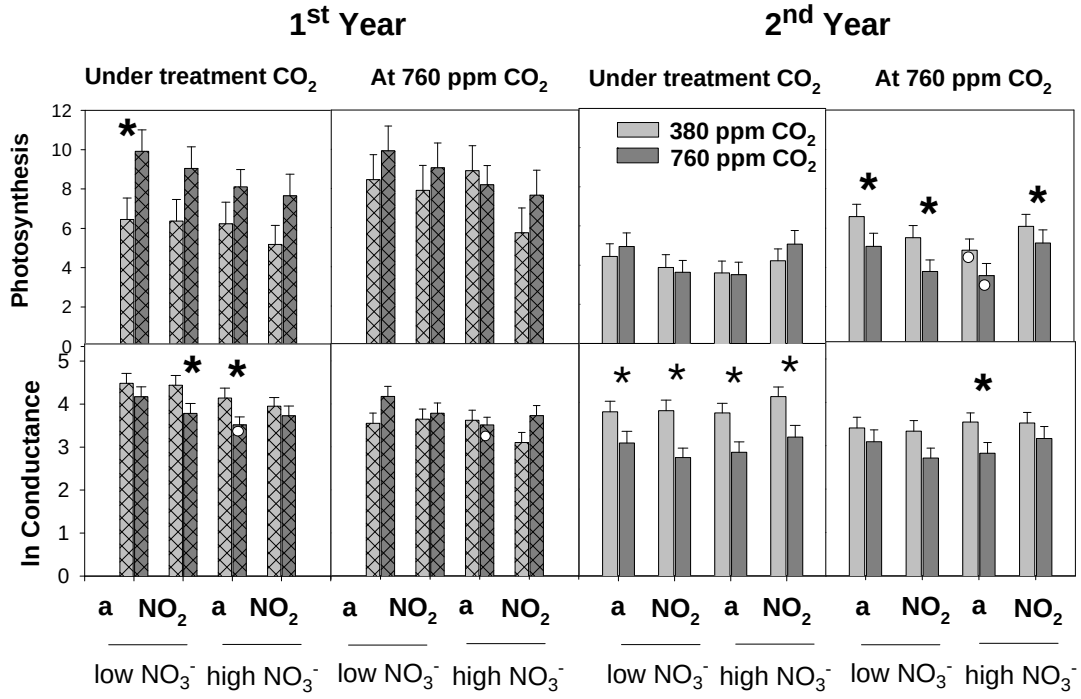

Figure 3.2: Gas exchange data of maple seedlings during the first and second growing seasons (hatched bars and open bars, respectively). The top panels are photosynthesis (μmol CO_2 m^{-2} s^{-1}) and the lower panels are ln of stomatal conductance (μmol H_2O m^{-2} s^{-1}). Light gray bars indicate ambient (380ppm) CO_2 and dark gray bars indicate elevated (760ppm) CO_2. In each panel, the four bars on the left were grown under ambient NO_3^- deposition (low NO_3^-) and the four bars on the right were grown under 30 kg ha^{-1} yr^{-1} NO_3^- deposition (high NO_3^-). On the x-axis, a = ambient NO_2 and NO_2 = 40ppb NO_2. Asterisks indicate a significant difference between the ambient and elevated CO_2 treatments. Open circles indicate a significant difference between that treatment and ambient NO_2 + ambient NO_3^- treatment in the same CO_2 group (e.g. an open circle on the bar of the elevated CO_2 + elevated NO_2 treatment indicates it is significantly different from the CO_2 alone treatment).

Allocation

Only maples altered their allocation of biomass between the root, stem, and leaf tissues (Fig 3.3). After the first season of treatment, elevated CO_2 shifted allocation from root to leaf biomass resulting in a decreased root:shoot (significant in every case except NO_2 + NO_3^- vs CO_2 + NO_2 + NO_3^- where p = 0.08). When NO_2 was elevated under ambient NO_3^-, it eliminated the effect of CO_2. After two seasons, N

was a stronger driver of allocation than CO_2. Nitrogen addition as either NO_2 or NO_3^- reduced allocation to roots causing a decrease in root:shoot relative to the control regardless of CO_2 concentration. Elevated CO_2 alone also weakly decreased root allocation ($p = 0.18$) and root:shoot ($p = 0.16$) so the combinations of N and CO_2 were

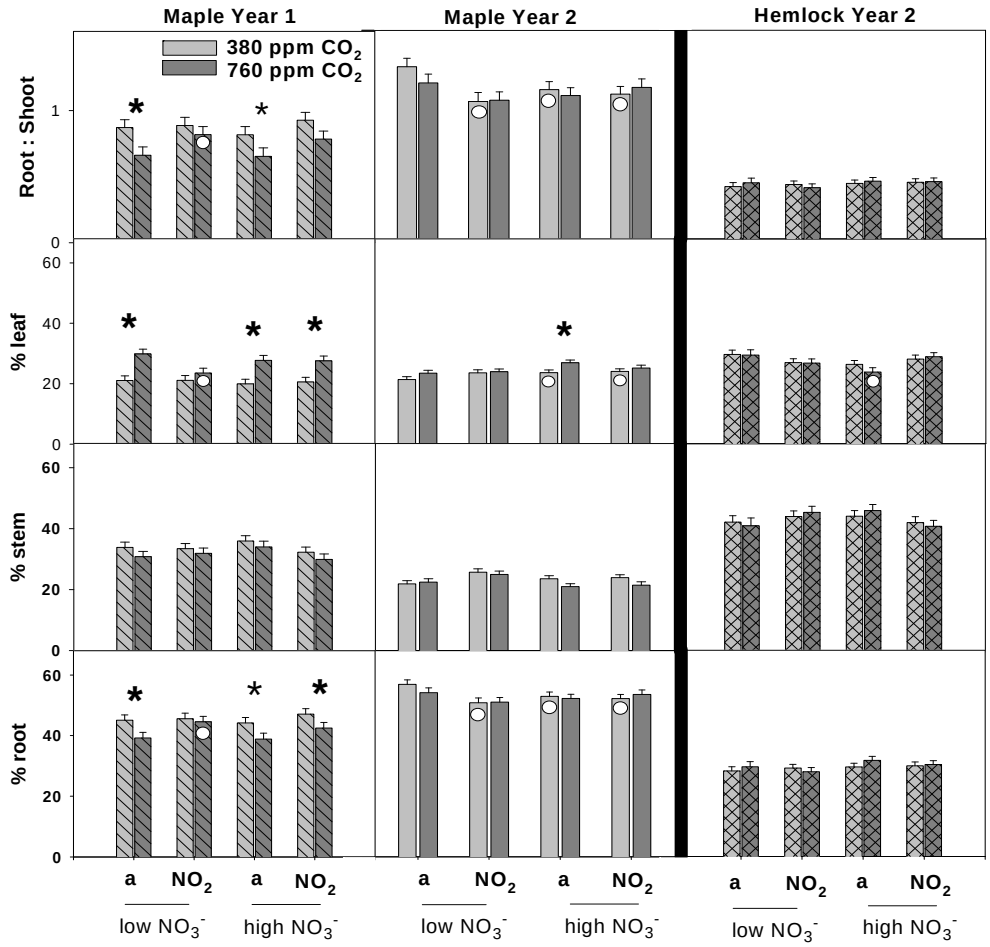

Figure 3.3: Mean root:shoot, and percent biomass found in the leaves, stems, and roots based on dry mass. Light gray bars indicate ambient (380ppm) CO_2 and dark gray bars indicate elevated (760ppm) CO_2. In each panel, the four bars on the left were grown under ambient NO_3^- deposition (low NO_3^-) and the four bars on the right were grown under 30 kg ha^{-1} yr^{-1} NO_3^- deposition (high NO_3^-). On the x-axis, a = ambient NO_2 and NO_2 = 40ppb NO_2. Asterisks indicate a significant difference between the ambient and elevated CO_2 treatments. Open circles indicate a significant difference between that treatment and ambient NO_2 + ambient NO_3^- treatment in the same CO_2 group (e.g. an open circle on the bar of the elevated CO_2 + elevated NO_2 treatment indicates it is significantly different from the CO_2 alone treatment).

not different from the CO_2 alone treatment. Elevated CO_2 increased allocation to leaves when NO_3^- alone was also elevated. No treatments altered allocation to the stem for either species.

Leaf changes

Elevated CO_2 changed the structure and chemistry of leaves in both maple and hemlock seedlings regardless of treatment duration (Fig 3.4). Specific leaf area (SLA) was decreased by elevated CO_2 in almost every case [not significant for 2nd year maples under ambient N ($p = 0.13$) or hemlocks when NO_2 and NO_3^- were both elevated ($p = 0.06$)]. The combination of NO_2 and NO_3^- also resulted in lower SLA, but only when CO_2 was ambient for hemlocks and 1st year maples and when CO_2 was elevated for 2nd year maples. In the 1st year maples, total seedling leaf area was increased by elevated CO_2 (except when NO_2 alone was also elevated) causing the seedlings to have both thicker leaves and increased total photosynthetic surface.

Elevated CO_2 also decreased leaf % N and increased C:N in most cases [Fig 3.5; in 2nd year maples C:N was not significant when NO_3^- alone was elevated ($p = 0.09$)]. In 2nd year maple seedlings, leaf % C was increased by $NO_2 + NO_3^-$ when CO_2 was ambient and by CO_2 when NO_3^- was elevated. Leaf % C in hemlocks was decreased by the presence of elevated CO_2 suggesting that elevated CO_2 caused the production of more oxygen-rich molecules like condensed tannins (King et al. 2001) since oxygen, carbon, and nitrogen make up most of the elemental mass in plant tissue. Unlike maples, hemlocks were additionally influenced by elevated NO_3^-. Elevated NO_3^- increased leaf % N and decreased C:N in all the combinations to which it was added. The combination of elevated CO_2 and NO_3^- balanced each other such that the hemlocks' leaf % N and C:N in these treatments were similar to those in the ambient treatment.

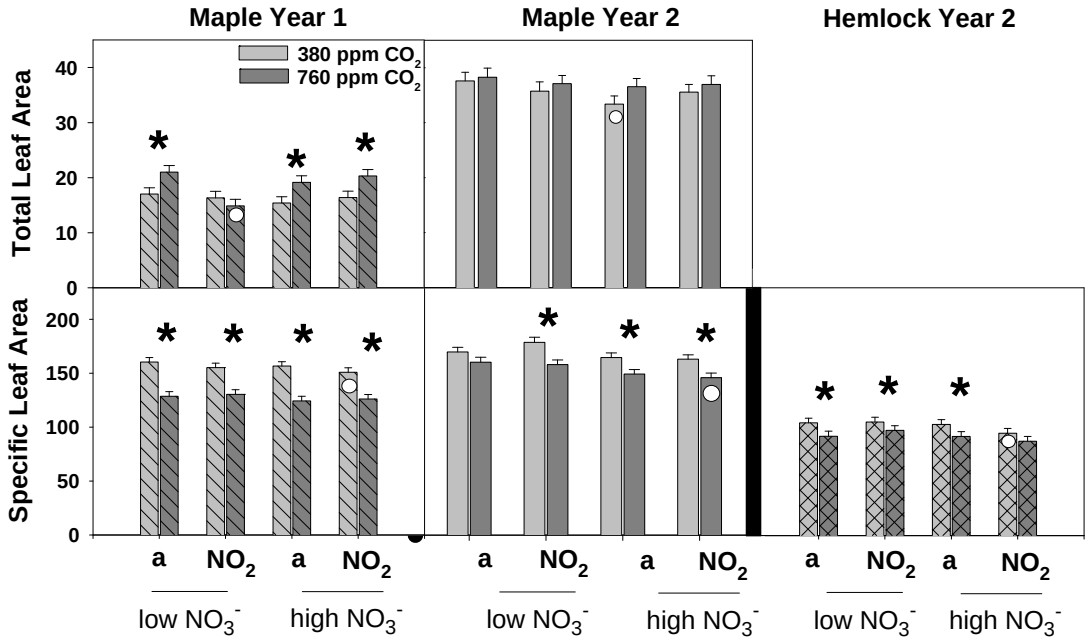

Figure 3.4: Total leaf area in cm^2 for maple seedlings under treatment for 1 and 2 years. Specific leaf area in $cm^2\ g^{-1}$ for all seedlings. Light gray bars indicate ambient (380ppm) CO_2 and dark gray bars indicate elevated (760ppm) CO_2. In each panel, the four bars on the left were grown under ambient NO_3^- deposition (low NO_3^-) and the four bars on the right were grown under 30 kg $ha^{-1}\ yr^{-1}$ NO_3^- deposition (high NO_3^-). On the x-axis, a = ambient NO_2 and NO_2 = 40ppb NO_2. Asterisks indicate a significant difference between the ambient and elevated CO_2 treatments. Open circles indicate a significant difference between that treatment and ambient NO_2 + ambient NO_3^- treatment in the same CO_2 group (e.g. an open circle on the bar of the elevated CO_2 + elevated NO_2 treatment indicates it is significantly different from the CO_2 alone treatment).

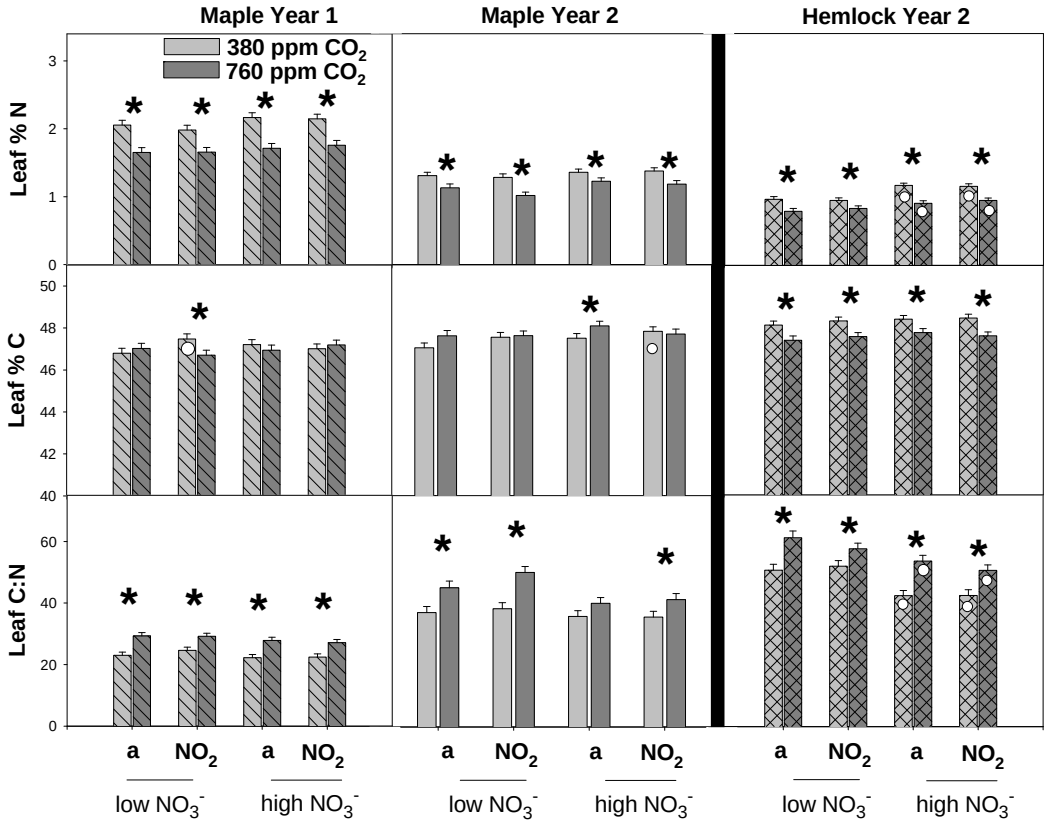

Figure 3.5: Elemental analysis of leaf tissue. Mean percent of leaf tissue composed of nitrogen and carbon and the ratio of carbon to nitrogen. Light gray bars indicate ambient (380ppm) CO_2 and dark gray bars indicate elevated (760ppm) CO_2. In each panel, the four bars on the left were grown under ambient NO_3^- deposition (low NO_3^-) and the four bars on the right were grown under 30 kg ha^{-1} yr^{-1} NO_3^- deposition (high NO_3^-). On the x-axis, a = ambient NO_2 and NO_2 = 40ppb NO_2. Asterisks indicate a significant difference between the ambient and elevated CO_2 treatments. Open circles indicate a significant difference between that treatment and ambient NO_2 + ambient NO_3^- treatment in the same CO_2 group (e.g. an open circle on the bar of the elevated CO_2 + elevated NO_2 treatment indicates it is significantly different from the CO_2 alone treatment).

Discussion

The plant responses to elevated CO_2, NO_2, and NO_3^- in this study can be broadly grouped into three categories. The first group of responses describes how overall growth and allocation of biomass within seedlings was altered by changing CO_2 and N regimes. Changes in the patterns of seedling growth and allocation are of

particular interest because they are necessary for generating accurate predictions of future forest growth and the carbon storage potential of growing forests. The second set of responses represents changes in foliar elemental composition following exposure to CO_2, NO_2, and NO_3^-. Leaves are an important food source for both insect and mammalian herbivores and leaves from deciduous trees provide the substrate for many soil microbial processes. As a result, changes in the C and N content of leaves can have ramifications for food webs and ecosystems processes. The final category is the response of gas exchange to elevated CO_2, NO_2, and NO_3^-. These data help to provide the mechanistic underpinning describing the observations in growth, allocation, and leaf chemistry.

Seedling responses to elevated CO_2 were species-specific when there was no additional reactive N added to the soil or air. After two years, only the hemlock seedlings had greater biomass under elevated CO_2, but the addition of N to either the soil or air eliminated the response. The combination of elevated CO_2 and additional N in the soil or air did not cause a change in the total biomass of either species. This suggests that sugar maple and hemlock seedlings growing under the increased CO_2 concentration and nitrogen deposition predicted under many future scenarios of global change may have similar growth rates as those growing under current atmospheric conditions. This is in contrast to the majority of studies that find increased growth under elevated CO_2 (Ainsworth and Long 2005, Norby et al. 1999, Curtis and Wang 1998), especially with the addition of N, but agrees with several studies that focus on sugar maples and observed little or no growth response to elevated CO_2, at least for the first few years of exposure (Lindroth et al. 1993, Gaucher et al. 2003, Karnosky et al. 2005). The addition of N as either soil NO_3^- or gaseous NO_2 did not increase growth in either species and caused decreases in the biomass of sugar maples under ambient CO_2 and hemlock under elevated CO_2. Although the responses of the two

species are similar, we suspect that the mechanisms behind of the responses may be different. Sugar maples under ambient CO_2 reduced their belowground production in response to higher N, possibly indicating that a smaller root mass was required to obtained the necessary N for the aboveground biomass. Conversely, the increased growth caused by elevated CO_2 in hemlocks was eliminated by the addition of N, which suggests that the N damaged the seedlings in the elevated CO_2 treatment.

Both soil NO_3^- and gaseous NO_2 caused a decline in sugar maple biomass after two years of treatment, but it appears to be related to altered resource allocation rather than to NO_2 oxidative damage or some primary or secondary toxic effect of soil NO_3^-. Sugar maple seedlings responded to high soil NO_3^- and/or high NO_2 with a reduction in below-ground tissue production (Fig 3.1); a response that could be driven by a reduction in the amount of root-foraging necessary to meet above-ground plant water and nutrient requirements. Additional evidence for reduced root biomass as a change in resource allocation rather than a response to damage lies in the observation that the above-ground growth of these seedlings appears to have been constrained by the size of the initial leaf flush. In both years, the sugar maple seedlings produced the majority of their new biomass during the initial period of spring growth; very few seedlings produced new leaves after the first spring flush. With additional N available for growth, biomass production could follow one of two general paths: 1) plants in high N treatments maintain the same amount of below-ground tissue as seedlings in low N treatments and use the additional N to increase above-ground production, or 2) plants in high N treatments maintain the same amount of above-ground biomass as seedlings in low N treatments, but reduce below-ground production to the new level required to support the above-ground tissues. In this study, the above-ground production of sugar maple seedlings did not increase in the high NO_3^- and/or NO_2 treatments (Fig 3.1) perhaps constrained by the size of the initial leaf-flush, and therefore, seedling root

systems did not explore as large a volume of soil to meet the N requirements of aboveground growth. This scenario may be a plausible explanation for the lower below-ground biomass production observed in seedlings grown under supplemental N (NO_3^- or NO_2) compared to seedlings grown under ambient N conditions.

Changes in gas exchange provide a partial explanation for the absence of a CO_2 growth enhancement and the observation that elevated CO_2 eliminated the reduction in sugar maple growth caused by NO_2 and/or NO_3^- (Fig 3.1). After two years, photosynthesis in sugar maples acclimated to the higher concentration of CO_2 in the atmosphere, causing the seedlings in the elevated and ambient CO_2 treatments to fix the same amount of C per unit leaf area (Fig 3.2). Photosynthetic acclimation to elevated CO_2 has been reported in a number of other studies (e.g. Kubiske et al. 1997, Curtis et al. 2000, Ainsworth and Long 2005, Sefcik et al. 2007) and with no extra carbon fixation, there can be no extra growth. Reduced stomatal conductance is another common response to elevated CO_2 (e.g. Ainsworth and Long 2005, Norby et al. 1999) and occurred in this study regardless of the concentration of NO_2 and/or NO_3^- (Fig 3.2). Reduced stomatal conductance can explain why neither NO_2 nor NO_3^- had an effect under elevated CO_2, although the effects are direct and indirect on NO_2 and NO_3^-, respectively. The entrance of NO_2 into leaves is predominately controlled by stomatal conductance (Eller and Sparks 2006), so decreased conductance would have physically limited the amount of NO_2 that the plants experienced. Even though the NO_2 concentration outside the leaves was higher than in the control, the amount inside the leaves may have been similar. Decreased stomatal conductance may have indirectly reduced the uptake of NO_3^- by reducing transpiration and decreasing the bulk flow of water from the soil through the plant. Many nutrients, including NO_3^-, enter plant roots via bulk flow transport and slower movement of water into the plant will slow the acquisition of these nutrients. The reduction in stomatal conductance

limited the ability of seedlings to take advantage of the extra available N, causing all the seedlings grown under elevated CO_2 to have similar quantities and distribution of biomass regardless of NO_2 and/or NO_3^- concentrations.

The duration of the treatment was an important factor in the growth responses of sugar maples to CO_2, NO_2, and NO_3^-. After only one growing season, there was no photosynthetic acclimation to elevated CO_2 and total biomass was increased by elevated CO_2 in nearly every case (Figs 3.1 and 3.2). However, the increased growth under elevated CO_2 was transient and disappeared in the second year as photosynthesis acclimated to elevated CO_2. After two years, NO_3^- and NO_2 had the same effect on biomass: causing reductions in root biomass. Conversely, during the first year NO_2 alone eliminated the CO_2 effect while increased soil NO_3^- had no effect on biomass. Based on the responses to one year of treatment, the conclusion would have been that sugar maples do not exhibit photosynthetic acclimation to elevated CO_2, their growth is stimulated by elevated CO_2, and they have no growth response to increased soil NO_3^-. These are very different conclusions from those we can draw after two years where elevated CO_2 did cause photosynthetic acclimation and did not stimulate growth, and increased NO_3^- and/or NO_2 caused reductions in biomass under ambient CO_2. The large difference in responses from one to two seasons under treatment is a reminder of the importance of duration when looking for responses in long-lived species. Even two growing seasons is a relatively short exposure for tree seedlings and we hope that future studies will be conducted for longer periods of time to determine whether the responses seen after two years are the same after three, four, five, or more years.

Hemlocks exhibited an increase in biomass under elevated CO_2, but the addition of N as either gaseous NO_2 or NO_3^- fertilizer applied to the soil surface eliminated this growth effect. It is difficult to explain why NO_2 and NO_3^- had no

effect when CO_2 was ambient, but appeared to have a negative when CO_2 was elevated. We did not measure gas exchange on the hemlock seedlings, but the increased growth under elevated CO_2 suggests that photosynthesis was increased. While many species show decreased stomatal conductance under elevated CO_2 the response is not universal (Curtis and Wang 1998) and the fact that hemlock needles had higher % N when NO_3^- was high suggests that conductance, and therefore bulk flow, was not reduced enough to impede the uptake of NO_3^-. Under ambient CO_2 an increase in photosynthesis is typically coupled with increased stomatal conductance. If the increased photosynthesis caused by elevated CO_2 in this case increased stomatal conductance, it would have increased the uptake of NO_2, which could have caused damage to the hemlock needles. While NO_2 probably had a direct effect on the hemlock needles, it is likely that the effect of NO_3^- on the hemlock seedlings was indirect. The additional NO_3^- may have depleted soil base cations, particularly Ca^+ and Mg^+, causing hemlocks to become nutrient limited and unable to benefit from extra CO_2. If the addition of NO_3^- caused nutrients other than N to limit seedling growth it would explain why the seedlings grown in high NO_3^- had similar biomass regardless of CO_2 concentration. As seen in the sugar maple seedlings, the combination of elevated CO_2 and additional N resulted in biomass production similar to the control even though NO_2 and NO_3^- were likely acting through different physiological mechanisms.

Elevated CO_2 caused rapid and substantial changes to leaf structure and chemistry similar to those seen in other studies (e.g. Curtis and Wang 1998, Norby et al. 1999, King et al. 2001, Ainsworth and Long 2005). In nearly every treatment combination elevated CO_2 caused decreased SLA and leaf % N, and increased leaf C:N in the hemlocks and in both the one- and two-year maples (Figs 3.4 and 3.5). It is surprising that both the one and two-year maples had the same responses in leaf

chemistry when their growth responses were so different. Maples grown under treatment for only one year increased their overall biomass under elevated CO_2, but without compensating for the dilution of N they ended up with lower leaf % N and greater production of heavy, low-N compounds in their leaves. After two years, there was no increase in maple photosynthesis or growth under elevated CO_2, suggesting there was no additional carbon fixation in these seedling. With no additional carbon fixation one would predict that there should be no increase in the C:N in the leaf tissue, but that is not what we found. The reduction in % N and increase in C:N observed under elevated CO_2 supports the theory that the decreased stomatal conductance caused by elevated CO_2 reduced the ability of maple seedlings to acquire N even when it was being added to the environment as NO_2 or NO_3^-. The increase in SLA suggests that seedlings responded to the lack of N by using the available C to produce greater quantities compounds like condensed tannins as seen in other studies (King et al. 2001). It appears that maples in the future high CO_2 environments will have lower SLA and % N, and higher C:N in both high and low NO_2 and NO_3^- environments regardless of whether or not there is CO_2-induced growth enhancement.

Hemlocks had decreased SLA, % N, and % C, and increased C:N in nearly every treatment combination that included elevated CO_2. They also had increased leaf % N and decreased C:N in all the treatments that included elevated NO_3^-, which caused the plants grown under both elevated CO_2 and NO_3^- to have % N and C:N values similar to the seedlings in the control (Figs 3.4 and 3.5). The CO_2-induced decrease in both leaf % N and % C in the leaves suggests that seedlings in these groups were producing greater quantities of compounds that contained high amounts of oxygen, which along with hydrogen, N, and C make up the most common elements found in leaves. As seen in the one year vs two year maple seedlings the hemlock leaf responses to elevated CO_2 were similar regardless of whether or not there was an

increase in biomass. In the case of the hemlocks, CO_2 alone increased biomass while CO_2 + NO_2 did not and yet the SLA, % N, % C, and C:N of the leaves in the two groups was similar. Unlike the maples, the hemlock seedlings did acquire additional N when soil NO_3^- was high and seedlings grown in the CO_2 + NO_3^- treatments were similar to the control suggesting that in the future, hemlocks growing in high NO_3^- environments will have leaves similar to those currently growing in low NO_3^- environments.

Perhaps the most surprising finding in this study was how few treatments resulted in increased whole-plant carbon acquisition. The only case of increased wood production was hemlock seedlings grown under elevated CO_2 with no additional NO_2 or NO_3^-. Many current models that predict future carbon storage by vegetation include algorithms to account for a "CO_2 fertilization" effect (e.g., Albani et al. 2006, Zaehle et al. 2007) and predict increased future net primary productivity (NPP). However, Zaehle et al. (2007) suggest that the CO_2 effect may be temporary and will be diminished by 2070 as a result of increased temperature. Further, Albani et al. (2006) found that when they compared their NPP predictions with measurements, the model that included a CO_2 fertilization effect overestimated the amount of carbon stored. While some species undoubtedly experience CO_2 fertilization, sugar maple, a dominant species in northeastern US forests had no response to elevated CO_2 and eastern hemlock, another common northeastern species, only showed a response in the absence of NO_2 or NO_3^-. Models that assume a general CO_2 fertilization effect and do not incorporate differing species responses and the effects of concomitant atmospheric changes are likely to overestimate the amount of carbon stored in tree biomass.

The responses of seedling biomass to the combination of elevated CO_2, NO_2, and NO_3^- in this study could not have been predicted by the addition of the single treatment responses. For both maples and hemlocks, treatments that had no effect

when applied singly often eliminated the effects of other treatments. In maples, adding CO_2 eliminated the NO_2 and NO_3^- effects and in hemlocks adding N as either NO_2 or NO_3^- eliminated the CO_2 effect. The effects of single gases were species-specific, but in both species, seedlings exposed to the full combination of treatments exhibited similar growth to those in the ambient treatment. This study is an example of why it is important use simultaneous application of multiple treatments and continue the treatments for more than one growing season to determine how plants may respond to the many concurrent changes happening in Earth's atmosphere.

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REFERENCES

Ainsworth EA, Long SP (2005) What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO_2. New phytologist 165: 351–372

Albani M, Medvigy D, Hurtt GC, Moorcroft PR (2006) The contributions of land-use change, CO2 fertilization, and climate variability to the Eastern US carbon sink. Global Change Biology 12(12):2370-2390

Boggs JL, McNulty SG, Gavazzi MJ, Myers JM (2005) Tree growth, foliar chemistry, and nitrogen cycling across a nitrogen deposition gradient in southern Appalachian deciduous forests. Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere 35:1901-1913

Curtis PS, Vogel CS, Wang XZ, Pregitzer KS, Zak DR, Lussenhop J, Kubiske M, Teeri JA (2000) Gas exchange, leaf nitrogen, and growth efficiency of Populus tremuloides in a CO2-enriched atmosphere. Ecological Applications 10(1):3-17

Curtis PS, Wang X (1998) A meta-analysis of elevated CO_2 effects on woody plant mass, form, and physiology. Oecologia 113:299-313

EEA, Copenhagen, (2008) http://themes.eea.europa.eu/IMS/IMS/ISpecs/I Specification 20080701123452/IAssessment1219309276318/view_content (5/17/2009)

Eller ASD, Sparks JP (2006) Predicting leaf-level fluxes of O-3 and NO2: the relative roles of diffusion and biochemical processes. Plant Cell and Environment 29:1742-1750

Fenn ME, Poth MA, Aber JD (1998) Nitrogen excess in North American ecosystems: Predisposing factors, ecosystem responses, and management strategies. Ecological Applications 8(3):706-733

Gaucher C, Costanzo C, Afif D, Mauffette Y, Chevrier N, Dizengremel P (2003) The impact of elevated ozone and carbon dioxide on young *Acer saccharum* seedlings. Physiologia Plantarum 117:392-402

Galloway JN, Aber JD, Erisman JW, Seitzinger SP, Howarth RW, Cowling EB, Cosby BJ (2003) The nitrogen cascade. Bioscience 53(4): 341-356

Housman DC, Naumburg E, Huxman TE, Charlet TN, Nowak RS, Smith SD (2006) Increases in desert shrub productivity under elevated carbon dioxide vary with water availability. Ecosystems 9:374-385

IPCC (2007). Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change [Solomon, S., D. Qin, M. Manning, Z. Chen, M. Marquis, K.B. Averyt, M.Tignor and H.L. Miller (eds.)]. Cambridge

Karnosky DF, Pregitzer KS, Zak DR, Kubiske ME, Hendrey GR, Weinstein D, Nosal M, Percy KE (2005) Scaling ozone responses of forest trees to the ecosystem level in a changing climate. Plant Cell and Environment 28:965-981

King JS, Pregitzer KS, Zak DR, Kubiske ME, Holmes WE (2001) Correlation of foliage and litter chemistry of sugar maple, *Acer saccharum*, as affected by elevated CO2 and varying N availability, and effects on decomposition. Oikos 94:403-416.

Kubiske ME, Pregitzer KS, Mikan CJ, Zak DR, Maziasz JL, Teeri JA (1997) *Populus tremuloides* photosynthesis and crown architecture in response to elevated CO2 and soil N availability. Oecologia 110(3):328-336

Lindroth RL, Kinney KK, Platz CL (1993) Responses of deciduous trees to elevated atmospheric CO_2: Productivity, phytochemistry, and insect performance. Ecology 74 (3):763-777

MacDonald JA, Dise NB, Matzner E, Armbruster M, Gunderson P, Forsius M (2002) Nitrogen input together with ecosystem nitrogen enrichment predict nitrate leaching from European forests. Global Change Biology 8:1028-1033.

Magill AH, Aber JD, Berntson GM, McDowell WH, Nadelhoffer KJ, Melillo JM, Steudler P (2000) Long-term nitrogen additions and nitrogen saturation in two temperate forests. Ecosystems 3:238-253

McNulty SG, Aber JD, Newman SD (1996) Nitrogen saturation in a high elevation New England spruce-fir stand. Forest Ecology and Management 84:109-121

NADP http://nadp.sws.uiuc.edu (05/17/2009)

NASA Visible Earth http://visibleearth. nasa.gov/view_rec.php?id=15000 (05/17/2009)

Norby RJ, Wullschleger SD, Gunderson CA, Johnson DW, Ceulemans R (1999) Tree responses to rising CO_2 in field experiments: implications for the future forest. Plant, Cell and Environment 22:683-714

Oren R, Ellsworth D, Johnson KH, Phillips N, Ewers BE, Maler C, Schafer KVR, McCarthy H, Hendrey G, McNulty S, Katul G (2001) Soil fertility limits carbon sequestration by forest ecosystems in a CO2-enriched atmosphere. Nature 411: 469-472.

Pregitzer KS, Burton AJ, Zak DR, AF Talhelm (2009) Simulated chronic nitrogen deposition increases carbon storage in Northern Temperate forests. Global Change Biology 14:142-153

Qiao Z, Murray F (1997) The effects of root nitrogen supplies on the absorption of atmospheric NO2 by soybean leaves. New Phytologist 136:239-243

Rennenberg H, Gessler A (1999) Consequences of N deposition to forest ecosystems - Recent results and future research needs. Water Air and Soil Pollution 116:47-64

Rowland AJ, Drew MC, Wellburn AR (1987) Foliar Entry and Incorporation of Atmospheric Nitrogen-Dioxide into Barley Plants of Different Nitrogen Status. New Phytologist 107: 357-371.

Sefcik LT, Zak DR, Ellsworth DS (2007) Seedling survival in a northern temperate forest understory is increased by elevated atmospheric carbon dioxide and atmospheric nitrogen deposition. Global Change Biology 13:132-146

Siegwolf RTW, Matyssek R, Saurer M, Maurer S, Gunthardt-Goerg MS, Schmutz P, Bucher JB (2001) Stable isotope analysis reveals differential effects of soil nitrogen and nitrogen dioxide on the water use efficiency in hybrid poplar leaves. New Phytologist 149:233-246

Sparks JP (2009) Ecological ramifications of the direct foliar uptake of nitrogen. Oecologia 159:1-13

Srivastava HS, Ormrod DP (1989) Nitrogen-Dioxide and Nitrate Nutrition Effects on Nitrate Reductase-Activity and Nitrate Content of Bean-Leaves. Environmental and Experimental Botany 29: 433-438.

Vallano DM, Sparks JP (2007) Quantifying foliar uptake of gaseous nitrogen dioxide using enriched foliar $\delta^{15}N$ values. New Phytologist 177:946-955

Vitousek PM, Howarth RW (1991) Nitrogen limitation on land in the sea: how can it occur? Biogeochemistry 13:87-115

Zaehle S, Bondeau A, Carter TR, Cramer W, Erhard M, Prentice IC, Reginster I, Rounsevell MDA, Sitch S, Smith B, Smith PC, Sykes M (2007) Projected changes in terrestrial carbon storage in Europe under climate and land-use change, 1990-2100. Ecosystems 10:380-401

Zak DR, Pregitzer KS, Curtis PS, Vogel CS, Holmes WE, Lussenhop J (2000) Atmospheric CO2, soil-N availability, and allocation of biomass and nitrogen by Populus tremuloides Ecological Applications 10:34-46

Chapter 4

Responses of tree seedlings to global change: single and combined effects of elevated carbon dioxide, nitrogen dioxide, and ozone

Abstract

Human activities have increased the atmospheric concentrations of carbon dioxide (CO_2), nitrogen dioxide (NO_2), and ozone (O_3) since the industrial revolution and these changes are known to affect the growth of plants. Responses of forests to these global changes are of particular interest because of the area of forested land coverage on earth and the ability of trees to remove CO_2 from the atmosphere and store it in wood. Previous studies have found that elevated CO_2 generally enhances tree seedling growth unless other factors like nutrient or water availability are limiting. In addition, other factors, like the oxidative effect of elevated ozone (O_3) or high temperatures have been suggested to decrease photosynthesis and eventually lead to a decline in growth in spite of any affects of elevated CO_2. Nitrogen dioxide has been less studied, but also has the potential to diminish growth through oxidative effects or to be a source of nutritive nitrogen to forest trees. Changes in stomatal conductance induced by elevated CO_2 are likely to alter the effects of O_3 and NO_2 by limiting the entry of these gases into leaves. In order to investigate the single and combined effects of elevated CO_2, NO_2, and O_3 on tree seedlings, we used open-top chambers to alter the atmosphere around sugar maple (*Acer saccharum*), eastern hemlock (*Tsuga canadensis*), red oak (*Quercus rubra*), and two clones of trembling aspen (*Populus tremuloides*). Seedlings were fumigated for two years with ambient or 560 ppm CO_2, ambient or 40 ppb NO_2, ambient or 100 ppb (5 days/week) O_3, and grown in ambient or 30 kg ha^{-1} yr^{-1} soil NO_3^-. We found very few effects on total seedling biomass and no cases of increased biomass under elevated CO_2 alone. In general, single gas treatments and combinations of multiple oxidative caused decreases in the biomass of

maple and hemlock seedlings. However, the combinatorial treatment most likely to simulate the future atmosphere ($CO_2 + NO_2 + O_3$) exhibited growth responses not different from the control suggesting that the future growth of these species may be similar to their current growth.

Introduction

The atmospheric concentrations of carbon dioxide (CO_2), nitrogen dioxide (NO_2), and ozone (O_3) are increasing, largely due to human activities. It is expected that the global CO_2 concentration will reach 560 ppm within the next 50 years (IPCC 2007) and areas around many cities frequently experience NO_2 levels up to 45 ppb (NASA visible earth) and O_3 levels above 80 ppb (EPA, AIRNOW). To understand how these changes will affect forested ecosystems, it is imperative to assess how trees will respond to these changes simultaneously. Tree seedlings provide the source material for future forests, so their responses to global change will ultimately determine the composition, health, and carbon storage potential of forested areas.

In general, tree seedlings grown under elevated CO_2 exhibit increased biomass in all tissue components, a result accompanied by increased photosynthesis, decreased leaf nitrogen (e.g. Ainsworth and Long 2005, Curtis and Wang 1998, Norby *et al.* 1999) and decreased stomatal conductance (Ainsworth and Long 2005). These general trends have been reported in several review papers and meta-analyses, but they are not universal. In particular, the stimulation of seedling growth was not statistically significant in FACE (free-air CO_2 enrichment) studies when nutrients were limiting (Ainsworth and Long 2005) and declines in stomatal conductance were inconclusive or not supported in meta-analyses that relied primarily on chamber and greenhouse studies (Curtis and Wang 1998, Norby *et al.* 1999).

Ozone is a strong oxidant that causes visible injury to plant leaves, accelerates leaf senescence (e.g. Greitner *et al.* 1994, Coleman *et al.* 1995), and decreases photosynthesis (Chappelka and Samuelson 1998). Despite visible damage and decreases in photosynthesis, many tree species do not exhibit declines in biomass, especially if the exposures are relatively short or the plants are grown under conditions where stomatal conductance is reduced (Chappelka and Samuelson 1998). Over the lifetime of the tree, the cumulative reduction in photosynthesis caused by elevated O_3 has been estimated to decrease hardwood tree growth by 3 – 22% (Ollinger et al 1997). Declines in tree growth is of particular concern to the global change community because of both impacts on overall forest health and also the ability of growing forests to remove CO_2 from the atmosphere and store it as woody biomass. Sensitivity to O_3 has been shown to vary between tree species and even between different clones of the same species, so it is important that studies hoping to make predictions about future forests examine multiple species (e.g. reviews by Chappelka and Samuelson 1998, Skärby *et al.* 1998).

The positive effects of elevated CO_2 are often balanced by the negative effects of O_3 and vice versa (e.g. Isebrands *et al.* 2001, Karnosky *et al.* 2003, King *et al.* 2005). In order for O_3 to damage plants, it must enter plant leaves through stomata and oxidize sensitive tissues like plasma membranes and other cell components. Therefore, decreased stomatal conductance induced by elevated CO_2 can limit the effective dose of O_3 that a plant experiences. Conversely, the increased carboxylation capacity of plants under elevated CO_2 generates more photosynthate that can be used to build new tissue, or replace tissue damaged by elevated O_3. This balance of increased carboxylation and oxidative damage is sometimes found to result in plants that have similar growth rates grown under elevated CO_2 and O_3 compared to those being grown under ambient conditions (Karnosky *et al.* 2003).

Increased gaseous NO_2 in the atmosphere can be directly incorporated through tree foliage and can theoretically increase or decrease photosynthesis and growth (Sparks 2009). When NO_2 enters plant leaves it disproportionates into nitrate and nitrite (Rennenberg and Geβler 1999, Siegwolf *et al.* 2001) and has been shown to contribute nitrogen to the formation of plant tissue, suggesting that plants can use it as a source of nitrogen (Vallano and Sparks 2007). Siegwolf *et al.* 2001 found a significant increase in biomass under 100 ppb NO_2 and as much as 15% of a plant's nitrogen has been shown to come from NO_2 (Vallano and Sparks 2007). Like increasing NO_3^- deposition, it is expected that in a nitrogen limited system, elevated NO_2 could stimulate plant growth. However, NO_2 is also an oxidant and, like O_3, when it enters plant leaves it has the potential to react with cell membranes and damage internal living tissue. At very high concentrations of NO_2, investigators have found reduced plant growth (Rowland *et al.* 1985) and increased mortality (Strivastava *et al.* 1992, Qiao and Murray 1997).

Elevated NO_2 has the potential to enhance growth under elevated CO_2, but elevated CO_2, through its influence on stomatal conductance, may simultaneously limit the influence of NO_2. As a source of additional nitrogen, NO_2 may alleviate the nitrogen-limitation that reduces a plant's ability to fix additional carbon under elevated CO_2 (Sparks 2009). However, NO_2, as a weak oxidant, may also cause damage in a way similar to O_3. Like O_3, the entry of NO_2 into plant leaves is largely controlled by stomatal conductance (Eller and Sparks 2006) and if elevated CO_2 reduces stomatal conductance, it will likely reduce the effect of NO_2 regardless of whether the effect is positive or negative. Because of its relatively weaker influence compared to CO_2 and ozone and its bifunctionality as both a potential nutrient and oxidant, NO_2 may alter the balance of positive and negative effects exerted by the combined $CO_2 + O_3$ treatments. In other words, one might predict that NO_2, although not a significant

factor on its own, would likely significantly alter combined CO_2 + O_3 treatments in ways that are challenging to predict.

The goal of this study was to determine how tree seedlings will respond to the CO_2 concentration expected in 2050 and how the air pollutants O_3 and NO_2 will attenuate those responses. The forests of the northeastern United States include areas of established and re-growing forests which are dominated by late- and early-successional species, respectively. To help to capture some of this successional diversity, we included the late-successional species sugar maple (*Acer saccharum*), eastern hemlock (*Tsuga canadensis*), and red oak (*Quercus rubra*) and two clones of the early-successional species, trembling aspen (*Populus tremuloides*). Because many northeastern US forests are limited by nitrogen, we also examined the effects of these gases under both low and high soil nitrogen availability.

Methods

Site description:

This study was conducted in an open field at the University of Michigan Biological Station (UMBS) in Pellston, MI USA (45°33′14″N 84°47′4″W). The average high and low June-August temperatures at the site were 23 and 13^{o} C, respectively. In order to reduce the ambient light level, a shade cloth was erected over the entire site, reducing the incoming light by 50%. The shade cloth was porous and allowed at least some natural precipitation to pass. The total summer (June-August) precipitation in 2006 and 2007 was 18 and 19 cm, respectively, and the seedlings were watered every three days (if there was no natural precipitation) to alleviate drought stress.

Plant material and chambers:

Bare-rooted seedlings of hemlock (*Tsuga canadensis*) and sugar maple (*Acer saccharum*) seedlings were purchased from Pikes Peak Nursery (Penn Run, PA). The seedlings were 2-4 years old and 24-48 cm tall when planted into the experiment an May 10th 2006. Red oak (*Quercus rubra*) seedlings were transplanted from nearby forests shortly after germination and planted on June 10th 2006. Cuttings from two clonal lines of trembling aspen (*Populus tremuloides*) used in the Rhinelander FACE experiment (Clone #259-O_3 sensitive and Clone #271-O_3 tolerant) were propagated in greenhouses at Cornell University in 2005 and transported to UMBS in 2006. The propagation soil medium was washed from the plant roots before they were planted on May 20th 2006.

Rootboxes were prepared by drilling approximately 30 2.5 cm holes into the bottoms of 160 plastic storage tubs (volume = 0.16 m^3). The boxes were buried so that the top of each box was level with the surrounding soil surface and the boxes were filled with sand and covered with 10 cm of topsoil (both from local sources). This sand-soil layering mimicked the soil structure in the surrounding forests.

In each rootbox, one hemlock, one sugar maple, one red oak, and three trembling aspen (2 O_3 tolerant + 1 O_3 sensitive in blocks 1, 2, 3, 4, 6, 8, and10 and 2 O_3 sensitive + 1 O_3 tolerant in blocks 5, 7, and 9) seedlings were randomly arranged and each seedling was inoculated with 50 cm^3 of forest topsoil removed from a predominantly sugar maple, hemlock, oak, or aspen stand to establish a microbial community similar to that of the local forests. Seedlings that died during the experiment were replaced so that the species composition in each box did not change, but only seedlings planted before July 15th 2006 were included in analyses.

One flow-through open-top chamber (0.8m x 0.8m x 1m) was placed over two rootboxes. The chamber frames were made of 1/2 inch PVC pipe and the frames were

wrapped with transparent 0.8 mm PVC film. Fans encased in metal blower boxes were connected to a perforated ring of PVC that was placed at the bottom of the chamber. The bulk flow from the blower box through the chamber was 600-700 lpm resulting in a turnover time of less than two minutes. A smoke test showed that the chambers became fully mixed in less than 10 seconds.

The chambers were assigned to a block based on their location in the field in order to minimize the effects of possible light, wind, and moisture differences across the field. Within each block, each chamber was randomly assigned to one of the eight possible treatment combinations.

Treatments:

Carbon dioxide was purchased from Airgas (Charlevoix, MI) as a liquid, NO_2 was purchased in 10,000 ppm tanks from Scott-Marin Specialty Gas (Riverside, CA), and O_3 was generated by pumping ambient air through a LG-7 CD Laboratory O_3 generator (Ozone Engineering, El Sobrente CA). The gas from each tank or generator was delivered to a manifold block where it was split into 40 lines, each to a needle valve with a flowmeter (Aalborg, Orangeburg, NY). Each needle valve controlled the flow of CO_2, NO_2, or O_3 to a single chamber. Opaque black PTFE tubing (Forberg Scientific, MI) was used for all NO_2 and O_3 lines and Poly Flo tubing (J.F. Good Company, OH) was used for CO_2 lines. Return lines placed in each chamber were used to bring air from the chamber to a solenoid system that automatically sampled each chamber every four hours for CO_2 and NO_2. O_3 in each chamber was monitored manually every other day with a ThermoEnvironmental Model 49 Chemiluminescence O_3 monitor. A LiCor 6252 IRGA was used for analysis of CO_2 concentration and an EcoPhysics CLD 760 with a PLC 660 NO_x converter was used for analysis of NO_2 and

NO concentrations. The analyzers were calibrated weekly using sequential dilution of certified calibration tanks (Scott-Marrin Specialty Gas, Riverside, CA).

In 2006, the elevated CO_2 treatment began June 8, the NO_2 treatment began June 20, and the O_3 treatment began on July 3. The O_3 and NO_2 treatments were turned off August 20 and CO_2 was turned off on September 15. In 2007, the CO_2 and NO_2 treatments began on May 9, the O_3 treatment began on May 26 and all treatments ended on September 5. Treatment chambers received 560ppm CO_2, 40ppb NO_2 or 100 ppb O_3. CO_2 and NO_2 were applied to the chambers from 7am and 7pm daily. The elevated O_3 treatment was applied 5 days/week from 10 am until 4 pm. The NO concentration in elevated NO_2 chambers was typically 3-7 ppb. Ambient CO_2 concentration was 365ppm, ambient O_3 was typically 30-40 ppb, and ambient NO_2 and NO concentrations were both < 1 ppb.

Once a week, all experimental plants received a ¼ strength Hoagland solution that was modified to include no nitrogen. Half the chambers were given additional soil nitrogen in the form of $NaNO_3$ at a rate of 30 kg N ha^{-1} yr^{-1}. The $NaNO_3$ was added to the Hoagland solution and applied every two weeks beginning June 23 in 2006 and June 11 in 2007.

Photosynthesis measurements:

The Li-Cor 6400 gas exchange system (Li-Cor, Lincoln, NE) was used for all gas exchange measurements. Photosynthesis and stomatal conductance were measured once a month on 3-5 seedlings in each treatment in June, July, and August 2007 resulting in 8-10 measurements from each treatment. Both photosynthesis and stomatal conductance were measured at the CO_2 level under which the seedlings were growing, (i.e. seedlings grown under ambient CO_2 were measured at ambient CO_2 and those grown under 760 ppm CO_2 were measured at 760 ppm CO_2) and then all the

seedlings were measured at 760 ppm. This allowed us to compare gas exchange under the different growing regimes and to look for acclimation of photosynthesis and stomatal conductance.

In August 2007, dark respiration was measured on 3-5 individuals from each treatment. The measurements were made between midnight and 4 am to ensure that there was no photosynthesis occurring.

Growth and Allocation Measurements:

At the time of planting, 10 seedlings of each species were destructively harvested and the wet and dry biomass of the leaf, stem, and root tissues were measured. The measurements from the harvested seedlings were used to determine the relationship between the wet and dry biomasses at the time of planting. Before each seedling was planted, its wet biomass was recorded and the relationship between wet and dry biomass was used to estimate of the initial dry biomass for every seedling planted in the study. Estimated initial biomass was used as a covariate in all the subsequent analyses of biomass.

Leaf production and senescence were monitored in eight of the ten total blocks. Every two weeks, colored thread was used to label all new leaves and the number of leaves in each color group was measured. This allowed us to keep track of the number of leaves grown and lost during each two-week period and calculate the total leaf biomass produced during the growing season. Total seasonal leaf production was estimated as the sum of the end of season leaf biomass and the estimated biomass lost through senescence. Senesced leaf biomass was calculated as the product of the number of leaves lost during the season and the average leaf dry weight within a treatment. At the end of the growing season, the percentage of the total leaf production lost and the timing of that loss was assessed by multiplying the % number of leaves

lost per time period by the total seasonal leaf production. For seedlings in blocks one and two (where leaf production was not monitored) the mean % number of leaves lost by seedlings in each treatment (blocks 3-10) was used to estimate the leaf biomass lost by the individuals in the same treatment. The estimated total leaf biomass was used in all the calculations of total seedling biomass and allocation of seedling biomass.

In September 2007, all of the seedlings were harvested by removing the rootboxes from the ground and placing the soil and seedlings onto a 2 mm mesh screen to rinse the soil off the roots. Once clean, the roots, stems, and leaves were separated, the roots frozen, and the leaves pressed. All samples were transported to Cornell University in Ithaca, NY where the roots were thawed and separated into three size classes: < 1 mm, 1-2 mm, and > 2 mm. All plant tissues were dried for 3 days at 50 ^{o}C and weighed to determine dry mass.

Total leaf area of maple, oak, and aspen seedlings was determined using a LI-3100 leaf area meter (Li-Cor Lincoln, NE). Leaf area was divided by total leaf biomass to determine specific leaf area (SLA).

Statistical Analysis:

In this randomized blocked design there were 3-10 replicates of each species in each treatment depending on seedling mortality. Most seedling mortality occurred early and was not related to treatment (with the exception of oaks). Treatment means were compared using the mixed model ANOVA and ANCOVA techniques with pairwise comparisons ($\alpha = 0.1$). All the analyses of mass included estimated initial biomass as a covariate. In some cases, there was increasing variance in the residuals and the data were square-root transformed for the statistical analyses. There were also some moderate cases of unequal variances between treatments, so the SAS ddfm SATTERTHWAITE technique was used. The ANOVA results are presented in

appendix A. All statistical analyses were done using the SAS statistical software (SAS Institute Inc., SAS Version 9.1.3, Cary, NC) and figures were generated in SigmaPlot (SPSS Science, Chicago, IL).

Results

Mortality:

Mortality was only considered if it occurred at least four weeks after the seedlings were transplanted into the experiment. In all the species except red oak, mortality ranged from 0% to 40% and there were no differences in mortality between treatments (data not shown). In red oak, the mortality generally occurred over the winter after the first growing season and was highest in the $NO_2 + O_3$ treatment when soil NO_3^- was low (Fig 4.1). The 70% mortality rate in that treatment left only three individuals surviving and resulted in very low statistical power for this species.

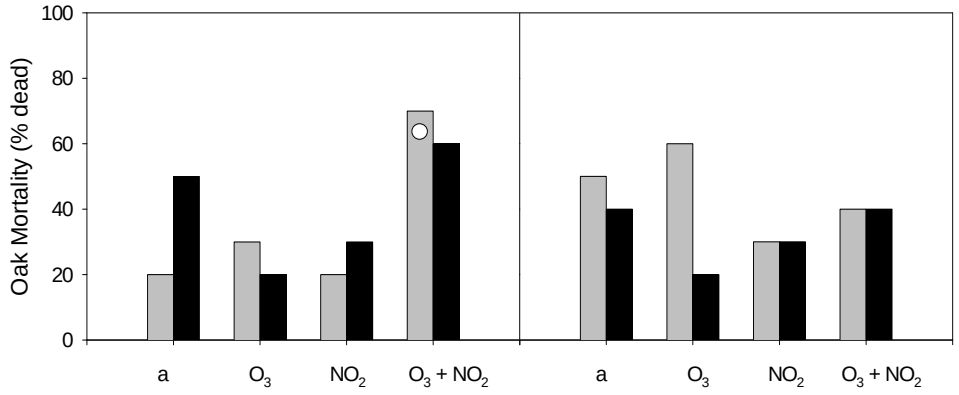

Figure 4.1: Cumulative oak seedling mortality expressed as % dead. Light gray bars indicate ambient (380ppm) CO_2 and black bars indicate elevated (560ppm) CO_2. The left-hand panel are seedlings grown in low soil NO_3^- and the right-hand panel are those grown in 30 kg NO_3^- ha^{-1} yr^{-1}. On the x-axis, a = ambient NO_2 + ambient O_3, O_3 = ambient NO_2 + elevated O_3, NO_2 = elevated NO_2 + ambient O_3, and $O_3 + NO_2$ = elevated NO_2 + elevated O_3. Asterisks indicate a significant difference between the ambient and elevated CO_2 treatments. Open circles indicate a significant difference between that treatment and ambient NO_2 + ambient O_3 treatment in the same CO_2 group.

Biomass:

In this study, there were very few cases where total seedling biomass responded to our treatments (Fig 4.2). The only cases where single treatments altered biomass were in the O_3 sensitive aspens where NO_2 and O_3 each reduced total biomass (Fig 4.2 i and j).

Maple and hemlock seedlings had similar trends in total biomass (Fig 4.2 a-d); when soil NO_3^- was low, there were no single gas effects and no treatment including elevated CO_2 differed from the ambient treatment. Conversely, when soil NO_3^- was high, there were no effects of single gases or of $CO_2 + NO_2$ or $CO_2 + O_3$, but the $CO_2 + NO_2 + O_3$ treatment resulted in greater biomass compared to the ambient treatment.

The two clones of aspen generally showed divergent responses to the treatments (Fig 4.2 g-j). The O_3 tolerant clone under both NO_3^- treatments and the O_3 sensitive clone under the high NO_3^- treatment exhibited no responses to single gases and no treatment combinations were significantly different from the ambient. In contrast, the O_3 sensitive seedlings grown in low soil NO_3^- exhibited decreased total biomass when either O_3 or NO_2 was elevated and although CO_2 had no effect on its own, it eliminated the effect of elevated NO_2 causing the $CO_2 + NO_2 + O_3$ to produce a similar quantity of biomass as the control.

In general, the biomass of < 2 mm roots, > 2 mm roots, stems, and leaves showed the same trends as total biomass (Tables 4.1 & 4.2). Maple seedlings showed no response to single-gas treatments. However, when soil NO_3^- was low, the $CO_2 + NO_2$ treatment caused a decrease in > 2 mm root biomass. Further, when soil NO_3^- was high, the combination of $CO_2 + NO_2 + O_3$ fumigation increased biomass in all tissue types. Under low soil NO_3^-, hemlocks also exhibited no single-gas responses in

Table 4.1: Mean dry biomass (g) of each tissue type for each species grown under low soil NO_3^-. Different letters indicate significantly different pairwise comparisons ($p < 0.1$) and standard error is shown in parentheses.

		Low soil NO_3^-							
	Treatment	**a**	**O_3**	**NO_2**	**$NO_2 + O_3$**	**CO_2**	**$CO_2 + O_3$**	**$CO_2 + NO_2$**	**$CO_2 + NO_2 + O_3$**
Sugar maple	Total biomass	22.56^{ab} (3.70)	16.33^{a} (3.98)	22.70^{ab} (3.40)	23.47^{ab} (3.34)	23.68^{ab} (3.69)	24.41^{b} (3.50)	18.79^{ab} (3.50)	23.15^{ab} (3.69)
	< 2 mm root	7.06^{ab} (1.28)	4.82^{a} (1.38)	6.58^{ab} (1.18)	7.33^{ab} (1.16)	6.62^{ab} (1.28)	7.44^{b} (1.22)	5.01^{b} (1.22)	5.88^{ab} (1.28)
	> 2mm root	7.32^{ab} (1.15)	4.84^{a} (1.24)	7.31^{ab} (1.05)	7.25^{ab} (1.03)	7.83^{b} (1.15)	7.55^{b} (1.08)	5.16^{a} (1.08)	7.44^{b} (1.15)
	Total root	7.32^{ab} (1.15)	4.84^{a} (1.24)	7.31^{ab} (1.05)	7.25^{ab} (1.03)	7.83^{b} (1.15)	7.55^{b} (1.08)	5.16^{a} (1.08)	7.44^{ab} (1.15)
	Stem	3.14^{ab} (0.52)	2.48^{a} (0.55)	3.49^{b} (0.47)	3.78^{b} (0.46)	2.73^{ab} (0.51)	3.22^{b} (0.49)	2.69^{ab} (0.49)	3.27^{ab} (0.51)
	Leaf	5.99^{ab} (1.35)	5.27^{a} (1.49)	6.79^{ab} (1.59)	6.30^{ab} (1.43)	7.39^{ab} (1.36)	7.36^{b} (1.39)	6.93^{ab} (1.33)	7.63^{b} (1.35)
Eastern Hemlock	Total biomass	16.16^{ab} (2.40)	12.84^{a} (2.00)	16.81^{ab} (2.21)	18.48^{b} (2.37)	17.08^{ab} (2.21)	16.64^{ab} (2.22)	19.68^{b} (2.09)	20.34^{b} (2.36)
	< 2 mm root	4.43^{ab} (0.87)	2.88^{a} (0.72)	3.95^{ab} (0.80)	4.84^{b} (0.86)	4.07^{ab} (0.80)	3.95^{ab} (0.81)	5.64^{b} (0.76)	4.55^{ab} (0.86)
	> 2mm root	1.94^{a} (0.29)	1.68^{a} (0.24)	2.18^{a} (0.26)	1.84^{a} (0.28)	2.14^{a} (0.26)	1.80^{a} (0.26)	2.16^{a} (0.25)	2.85^{b} (0.28)
	Total root	6.37^{ab} (1.04)	4.56^{a} (0.86)	6.13^{ab} (0.96)	6.69^{b} (1.03)	6.21^{ab} (0.95)	5.75^{ab} (0.96)	7.79^{b} (0.90)	7.39^{b} (1.02)
	Stem	7.05^{ab} (0.87)	6.20^{a} (0.72)	7.68^{ab} (0.80)	8.62^{b} (0.85)	7.50^{ab} (0.79)	7.62^{ab} (0.80)	8.18^{b} (0.75)	8.91^{b} (0.85)
	Leaf	1.55^{ab} (0.21)	1.31^{a} (0.18)	1.63^{ab} (0.20)	1.76^{b} (0.21)	1.72^{b} (0.20)	1.72^{b} (0.20)	1.83^{b} (0.19)	1.98^{b} (0.21)
Red Oak	Total biomass	2.27^{a} (0.55)	1.79^{a} (0.64)	1.38^{a} (0.59)	1.35^{a} (0.91)	1.74^{a} (0.70)	2.80^{a} (0.59)	1.80^{a} (0.55)	1.56^{a} (0.64)
	< 2 mm root	0.47^{a} (0.12)	0.28^{a} (0.14)	0.31^{a} (0.13)	0.23^{a} (0.19)	0.36^{a} (0.15)	0.48^{a} (0.13)	0.38^{a} (0.12)	0.24^{a} (0.14)
	> 2mm root	1.10^{a} (0.31)	0.95^{a} (0.36)	0.70^{a} (0.33)	0.65^{a} (0.51)	0.86^{a} (0.39)	1.52^{a} (0.34)	0.99^{a} (0.31)	0.78^{a} (0.36)
	Total root	1.57^{a} (0.42)	1.23^{a} (0.49)	1.00^{a} (0.45)	0.89^{a} (0.69)	1.21^{a} (0.53)	2.00^{a} (0.45)	1.37^{a} (0.42)	1.02^{a} (0.48)
	Stem	0.30^{a} (0.05)	0.30^{a} (0.06)	0.16^{a} (0.06)	0.20^{a} (0.08)	0.21^{a} (0.07)	0.26^{a} (0.06)	0.20^{a} (0.05)	0.24^{a} (0.06)
	Leaf	0.41^{a} (0.11)	0.34^{a} (0.13)	0.24^{a} (0.12)	0.19^{a} (0.19)	0.31^{a} (0.14)	0.48^{a} (0.12)	0.25^{a} (0.11)	0.32^{a} (0.13)

Table 4.1 (continued):

O_3 tolerant Trembling aspen	Total biomass	14.13^{ab} (2.59)	11.89^{a} (2.70)	18.87^{b} (2.54)	12.78^{a} (2.52)	10.53^{a} (2.54)	13.61^{ab} (2.40)	14.57^{ab} (2.53)	14.55^{ab} (2.52)
	< 2 mm root	4.42^{a} (0.78)	3.05^{a} (0.82)	6.19^{b} (0.77)	3.42^{a} (0.77)	3.78^{a} (0.77)	4.27^{ab} (0.74)	4.75^{ab} (0.77)	4.65^{ab} (0.77)
	> 2mm root	2.78^{a} (0.69)	2.21^{a} (0.73)	3.30^{a} (0.68)	2.37^{a} (0.68)	2.28^{a} (0.68)	3.11^{a} (0.65)	2.93^{a} (0.68)	2.76^{a} (0.68)
	Total root	7.20^{ab} (1.42)	5.28^{a} (1.50)	9.50^{b} (1.41)	5.79^{a} (1.40)	6.05^{ab} (1.40)	7.38^{ab} (1.34)	7.68^{ab} (1.40)	7.41^{ab} (1.40)
	Stem	3.03^{a} (0.65)	2.83^{a} (0.68)	4.56^{b} (0.64)	2.68^{a} (0.63)	2.18^{a} (0.64)	2.93^{ab} (0.60)	3.17^{ab} (0.64)	3.16^{ab} (0.63)
	Leaf	3.45^{ab} (0.63)	3.71^{ab} (0.66)	4.90^{a} (0.62)	3.66^{ab} (0.61)	2.60^{b} (0.62)	3.58^{ab} (0.59)	3.58^{ab} (0.62)	3.91^{ab} (0.61)
O_3 sensitive Trembling aspen	Total biomass	22.87^{d} (3.48)	15.54^{abc} (3.50)	13.49^{ab} (3.89)	11.91^{a} (3.53)	18.05^{abcd} (3.73)	18.11^{cd} (3.42)	21.69^{d} (3.88)	19.70^{cd} (3.59)
	< 2 mm root	5.90^{c} (0.93)	3.89^{ab} (0.93)	3.92^{ab} (1.03)	2.62^{a} (0.94)	5.24^{abc} (0.99)	4.67^{bc} (0.91)	5.87^{c} (1.03)	5.26^{c} (0.95)
	> 2 mm root	6.39^{c} (1.21)	4.18^{ab} (1.17)	3.11^{ab} (1.39)	2.60^{a} (1.18)	4.73a^{bc} (1.24)	4.98^{bc} (1.16)	6.55^{c} (1.33)	4.68^{bc} (1.24)
	Total root	12.31^{c} (2.05)	8.11^{ab} (2.04)	7.20^{ab} (0.28)	5.27^{a} (2.06)	10.03^{bc} (2.16)	9.68^{bc} (2.00)	12.45^{c} (2.28)	9.96^{bc} (2.11)
	Stem	4.38^{b} (0.72)	2.72^{a} (0.70)	2.36^{a} (0.78)	2.40^{a} (0.71)	3.22^{ab} (0.74)	3.40^{ab} (0.69)	3.91^{b} (0.79)	3.72^{b} (0.74)
	Leaf	6.13^{c} (0.86)	4.64^{ac} (0.88)	3.81^{a} (0.97)	4.04^{ab} (0.88)	4.78^{ac} (0.94)	5.01^{ac} (0.85)	5.36^{bc} (0.96)	5.81^{c} (0.89)

Table 4.2: Mean dry biomass (g) of each tissue type for each species grown under 30 kg ha^{-1} yr^{-1} N. Different letters indicate significantly different pairwise comparisons ($p < 0.1$) and standard error is shown in parentheses.

		High soil NO_3^-							
		a	O_3	NO_2	NO_2 + O_3	CO_2	CO_2 + O_3	CO_2 + NO_2	CO_2 + NO_2 + O_3
Sugar maple	Total biomass	18.56^{a} (3.14)	18.02^{a} (3.15)	19.70^{ab} (3.12)	20.87^{ab} (3.34)	18.69^{a} (3.50)	22.19^{ab} (3.34)	16.20^{a} (3.13)	27.55^{b} (3.29)
	< 2 mm root	4.73^{a} (0.89)	4.98^{a} (0.90)	5.16^{ab} (0.89)	4.94^{ab} (0.95)	4.53^{a} (1.00)	6.04^{ab} (0.95)	4.01^{a} (0.89)	7.42^{b} (0.94)
	> 2mm root	5.90^{a} (1.01)	5.70^{a} (1.01)	6.36^{ab} (1.00)	6.51^{ab} (1.07)	5.57^{a} (1.13)	5.65^{ab} (1.07)	5.37^{a} (1.01)	8.25^{b} (1.06)
	Total root	5.90^{a} (1.01)	5.70^{a} (1.01)	6.36^{ab} (1.00)	6.51^{ab} (1.07)	5.57^{a} (1.13)	5.65^{ab} (1.07)	5.37^{a} (1.01)	8.25^{b} (1.06)
	Stem	3.15^{ab} (0.59)	2.49^{a} (0.59)	2.81^{abc} (0.59)	3.70^{bc} (0.63)	2.72^{ab} (0.66)	3.78^{bc} (0.63)	2.39^{ab} (0.59)	4.52^{c} (0.62)
	Leaf	4.78^{a} (1.03)	4.84^{a} (1.03)	5.37^{ab} (1.03)	5.74^{ab} (1.10)	5.91^{ab} (1.15)	6.71^{ab} (1.10)	4.43^{a} (1.03)	7.34^{b} (1.08)

Table 4.2 (continued):

Eastern Hemlock	Total biomass	12.73^{a} (1.82)	12.27^{a} (1.89)	15.02^{ab} (2.04)	18.85^{b} (2.05)	15.70^{ab} (2.05)	15.55^{ab} (1.78)	16.51^{abc} (1.90)	20.52^{c} (1.89)
	< 2 mm root	2.81^{a} (0.62)	3.20^{a} (0.64)	3.60^{ab} (0.69)	5.33^{b} (0.70)	3.82^{ab} (0.70)	4.04^{ab} (0.61)	4.71^{b} (0.65)	4.94^{b} (0.64)
	> 2mm root	1.71^{ab} (0.24)	1.49^{a} (0.25)	1.69^{ab} (0.27)	2.19^{b} (0.27)	1.81^{ab} (0.27)	1.68^{ab} (0.24)	2.05^{ab} (0.25)	2.27^{b} (0.25)
	Total root	4.54^{a} (0.79)	4.69^{a} (0.82)	5.25^{ab} (0.88)	7.53^{c} (0.89)	5.64^{abc} (0.89)	5.72^{abc} (0.77)	6.74^{b} (0.83)	7.22^{b} (0.82)
	Stem	6.01^{ab} (0.71)	5.57^{a} (0.74)	6.93^{abc} (0.80)	7.82^{cd} (0.80)	6.68^{ab} (0.80)	7.34^{bcd} (0.70)	7.21^{abc} (0.74)	9.39^{d} (0.74)
	Leaf	2.27^{abc} (0.50)	2.00^{ab} (0.53)	2.90^{abcd} (0.57)	3.37^{cd} (0.57)	3.24^{bcd} (0.57)	2.00^{a} (0.53)	2.51^{abc} (0.53)	3.93^{d} (0.53)
Red Oak	Total biomass	2.34^{ab} (0.80)	2.74^{ab} (0.90)	2.93^{a} (0.72)	2.83^{a} (0.72)	2.63^{ab} (0.90)	1.11^{a} (0.60)	2.29^{ab} (0.67)	1.96^{ab} (0.73)
	< 2 mm root	0.38^{ab} (0.15)	0.63^{a} (0.17)	0.40^{a} (0.14)	0.65^{ab} (0.14)	0.36^{ab} (0.17)	0.19^{b} (0.11)	0.43^{ab} (0.12)	0.38^{ab} (0.14)
	> 2mm root	1.20^{a} (0.41)	1.38^{ab} (0.46)	1.69^{a} (0.37)	1.26^{ab} (0.37)	1.15^{ab} (0.46)	0.45^{b} (0.31)	1.09^{ab} (0.34)	0.99^{ab} (0.37)
	Total root	1.58^{ab} (0.53)	2.02^{a} (0.60)	2.09^{a} (0.48)	1.92^{a} (0.48)	1.51^{ab} (0.60)	0.64^{b} (0.40)	1.52^{ab} (0.44)	1.36^{ab} (0.49)
	Stem	0.28^{a} (0.09)	0.27^{a} (0.10)	0.29^{a} (0.08)	0.32^{a} (0.08)	0.35^{a} (0.10)	0.21^{a} (0.07)	0.28^{a} (0.08)	0.23^{a} (0.08)
	Leaf	0.48^{ab} (0.21)	0.45^{ab} (0.24)	0.55^{ab} (0.19)	0.59^{ab} (0.19)	0.77^{a} (0.24)	0.26^{b} (0.16)	0.48^{ab} (0.18)	0.37^{ab} (0.19)
O3 tolerant Trembling aspen	Total biomass	22.06^{ab} (6.48)	26.68^{a} (6.02)	20.60^{ab} (5.71)	16.12^{b} (5.68)	32.17^{a} (5.41)	31.96^{a} (5.39)	31.28^{a} (5.39)	31.36^{a} (5.57)
	< 2 mm root	5.85^{ab} (1.68)	7.33^{a} (1.57)	5.64^{ab} (1.49)	4.50^{b} (1.48)	8.77^{a} (1.41)	9.14^{a} (1.40)	8.76^{a} (1.41)	8.46^{a} (1.46)
	> 2mm root	5.35^{ac} (2.09)	5.21^{ac} (1.95)	3.92^{bc} (1.85)	3.21^{c} (1.84)	8.81^{a} (1.75)	7.71^{ab} (1.75)	8.17^{ab} (1.75)	7.15^{ab} (1.81)
	Total root	11.17^{ab} (3.67)	15.55^{ab} (3.42)	9.60^{ab} (3.25)	7.73^{a} (3.23)	17.58^{b} (3.07)	16.85^{b} (3.06)	16.93^{b} (3.07)	15.59^{b} (3.17)
	Stem	5.51^{ab} (1.70)	6.96^{a} (1.58)	5.23^{ab} (1.51)	4.17^{b} (1.50)	7.47^{a} (1.43)	7.44^{a} (1.42)	7.50^{a} (1.42)	7.99^{a} (1.47)
	Leaf	5.15^{ab} (1.28)	7.24^{a} (1.19)	5.71^{ab} (1.13)	4.31^{b} (1.12)	7.03^{a} (1.07)	7.75^{a} (1.07)	6.87^{a} (1.07)	7.78^{a} (1.10)

Table 4.2 (continued):

O3 sensitive Trembling aspen	Total biomass	19.52[a] (4.33)	14.09[a] (4.49)	14.69[a] (4.65)	20.42[a] (4.61)	15.46[a] (4.86)	13.97[a] (4.56)	17.54[a] (4.72)	18.94[a] (4.59)
	< 2 mm root	5.25[a] (1.26)	4.05[a] (1.33)	6.28[a] (1.31)	5.64[a] (1.37)	4.96[a] (1.44)	4.36[a] (1.35)	5.13[a] (1.39)	5.40[a] (1.30)
	> 2mm root	5.53[a] (1.88)	4.32[a] (1.98)	7.79[a] (2.00)	6.35[a] (2.12)	5.15[a] (2.15)	4.47[a] (2.02)	5.43[a] (2.09)	5.28[a] (1.93)
	Total root	11.05[a] (3.10)	8.60[a] (3.26)	13.88[a] (3.21)	11.88[a] (3.36)	10.35[a] (3.54)	9.11[a] (3.31)	10.82[a] (3.42)	10.99[a] (3.18)
	Stem	3.47[a] (0.77)	2.35[a] (0.87)	2.50[a] (0.87)	3.36[a] (0.88)	2.30[a] (0.93)	2.16[a] (0.87)	2.94[a] (0.93)	2.97[a] (0.78)
	Leaf	5.20[a] (1.14)	3.80[a] (1.19)	3.89[a] (1.22)	5.78[a] (1.21)	3.49[a] (1.28)	3.48[a] (1.20)	4.43[a] (1.26)	5.42[a] (1.19)

any tissue type. Similarly, there were no effects of $CO_2 + NO_2$ or $CO_2 + O_3$. In some cases the combination of $CO_2 + NO_2 + O_3$ caused an increase in biomass. This increases was seen in the < 2 mm roots, stem, and leaf biomass when soil NO_3^- was high and in the > 2 mm root biomass when soil NO_3^- was low. Oaks showed no alteration in any tissue type under any single gas or combinatorial treatments that included elevated CO_2. Under low soil NO_3^-, O_3 tolerant aspens had increased < 2 mm root and stem biomass under elevated NO_2 alone, but these effects were eliminated by elevated CO_2. When soil NO_3^- was high, there were no effects of single gases, or of any treatments including elevated CO_2. The O_3 sensitive aspens showed no response in biomass to any treatment when soil NO_3^- was high, but when soil NO_3^- was low, O_3 fumigation decreased the biomass of < 2 mm roots, > 2 mm roots, and stems and NO_2 fumigation decreased < 2 mm root, > 2 mm root, stem, and leaf biomass. The combination of NO_2 and O_3 had the same effect as NO_2 alone, but the addition of elevated CO_2 eliminated all the effects.

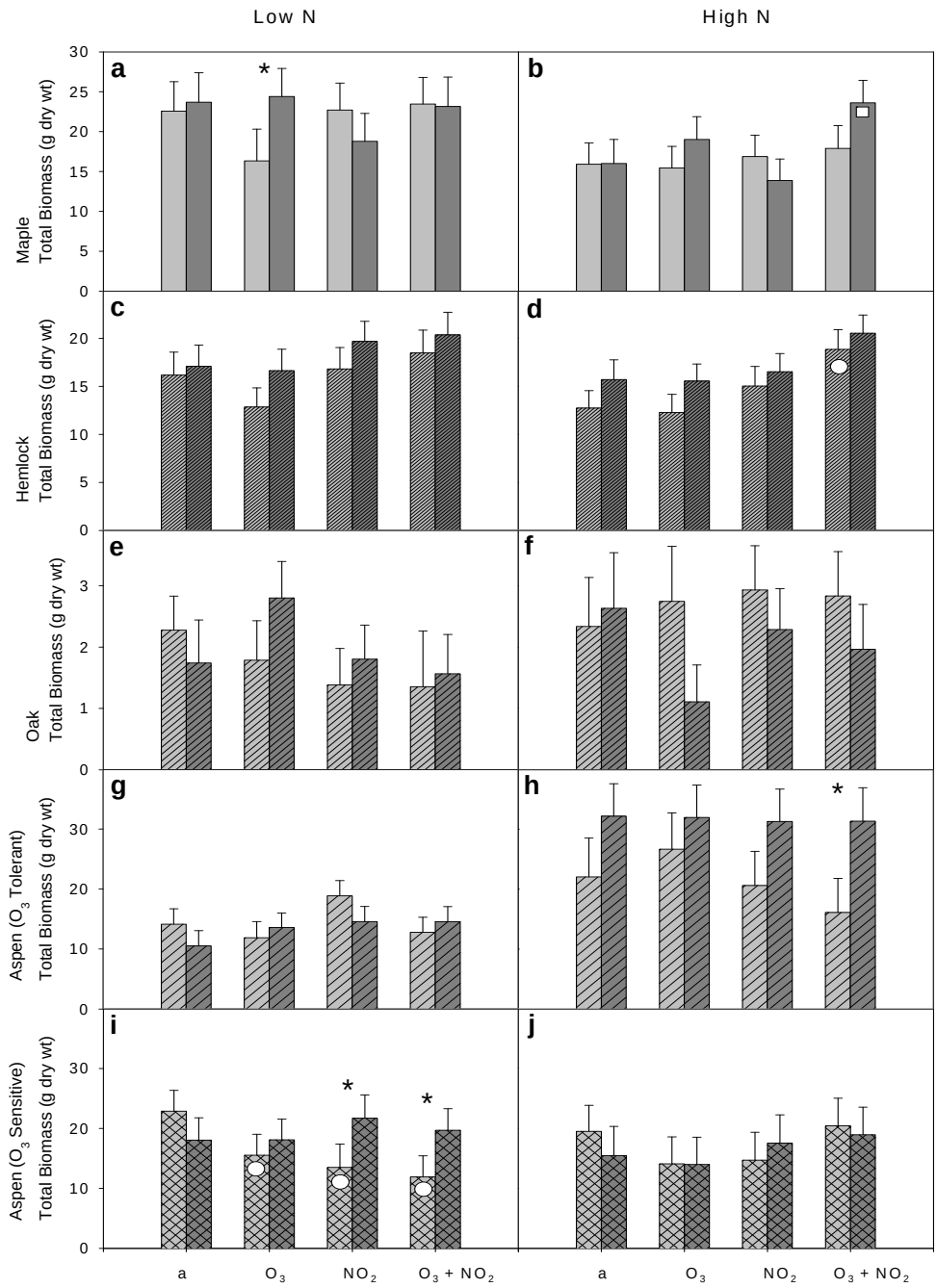

Figure 4.2: Mean total seedling biomass reported as dry mass in grams. Light gray bars indicate ambient (380ppm) CO_2 and dark gray bars indicate elevated (560ppm) CO_2. The left-hand panels are the seedlings grown in low soil NO_3^- and the right-hand panels are those grown in 30 kg NO_3^- ha^{-1} yr^{-1}. On the x-axis, a = ambient NO_2 + ambient O_3, O_3 = ambient NO_2 + elevated O_3, NO_2 = elevated NO_2 + ambient O_3, and $O_3 + NO_2$ = elevated NO_2 + elevated O_3. Asterisks indicate a significant difference between the ambient and elevated CO_2 treatments. Open circles indicate a significant difference between that treatment and ambient NO_2 + ambient O_3 treatment in the same CO_2 group.

Gas exchange:

Net carbon assimilation in sugar maples (A, the sum of photosynthesis and respiration) exhibited acclimation to elevated CO_2 (Tables 4.3 & 4.4). When instantaneous A was measured at the CO_2 concentration under which the plants were growing, elevated CO_2 never increased A. Nitrogen dioxide and O_3 had no effect singly or in combination when CO_2 was ambient regardless of soil NO_3^-. When CO_2 was elevated, O_3 decreased A in low NO_3^- and $NO_2 + O_3$ decreased it when soil NO_3^- was high. When all the plants were compared at 560 ppm CO_2, elevated CO_2 treatments generally had lower A than their ambient counterparts although this was only significant when soil NO_3^- was low and O_3 was high (with or without NO_2). There were no differences in oak A when measured at either CO_2 concentration.

There was no apparent acclimation of photosynthesis to elevated CO_2 in either the O_3 tolerant or sensitive aspens. When measured at treatment level CO_2, both the elevated CO_2 and the $CO_2 + NO_2$ treatments had significantly higher A than their ambient CO_2 counterparts (Table 4.3 & 4.4). There were no differences between the ambient/elevated CO_2 treatment pairs when all the trees were measured at 560 ppm CO_2, a clear indication that the changes in photosynthesis observed when seedlings were measured in their treatment were the same as the instantaneous responses to increased CO_2.

Although there was no evidence of photosynthetic acclimation in the aspens, the addition of NO_2 and/or O_3 in the high soil NO_3^- group reduced A when CO_2 was elevated, although the elevated CO_2 treatments were still higher than their ambient CO_2 counterpart. (table 4.3 & 4.4). There were no differences between the $NO_2 + O_3$ and the $CO_2 + NO_2 + O_3$ treatments, but this was due to higher A in the $NO_2 + O_3$ treatments rather than decreased A in the $CO_2 + NO_2 + O_3$ treatments.

Table 4.3: Means of each gas exchange parameter for each species grown under ambient soil NO_3^-. A and respiration are in μmol CO_2 m^{-2} s^{-1} and stomatal conductance in mmol H_2O m^{-2} s^{-1}. Different letters indicate significantly different pairwise comparisons ($p < 0.1$) and standard error is shown in parentheses.

		Low soil NO_3^-							
	Treat-ment	**a**	**O_3**	**NO_2**	**NO_2 + O_3**	**CO_2**	**CO_2 + O_3**	**CO_2 + NO_2**	**CO_2 + NO_2 + O_3**
Sugar maple	A (treatment CO_2)	3.73^{a} (0.58)	3.00^{ab} (0.58)	3.51^{ab} (0.58)	3.58^{a} (0.58)	3.93^{a} (0.58)	2.19^{b} (0.58)	4.26^{a} (0.58)	3.28^{ab} (0.58)
	A (560 ppm CO_2)	4.08^{bc} (0.73)	4.18^{bc} (0.67)	5.29^{c} (0.82)	5.52^{c} (0.67)	4.08^{bc} (0.73)	2.19^{a} (0.67)	4.26^{bc} (0.67)	3.28^{ab} (0.67)
	Stomatal conductance	0.034^{cd} (0.005)	0.030^{bcd} (0.005)	0.034^{cd} (0.005)	0.039^{d} (0.005)	0.029^{bcd} (0.005)	0.015^{a} (0.005)	0.026^{abc} (0.005)	0.020^{ab} (0.005)
	Dark respiration	-0.67^{a} (0.10)	-0.35^{cd} (0.10)	-0.53^{abc} (0.09)	-0.59^{ab} (0.10)	-0.34^{cd} (0.10)	-0.29^{d} (0.10)	-0.56^{ab} (0.10)	-0.41^{bd} (0.10)
Red Oak	A (treatment CO_2)	5.01^{a} (1.39)	6.14^{a} (1.21)	6.53^{a} (1.24)	5.14^{a} (1.50)	6.63^{a} (1.38)	6.63^{a} (1.55)	5.54^{a} (1.33)	4.66^{a} (1.53)
	A (560 ppm CO_2)	6.42^{a} (1.63)	7.84^{a} (1.42)	8.31^{a} (1.45)	6.27^{a} (1.78)	6.24^{a} (1.59)	6.67^{a} (1.82)	5.56^{a} (1.57)	4.62^{a} (1.81)
	Stomatal conductance	0.072^{ab} (0.021)	0.072^{ab} (0.019)	0.101^{a} (0.019)	0.065^{ab} (0.023)	0.076^{ab} (0.021)	0.062^{ab} (0.023)	0.044^{b} (0.020)	0.051^{b} (0.023)
	Dark respiration	-0.69^{ab} (0.12)	-0.49^{a} (0.12)	-0.54^{a} (0.11)	-0.76^{b} (0.11)	-0.52^{a} (0.12)	-0.49^{a} (0.12)	-0.45^{a} (0.14)	-0.66^{ab} (0.12)
O_3 tolerant aspen	A (treatment CO_2)	8.43^{ab} (1.40)	10.43^{bc} (1.35)	7.10^{a} (1.37)	8.05^{ab} (1.42)	11.61^{c} (1.46)	10.74^{bc} (1.73)	11.33^{c} (1.31)	9.97^{bc} (1.47)
	A (560 ppm CO_2)	12.50^{ab} (1.52)	13.00^{a} (1.62)	9.93^{b} (1.59)	10.69^{ab} (1.67)	11.61^{ab} (1.72)	10.77^{ab} (1.62)	10.38^{ab} (1.58)	10.08^{ab} (1.74)
	Stomatal conductance	0.224^{a} (0.026)	0.204^{a} (0.024)	0.189^{a} (0.025)	0.221^{a} (0.026)	0.189^{a} (0.028)	0.186^{a} (0.025)	0.196^{a} (0.023)	0.218^{a} (0.028)
	Dark respiration	-1.21^{ab} (0.16)	-1.21^{ab} (0.19)	-0.99^{ac} (0.15)	-1.14^{ab} (0.17)	-0.95^{bc} (0.18)	-0.78^{c} (0.17)	-0.99^{ab} (0.17)	-1.27^{a} (0.17)
O_3 sensitive aspen	A (treatment CO_2)	9.09^{a} (1.55)	9.75^{a} (1.54)	8.83^{a} (1.46)	11.48^{ab} (1.49)	13.76^{b} (1.37)	9.35^{a} (1.49)	12.01^{ab} (1.57)	11.59^{ab} (1.53)
	A (560 ppm CO_2)	12.51^{ab} (1.71)	12.86^{ab} (1.75)	11.78^{ab} (1.66)	13.51^{a} (1.68)	13.62^{a} (1.56)	9.52^{b} (1.70)	12.00^{ab} (1.79)	11.61^{ab} (1.77)
	Stomatal conductance	0.243^{ac} (0.048)	0.226^{bc} (0.047)	0.232^{bc} (0.044)	0.346^{a} (0.046)	0.287^{ab} (0.040)	0.185^{c} (0.046)	0.229^{ac} (0.049)	0.239^{ac} (0.046)
	Dark respiration	-1.35^{a} (0.12)	-0.97^{bc} (0.13)	-1.12^{ab} (0.12)	-0.98^{bc} (0.11)	-0.75^{c} (0.12)	-0.80^{bc} (0.12)	-0.89^{bc} (0.13)	-1.09^{ab} (0.12)

Table 4.4: Means of each gas exchange parameter for each species grown under 30 kg ha^{-1} yr^{-1} N. A and respiration are in µmol CO_2 m^{-2} s^{-1} and stomatal conductance in mmol H_2O m^{-2} s^{-1}. Different letters indicate significantly different pairwise comparisons ($p < 0.1$) and standard error is shown in parentheses.

		High soil NO_3^-							
	Treat-ment	**a**	**O_3**	**NO_2**	**NO_2 + O_3**	**CO_2**	**CO_2 + O_3**	**CO_2 + NO_2**	**CO_2 + NO_2 + O_3**
Sugar maple	A (treatment CO_2)	3.68^{ab} (0.68)	3.84^{a} (0.62)	3.66^{ab} (0.62)	3.03^{ab} (0.68)	4.02^{a} (0.62)	3.66^{ab} (0.62)	3.80^{ab} (0.62)	2.39^{b} (0.62)
	A (560 ppm CO_2)	4.25 (0.82)	4.96 (0.74)	4.99 (0.74)	3.43 (0.74)	4.02 (0.74)	3.66 (0.74)	3.80 (0.74)	2.03 (0.82)
	Stomatal conductance	0.034^{ac} (0.006)	0.040^{a} (0.005)	0.043^{a} (0.005)	0.028^{bc} (0.006)	0.023^{c} (0.005)	0.036^{ab} (0.005)	0.025^{bc} (0.005)	0.020^{c} (0.005)
	Dark respiration	-0.60^{ab} (0.10)	-0.52^{ac} (0.10)	-0.60^{a} (0.09)	-0.44^{ac} (0.10)	-0.33^{c} (0.10)	-0.38^{bc} (0.10)	-0.44^{ac} (0.10)	-0.63^{a} (0.10)
Red Oak	A (treatment CO_2)	6.45^{a} (1.21)	5.00^{a} (1.07)	3.83^{a} (1.21)	4.86^{a} (1.40)	6.17^{a} (1.54)	4.63^{a} (1.30)	5.45^{a} (1.10)	5.02^{a} (1.21)
	A (560 ppm CO_2)	7.95^{a} (1.42)	6.56^{a} (1.24)	4.71^{a} (1.42)	6.09^{a} (1.64)	6.16^{a} (1.79)	4.62^{a} (1.51)	5.43^{a} (1.29)	5.01^{a} (1.42)
	Stomatal conductance	0.113^{a} (0.017)	0.061^{b} (0.016)	0.052^{b} (0.017)	0.062^{b} (0.020)	0.033^{b} (0.023)	0.048^{b} (0.019)	0.042^{b} (0.016)	0.056^{b} (0.017)
	Dark respiration	-0.84^{a} (0.12)	-0.51^{b} (0.14)	-0.58^{b} (0.11)	-0.53^{b} (0.14)	-0.47^{b} (0.14)	-0.55^{b} (0.12)	-0.58^{ab} (0.12)	-0.76^{ab} (0.14)
O_3 tolerant aspen	A (treatment CO_2)	8.99^{cd} (1.09)	9.09^{bcd} (1.08)	8.72^{d} (1.05)	10.62^{bcd} (1.05)	13.83^{a} (1.05)	11.28^{b} (1.05)	11.06^{bc} (1.11)	11.03^{bc} (1.03)
	A (560 ppm CO_2)	12.42^{ab} (1.05)	12.20^{ab} (1.03)	12.14^{ab} (1.05)	13.49^{a} (1.01)	13.72^{a} (1.05)	12.14^{ab} (1.05)	11.12^{b} (1.06)	11.01^{b} (0.98)
	Stomatal conductance	0.212^{a} (0.037)	0.206^{a} (0.037)	0.203^{a} (0.034)	0.250^{a} (0.035)	0.255^{a} (0.035)	0.230^{a} (0.035)	0.198^{a} (0.038)	0.225^{a} (0.035)
	Dark respiration	-1.01^{ab} (0.26)	-0.95^{ab} (0.25)	-0.93^{ab} (0.23)	-1.55^{c} (0.24)	-0.79^{a} (0.25)	-1.27^{bc} (0.26)	-1.29^{bc} (0.25)	-1.17^{ac} (0.25)
O_3 sensitive aspen	A (treatment CO_2)	8.29^{b} (1.86)	9.67^{ac} (1.86)	6.24^{c} (1.86)	12.13^{a} (1.96)	14.05^{a} (1.92)	11.53^{ab} (1.96)	12.47^{a} (1.86)	13.16^{a} (1.91)
	A (560 ppm CO_2)	10.45^{ab} (1.07)	12.01^{ac} (1.27)	10.40^{ab} (1.16)	13.11^{ac} (1.35)	13.43^{c} (1.19)	10.16^{a} (1.11)	13.07^{bc} (1.16)	12.95^{bc} (1.11)
	Stomatal conductance	0.237^{ab} (0.049)	0.240^{ab} (0.049)	0.162^{a} (0.049)	0.302^{b} (0.053)	0.272^{b} (0.053)	0.292^{b} (0.053)	0.271^{b} (0.049)	0.253^{b} (0.053)
	Dark respiration	-1.03^{ab} (0.11)	-1.09^{ab} (0.13)	-1.17^{ab} (0.12)	-1.22^{a} (0.13)	-0.98^{ac} (0.15)	-0.74^{c} (0.13)	-1.21^{ac} (0.15)	-0.89^{bc} (0.12)

Net carbon assimilation in O_3 tolerant and sensitive aspens did not appear to acclimate to elevated CO_2 (Table 4.3 & 4.4). When all the seedlings were measured at 560 ppm CO_2, A in the elevated CO_2 group was rarely lower than its ambient CO_2 counterpart, giving little evidence for acclimation of A to elevated CO_2.

Stomatal conductance in maples and oaks was generally more responsive to the gas fumigation treatments compared to aspens (Table 4.3 & 4.4). In oaks, decreased conductance was seen under elevated NO_2 and/or O_3 when soil NO_3^- was high and under elevated CO_2 or NO_2 when soil NO_3^- was low. In maple seedlings, the combination of CO_2 and O_3 decreased stomatal conductance regardless of NO_2 in low soil NO_3^-. When soil NO_3^- was high, CO_2 decreased conductance under elevated NO_2, and the $CO_2 + O_3$ treatment exhibited higher stomatal conductance than CO_2 alone. Also in maples, the combination of $CO_2 + NO_2 + O_3$ always had lower stomatal conductance than the ambient treatment. O_3 tolerant aspens did not respond in terms of stomatal conductance to any of the gas treatments. In contrast, O_3 sensitive aspens expressed decreases stomatal conductance in response to the combination of low soil NO_3^- elevated CO_2, and increased. In addition, when soil NO_3^- high, O_3 tolerant aspens under the $CO_2 + NO_2$ treatment had higher conductance than those fumigated only with NO_2.

Respiration in maples and oaks was decreased by elevated CO_2 under all conditions except for oaks when under low soil NO_3^- (Table 4.3 & 4.4). In maples grown in low soil NO_3^-, O_3 alone decreased respiration and the CO_2 effect was eliminated by NO_2, while in high soil NO_3^- the combination of $NO_2 + O_3$ eliminated the CO_2 effect. In oaks, no treatments had an effect when soil NO_3^- was low and although NO_2 and/or O_3 decreased respiration under ambient CO_2, they did not cause a decrease under elevated CO_2. In both oaks and maples in high soil NO_3^-, the

combination of $CO_2 + NO_2 + O_3$ resulted in respiration rates similar to the ambient even though CO_2 alone reduced respiration in both cases.

Respiration in the two aspen clones responded differently to elevated CO_2 although in both cases the responses depended on the NO_2, O_3, and NO_3^- applications (Tables 4.3 & 4.4). In the O_3 tolerant clone, elevated CO_2 only decreased respiration in the elevated O_3 treatment when soil NO_3^- was low and adding NO_2 eliminated the effect. In both NO_3^- groups, respiration in the $CO_2 + NO_2 + O_3$ was not different from the ambient. When soil NO_3^- was high, the combination of NO_2 and O_3 increased respiration under ambient CO_2 while NO_2 and O_3 individually increased respiration when CO_2 was elevated. In the O_3 sensitive aspens grown in low soil NO_3^-, elevated CO_2 decreased respiration relative to the control unless NO_2 and O_3 were also elevated. When CO_2 was ambient, elevated O_3 decreased respiration regardless of NO_2 concentration. In the high soil NO_3^- treatment, neither NO_2 nor O_3 had an effect under ambient CO_2, but O_3 decreased respiration when CO_2 was elevated. In both clones, the combination of $CO_2 + NO_2 + O_3$ resulted in respiration rates similar to the ambient treatment regardless of soil NO_3^-.

Allocation:

Application of the treatments caused few changes in the allocation of biomass in the slow growing species (maple, hemlock, and oak) (Fig 4.3 a-f). No treatments had an effect on oaks or maples grown under high soil NO_3^-, or hemlocks grown under low soil NO_3^-. However, maples grown in low soil NO_3^-, had decreased root:shoot when CO_2 and NO_2 were applied in combination with or without elevated levels of CO_2 and hemlocks in high soil NO_3^- had decreased root:shoot in the $CO_2 + O_3$ treatments.

In both of the aspen clones, elevated CO_2 tended to increase root:shoot except for O_3 sensitive aspens in low soil NO_3^- (Fig 4.3 g-j). Nitrogen dioxide eliminated the effect of CO_2 in all cases such that $CO_2 + NO_2$ and $CO_2 + NO_2 + O_3$ were not different from the ambient treatment. The O_3 sensitive clone of aspen had fewer changes in root:shoot compared to the O_3 tolerant clone. When soil NO_3^- was low and CO_2 was ambient, $NO_2 + O_3$ reduced root:shoot. When soil NO_3^- was high, CO_2 alone increased root:shoot. In both clones, the root:shoot in the $CO_2 + NO_2 + O_3$ treatment was the same as in the control regardless of soil NO_3^-.

Changes in root:shoot were primarily driven by changes in allocation to roots and leaves, without changes in allocation to the stem, but was variable depending upon species identity and soil NO_3^- conditions (Table 4.5 & 4.6). In maple seedlings grown under low soil NO_3^-, the addition of CO_2 to the NO_2 or $NO_2 + O_3$ treatments caused a decrease in % root biomass and a concurrent increase in % leaf biomass. The addition of CO_2 to the ambient or $NO_2 + O_3$ treatments also caused a decrease in % stem biomass. When maples were grown under high soil NO_3^-, maples given CO_2, O_3, or NO_2 singly had lower % stem biomass with only small changes in % root biomass, but elevated CO_2 eliminated the effects and the combinatorial treatments exhibited root:shoot similar to the ambient treatment. Hemlock seedlings had no changes in root:shoot when soil NO_3^- was low, but when hemlocks were grown under high soil NO_3^-, the decrease in root:shoot caused by elevated CO_2 in the O_3 and $NO_2 + O_3$ treatments was caused primarily by decreased biomass in the roots. Also in hemlocks, $CO_2 + O_3$ fumigated plants exhibited decreased biomass in the leaves and increased biomass of the stem relative to the CO_2 alone treatment. There were no differences in allocation to tissue types in $CO_2 + NO_2 + O_3$ relative to the ambient treatments.

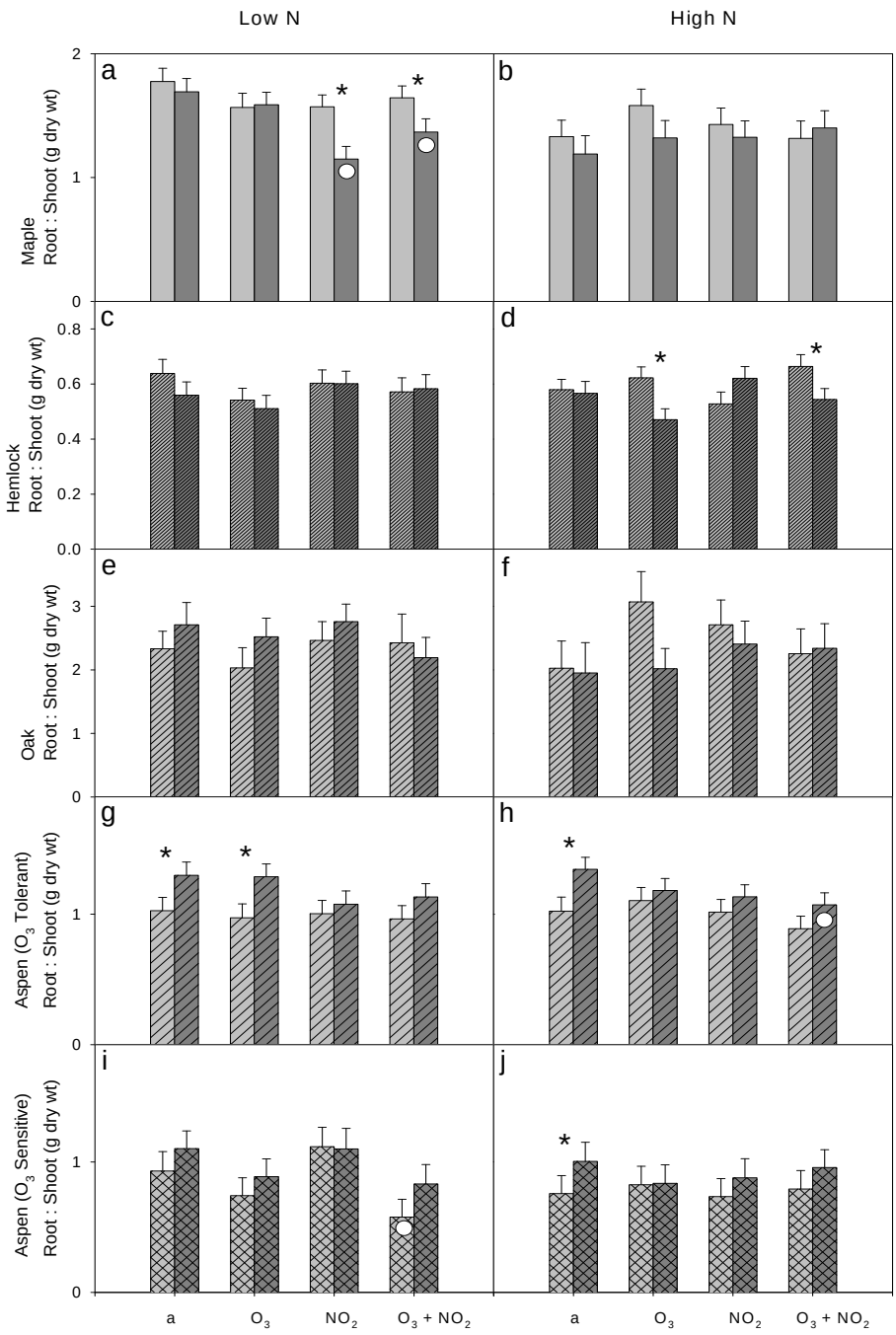

Figure 4.3: Ratio of belowground biomass (dry wt in g) to aboveground biomass (dry wt in g). Light gray bars indicate ambient (380ppm) CO_2 and black bars indicate elevated (560ppm) CO_2. The left-hand panels are the seedlings grown in low soil NO_3^- and the right-hand panels are those grown in 30 kg NO_3^- ha^{-1} yr^{-1}. On the x-axis, a = ambient NO_2 + ambient O_3, O_3 = ambient NO_2 + elevated O_3, NO_2 = elevated NO_2 + ambient O_3, and $O_3 + NO_2$ = elevated NO_2 + elevated O_3. Asterisks indicate a significant difference between the ambient and elevated CO_2 treatments. Open circles indicate a significant difference between that treatment and ambient NO_2 + ambient O_3 treatment in the same CO_2 group.

Table 4.5: Allocation of dry biomass within seedlings for each species grown under low soil NO_3^-. Different letters indicate significantly different pairwise comparisons ($p < 0.1$) and standard error is shown in parentheses.

		Low soil NO_3^-							
	Treat-ment	**a**	**O_3**	**NO_2**	**NO_2 + O_3**	**CO_2**	**CO_2 + O_3**	**CO_2 + NO_2**	**CO_2 + NO_2 + O_3**
Sugar maple	root:shoot	1.77^{a} (0.11)	1.57^{ab} (0.11)	1.57^{ab} (0.10)	1.64^{ab} (0.10)	1.69^{a} (0.11)	1.59^{ab} (0.10)	1.15^{c} (0.10)	1.37bc (0.11)
	% < 2 mm root	28.74^{ab} (2.07)	28.10^{ab} (2.26)	26.07^{a} (2.21)	28.98^{ab} (2.06)	27.19^{ab} (2.08)	29.87^{b} (2.06)	25.52^{a} (2.00)	25.52^{a} (2.07)
	% > 2mm root	36.34^{a} (2.29)	34.25^{ac} (2.53)	35.86^{ab} (2.68)	34.31^{ac} (2.42)	36.52^{a} (2.30)	32.52^{bc} (2.36)	28.68^{c} (2.24)	33.37^{ab} (2.29)
	% Total root	63.82^{a} (1.69)	60.72^{abc} (1.81)	60.05^{bc} (1.52)	61.68^{ab} (1.52)	62.45^{ab} (1.69)	60.83^{abc} (1.60)	52.89^{d} (1.60)	57.49^{cd} (1.69)
	% Stem	12.96^{ab} (1.63)	12.62^{ab} (1.80)	12.52^{a} (1.91)	14.88^{b} (1.73)	10.84^{a} (1.64)	11.68^{a} (1.69)	13.12^{ab} (1.61)	11.41^{a} (1.63)
	% Leaf	21.93^{ab} (1.50)	24.86^{ac} (1.61)	25.48^{c} (1.35)	21.78^{a} (1.35)	25.22^{bc} (1.51)	25.93^{c} (1.42)	32.59^{d} (1.42)	29.68^{d} (1.51)
Eastern Hemlock	root:shoot	0.64^{a} (0.05)	0.54^{ab} (0.04)	0.60^{ab} (0.05)	0.57^{ab} (0.05)	0.56^{ab} (0.05)	0.51^{b} (0.05)	0.60^{ab} (0.05)	0.58^{ab} (0.05)
	% < 2 mm root	27.90^{a} (2.63)	21.00^{c} (2.20)	23.59^{ac} (2.46)	25.29^{ac} (2.63)	21.72^{bc} (2.46)	22.80^{ac} (2.46)	26.82^{ab} (2.32)	22.86^{ac} (2.63)
	% > 2 mm root	11.15^{ac} (1.40)	13.68^{a} (1.19)	13.59^{ab} (1.31)	10.74^{bc} (1.39)	13.52^{ab} (1.31)	10.35^{c} (1.31)	10.50^{c} (1.24)	13.58^{ab} (1.40)
	% Total root	38.79^{a} (2.15)	34.68^{ab} (1.81)	37.27^{ab} (2.02)	36.00^{ab} (2.15)	35.28^{ab} (2.02)	33.23^{b} (2.02)	37.16^{ab} (1.90)	36.38^{ab} (2.15)
	% Stem	42.85^{a} (3.52)	49.74^{a} (2.97)	46.79^{a} (3.30)	47.89^{a} (3.52)	47.35^{a} (3.30)	48.55^{a} (3.30)	43.44^{a} (3.13)	43.47^{a} (3.52)
	% Leaf	18.50^{a} (2.73)	15.58^{a} (2.30)	15.97^{a} (2.56)	16.16^{a} (2.73)	17.36^{a} (2.56)	18.20^{a} (2.56)	19.42^{a} (2.42)	20.20^{a} (2.73)
Red Oak	root:shoot	2.33^{ab} (0.28)	2.03^{a} (0.32)	2.46^{ab} (0.30)	2.43^{ab} (0.45)	2.71^{ab} (0.35)	2.52^{ab} (0.30)	2.76^{b} (0.78)	2.19^{ab} (0.32)
	% < 2 mm root	19.77^{ab} (2.51)	17.96^{ab} (2.90)	22.65^{a} (2.69)	14.46^{b} (4.11)	20.12^{ab} (3.18)	17.21^{ab} (2.69)	19.12^{ab} (2.51)	15.04^{b} (2.90)
	% > 2 mm root	49.04^{a} (2.72)	47.60^{a} (3.14)	48.19^{a} (2.90)	54.90^{a} (4.44)	50.54^{a} (3.44)	53.22^{a} (2.90)	52.75^{a} (2.72)	51.68^{a} (3.14)
	% Total root	68.81^{a} (2.64)	65.56^{a} (3.05)	70.84^{a} (2.82)	69.37^{a} (4.31)	70.66^{a} (3.34)	70.42^{a} (2.82)	71.87^{a} (2.64)	66.72^{a} (3.05)
	% Stem	15.00^{ab} (1.98)	18.24^{a} (2.83)	12.68^{b} (2.12)	17.30^{ab} (3.22)	11.87^{b} (2.50)	11.89^{b} (2.12)	15.40^{ab} (1.98)	16.58^{ab} (2.28)
	% Leaf	16.18^{a} (2.11)	15.96^{a} (2.44)	16.34^{a} (2.26)	13.60^{a} (3.45)	17.09^{a} (2.67)	17.54^{a} (2.26)	12.83^{a} (2.11)	16.64^{a} (2.44)

Table 4.5 (continued):

O_3 tolerant Trembling aspen	root:shoot	1.03^{a} (0.10)	0.97^{a} (0.11)	1.00^{a} (0.10)	0.96^{ab} (0.10)	1.30^{b} (0.10)	1.29^{b} (0.10)	1.08^{ab} (0.10)	1.13^{ab} (0.10)
	% < 2 mm root	35.31^{c} (1.93)	27.64^{a} (2.03)	32.88^{bc} (1.93)	30.56^{ab} (1.93)	33.85^{bc} (1.93)	33.69^{bc} (1.84)	33.83^{bc} (1.93)	32.17^{bc} (1.93)
	% > 2 mm root	14.44^{a} (1.90)	19.43^{c} (2.00)	16.75^{ab} (1.90)	16.78^{ab} (1.90)	22.25^{c} (1.90)	21.05^{c} (1.81)	17.37^{ab} (1.90)	19.81^{bc} (1.90)
	% Total root	49.79^{b} (2.33)	47.08^{a} (2.44)	49.59^{ab} (2.33)	47.51^{ab} (2.33)	56.17^{c} (2.33)	54.74^{c} (2.24)	51.09^{ab} (2.33)	52.16^{bc} (2.33)
	% Stem	21.03^{ab} (1.45)	23.21^{ab} (1.54)	23.64^{a} (1.45)	21.71^{ab} (1.45)	19.77^{b} (1.45)	19.29^{b} (1.38)	20.89^{ab} (1.45)	21.65^{ab} (1.45)
	% Leaf	29.15^{bcd} (1.68)	29.81^{cd} (1.76)	26.77^{abc} (1.69)	30.89^{d} (1.69)	24.17^{a} (1.68)	25.97^{c} (1.62)	27.95^{bcd} (1.68)	26.31^{ab} (1.69)
O_3 sensitive Trembling aspen	root:shoot	0.93^{ab} (0.15)	0.74^{bc} (0.14)	1.12^{a} (0.15)	0.58^{c} (0.14)	1.10^{a} (0.14)	0.89^{ab} (0.14)	1.10^{a} (0.16)	0.83^{bc} (0.15)
	% < 2 mm root	24.07^{b} (2.88)	20.53^{b} (2.87)	31.58^{a} (3.00)	14.23^{c} (2.76)	24.13^{b} (2.87)	22.35^{b} (2.87)	23.46^{b} (3.17)	23.97^{b} (3.00)
	% > 2 mm root	32.23^{ab} (2.92)	28.94^{ab} (2.91)	29.64^{ab} (3.14)	28.51^{ab} (2.80)	30.61^{ab} (2.92)	33.05^{a} (2.92)	32.72^{ab} (3.21)	27.24^{b} (3.03)
	% Total root	55.10^{a} (3.40)	47.99^{b} (3.24)	58.38^{a} (3.49)	41.41^{c} (3.18)	53.62^{ab} (3.24)	54.29^{a} (3.24)	55.07^{a} (3.70)	50.30^{ab} (3.44)
	% Stem	19.96^{ac} (1.56)	19.49^{ac} (1.51)	17.67^{bc} (1.60)	21.55^{a} (1.49)	20.37^{a} (1.52)	17.59^{c} (1.52)	19.88^{ac} (1.69)	20.44^{ab} (1.55)
	% Leaf	23.15^{a} (3.14)	30.57^{bc} (3.10)	22.12^{a} (3.26)	35.25^{c} (2.99)	24.25^{a} (3.10)	26.36^{ab} (3.10)	23.45^{a} (3.44)	28.03^{ab} (3.24)

Table 4.6: Allocation of dry biomass within seedlings for each species grown under low soil NO_3^-. Different letters indicate significantly different pairwise comparisons ($p < 0.1$) and standard error is shown in parentheses.

		High soil NO_3^-							
		a	O_3	NO_2	$NO_2 + O_3$	CO_2	$CO_2 + O_3$	$CO_2 + NO_2$	$CO_2 + NO_2 + O_3$
Sugar maple	root:shoot	1.33^{ab} (0.13)	1.58^{a} (0.13)	1.43^{ab} (0.13)	1.32^{ab} (0.14)	1.19^{b} (0.15)	1.32^{ab} (0.14)	1.32^{ab} (0.13)	1.40^{ab} (0.14)
	% < 2 mm root	25.50^{a} (1.85)	26.64^{a} (1.85)	26.08^{a} (1.85)	24.73^{a} (1.95)	23.83^{a} (2.07)	27.65^{a} (1.95)	24.15^{a} (1.85)	26.59^{a} (1.95)
	% > 2mm root	29.85 (2.22)	33.04 (2.22)	31.93 (2.22)	31.54 (2.34)	28.56 (2.48)	27.99 (2.34)	31.40 (2.22)	30.37 (2.34)

Table 4.6 (continued):

Sugar Maple	% Total root	55.35^{ab} (2.71)	59.67^{a} (2.71)	58.00^{ab} (2.71)	56.29^{ab} (2.86)	52.43^{b} (3.03)	55.65^{ab} (2.86)	55.55^{ab} (2.71)	56.90^{ab} (2.86)
	% Stem	16.55^{ab} (1.18)	13.65^{ab} (1.18)	13.34^{a} (1.18)	16.16^{b} (1.25)	15.75^{ab} (1.32)	16.34^{b} (1.25)	15.22^{ab} (1.18)	16.41^{b} (1.25)
	% Leaf	28.10^{a} (2.32)	26.68^{a} (2.32)	28.65^{a} (2.32)	27.55^{a} (2.44)	31.82^{a} (2.59)	28.02^{a} (2.44)	29.23^{a} (2.32)	26.69^{a} (2.44)
Eastern Hemlock	root:shoot	0.58^{bc} (0.04)	0.62^{bc} (0.04)	0.53^{ac} (0.04)	0.66^{c} (0.04)	0.57^{bc} (0.04)	0.47^{a} (0.04)	0.62^{bc} (0.04)	0.54^{ab} (0.04)
	% < 2 mm root	22.45^{a} (1.95)	26.70^{ac} (2.09)	23.51^{ab} (2.25)	28.24^{bc} (2.25)	24.29^{ac} (2.25)	22.88^{a} (1.95)	29.01^{c} (2.09)	24.36^{ac} (2.09)
	% > 2mm root	13.93^{a} (1.05)	11.43^{b} (1.12)	10.91^{b} (1.20)	11.34^{b} (1.20)	11.55^{ab} (1.20)	11.18^{b} (1.05)	11.37^{b} (1.12)	10.84^{b} (1.12)
	% Total root	36.58^{bcd} (1.66)	38.00^{bcd} (1.76)	34.35^{ab} (1.90)	39.57^{cd} (1.90)	36.08^{bc} (1.90)	31.48^{a} (1.76)	40.15^{d} (1.76)	35.07^{ab} (1.76)
	% Stem	47.07^{ab} (3.01)	45.47^{ab} (3.22)	47.06^{ab} (3.47)	41.67^{a} (3.47)	41.17^{a} (3.47)	52.44^{b} (3.01)	42.73^{a} (3.22)	44.63^{a} (3.22)
	% Leaf	16.57^{bc} (2.41)	16.49^{bc} (2.58)	18.39^{ac} (2.78)	18.86^{ac} (2.78)	23.10^{a} (2.78)	13.41^{c} (2.41)	16.90^{bc} (2.58)	20.31^{ab} (2.58)
Red Oak	root:shoot	2.03^{ab} (0.43)	3.07^{a} (0.48)	2.71^{ab} (0.39)	2.26^{ab} (0.39)	1.95^{ab} (0.48)	2.02^{b} (0.32)	2.41^{ab} (0.36)	2.34^{ab} (0.39)
	% < 2 mm root	16.28^{ab} (3.24)	26.44^{c} (3.62)	14.65^{a} (2.96)	21.76^{b} (2.96)	21.54^{ac} (3.62)	16.72^{ab} (2.42)	19.64^{ac} (2.74)	16.57^{ab} (2.96)
	% > 2mm root	49.48^{ab} (5.41)	48.38^{ab} (6.05)	57.21^{a} (4.94)	44.14^{b} (4.94)	40.63^{b} (6.05)	47.69^{ab} (4.04)	47.66^{ab} (4.58)	52.40^{ab} (4.94)
	% Total root	66.72^{ab} (4.40)	75.65^{a} (4.92)	71.74^{ab} (4.03)	65.86^{ab} (4.03)	62.67^{b} (4.92)	64.32^{b} (3.30)	66.90^{ab} (3.73)	69.05^{ab} (4.03)
	% Stem	12.53^{ab} (2.23)	9.55^{a} (2.49)	11.54^{a} (2.04)	14.70^{ab} (2.04)	12.35^{ab} (2.49)	17.07^{b} (1.66)	12.25^{a} (1.88)	14.01^{ab} (2.04)
	%Leaf	21.43^{ab} (3.29)	15.36^{a} (3.67)	16.53^{a} (3.00)	19.17^{ab} (3.00)	25.64^{b} (3.67)	18.58^{ab} (2.45)	20.85^{ab} (2.78)	17.35^{b} (3.00)
O_3 tolerant aspen	root:shoot	1.03^{ab} (0.11)	1.11^{b} (0.10)	1.02^{ab} (0.10)	0.89^{c} (0.10)	1.35^{a} (0.09)	1.18^{ab} (0.09)	1.14^{b} (0.09)	1.07^{bc} (0.09)
	% < 2 mm root	28.37^{a} (1.71)	27.19^{a} (1.60)	28.05^{a} (1.51)	27.58^{a} (1.51)	30.51^{a} (1.44)	28.85^{a} (1.44)	28.49^{a} (1.44)	27.11^{a} (1.44)
	% > 2mm root	19.82^{bc} (2.24)	18.99^{bc} (2.10)	18.26^{bc} (1.99)	15.60^{c} (1.99)	24.82^{a} (1.89)	22.01^{ab} (1.89)	22.30^{ab} (1.89)	21.97^{ab} (1.89)
	% Total root	48.17^{bc} (2.47)	46.25^{bc} (2.32)	46.26^{bc} (2.20)	43.22^{c} (2.20)	55.32^{a} (2.10)	50.85^{ab} (2.10)	50.79^{b} (2.10)	49.08^{b} (2.10)
	% Stem	24.81^{a} (1.49)	25.67^{a} (1.41)	24.62^{a} (1.34)	25.08a (1.34)	21.76^{b} (1.28)	23.46^{ab} (1.28)	29.96^{ab} (1.28)	24.88^{a} (1.28)
	% Leaf	27.05^{bc} (2.13)	28.04^{ab} (2.00)	29.14^{ab} (1.90)	31.65^{a} (1.90)	22.92^{c} (1.81)	25.68^{bc} (1.81)	25.24^{bc} (1.81)	26.05^{bc} (1.81)

Table 4.6 (continued):

O3 sensitive Trembling aspen	root:shoot	0.76[a] (0.14)	0.83[ac] (0.14)	0.73[a] (0.14)	0.79[ab] (0.14)	1.01[c] (0.15)	0.84[ac] (0.14)	0.88[ac] (0.15)	0.96[bc] (0.14)
	% < 2 mm root	30.25[a] (2.94)	27.22[a] (3.10)	30.20[a] (2.95)	28.33[a] (3.09)	31.16[a] (3.27)	29.80[a] (3.10)	27.16[a] (3.27)	31.56[a] (2.94)
	% > 2mm root	26.20[a] (2.56)	27.07[a] (2.60)	27.40[a] (2.60)	26.56[a] (2.66)	29.84[a] (2.77)	28.18[a] (2.71)	31.38[a] (2.77)	28.74[a] (2.78)
	% Total root	52.83[ac] (3.56)	50.54[bc] (3.58)	52.32[ac] (3.54)	49.46[c] (3.58)	56.89[a] (3.73)	54.11[ac] (3.67)	55.23[ab] (3.70)	56.30[a] (3.83)
	% Stem	18.21[ac] (1.29)	19.09[ab] (1.37)	19.92[a] (1.29)	17.98[ac] (1.37)	19.76[a] (1.46)	16.43[bc] (1.37)	18.06[ac] (1.46)	16.06[c] (1.29)
	% Leaf	27.57[ab] (2.78)	29.26[ab] (2.80)	26.55[bc] (2.77)	31.22[a] (2.81)	22.05[c] (2.95)	28.05[ac] (2.89)	25.90[bc] (2.93)	25.83[bc] (3.02)

In both aspen clones, elevated CO_2 had a tendency to increase root:shoot. In O_3 tolerant aspens, the increase in root:shoot caused by elevated CO_2 alone was caused by an increase in the biomass of % > 2mm roots coupled with a decrease in % leaf biomass when NO_3^- was low or % stem biomass when NO_3^- was high. When soil NO_3^- was low, many of the treatment combinations cancelled each other; the only case where $CO_2 + NO_2 + O_3$ differed from the ambient was the increased % > 2 mm roots. When soil NO_3^- was high there were no differences between plants fumigated with $CO_2 + NO_2 + O_3$ and the ambient treatment for any tissue type. Ozone sensitive aspens in low NO_3^- and ambient CO_2 tended to shift biomass from the roots to the leaves when O_3 was elevated. However, this shift only resulted in a change in total root:shoot when NO_2 and O_3 were both elevated. The addition of elevated CO_2 eliminated these effects. Unlike in the O_3 tolerant aspens, the increased root:shoot in the CO_2 alone treatment under high soil NO_3^- was not caused by an increase in % root biomass, but by a decrease in % leaf biomass. Across all the tissue types and both clones, the only difference between the ambient treatment and the $CO_2 + NO_2 + O_3$ was in O_3 tolerant aspens where there was higher % > 2 mm root biomass in the $CO_2 + NO_2 + O_3$ treatment when soil NO_3^- was low compared to control.

Leaf characteristics:

Total leaf area generally followed the same trends as total biomass for all species (Fig 4.4). Under high soil NO_3^-, total leaf area in maples increased under the combined treatment of elevated CO_2 and O_3 and increased further in the $CO_2 + NO_2 + O_3$ treatment. In O_3 tolerant aspens, seedlings in the $NO_2 + O_3$ treatment had the lowest total leaf area, but adding elevated CO_2 eliminated the effect. In O_3 sensitive aspens growing in low soil NO_3^-, elevated CO_2 and NO_2 alone each decreased total leaf area resulting in seedlings in the $CO_2 + NO_2 + O_3$ treatment having less leaf area than the ambient.

The number of the leaves lost during the growing season was low for maples (< 10 %), but high for both aspen clones (~40 % in some treatments) (Fig 4.5). The O_3 tolerant clone was particularly prone to dropping its leaves as result of NO_2 and/or O_3 exposure. However, the combination of $CO_2 + NO_2 + O_3$ caused leaf losses similar to those observed in the control. O_3 sensitive seedlings in the $CO_2 + NO_2 + O_3$ treatment lost more leaves than the ambient when soil NO_3^- was high.

The most common change in SLA was a decrease when plants were grown under elevated CO_2. However, this response was not observed in all treatment combinations and was dependent on the concentrations of NO_2, O_3, and NO_3^- (Fig 4.6). In maples, fumigation with CO_2 or $CO_2 + NO_2$ decreased SLA relative to control when NO_3^- was high but the addition of O_3 reduced the effects. When soil NO_3^- was low, adding CO_2 to any treatment caused a decrease in SLA relative to control. The combination of $CO_2 + NO_2 + O_3$ caused lower SLA than the ambient treatment (not significant in low NO_3^- where $p = 0.11$). Oak seedlings exhibited few responses to the treatments; elevated NO_2 increased SLA under low NO_3^-, but the addition of CO_2 eliminated the effect. In O_3 tolerant aspens, irrespective of soil NO_3^- conditions CO_2

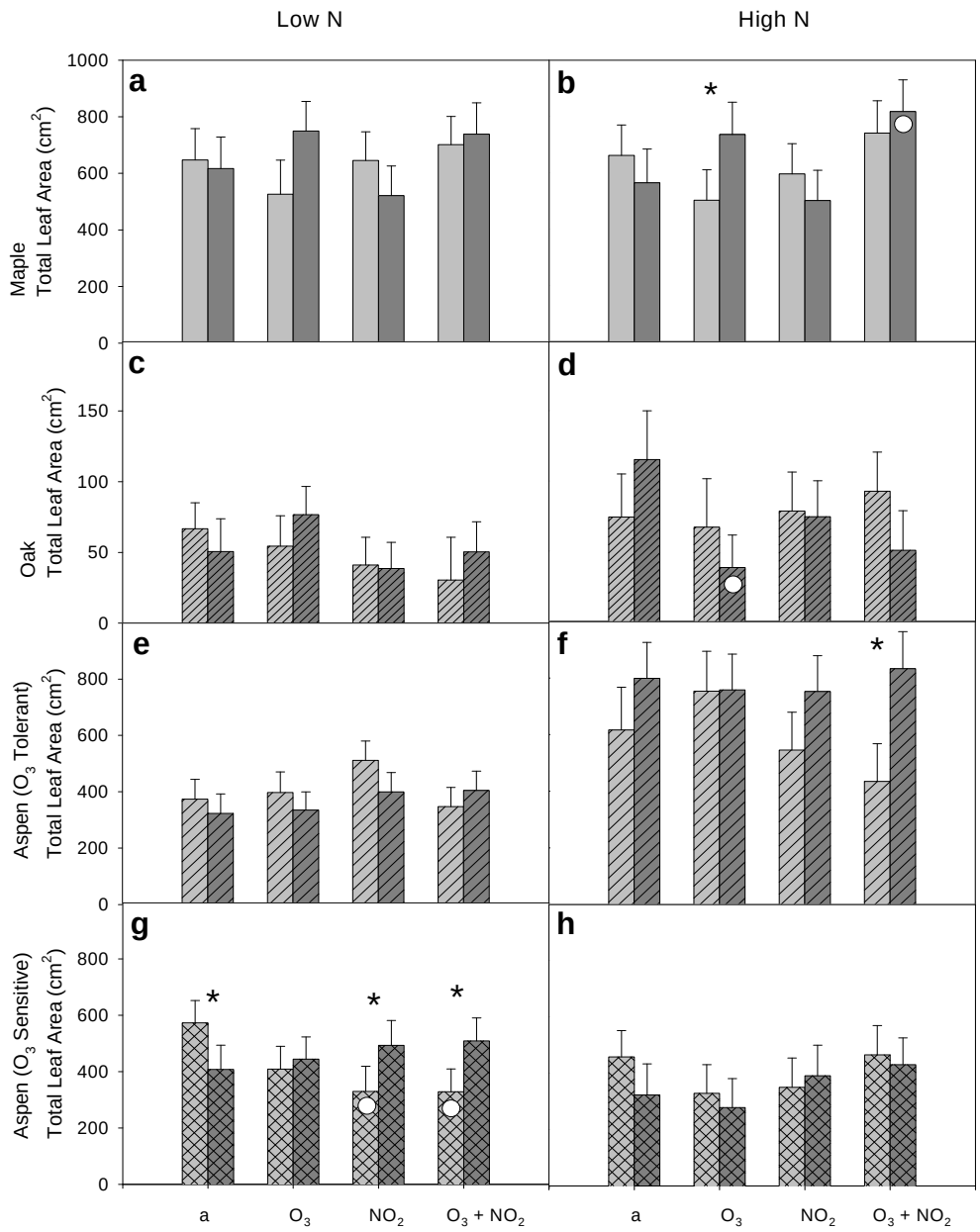

Figure 4.4: Mean total seedling leaf area (cm^2). Light gray bars indicate ambient (380ppm) CO_2 and black bars indicate elevated (560ppm) CO_2. The left-hand panels are the seedlings grown in low soil NO_3^- and the right-hand panels are those grown in 30 kg NO_3^- ha^{-1} yr^{-1}. On the x-axis, a = ambient NO_2 + ambient O_3, O_3 = ambient NO_2 + elevated O_3, NO_2 = elevated NO_2 + ambient O_3, and $O_3 + NO_2$ = elevated NO_2 + elevated O_3. Asterisks indicate a significant difference between the ambient and elevated CO_2 treatments. Open circles indicate a significant difference between that treatment and ambient NO_2 + ambient O_3 treatment in the same CO_2 group.

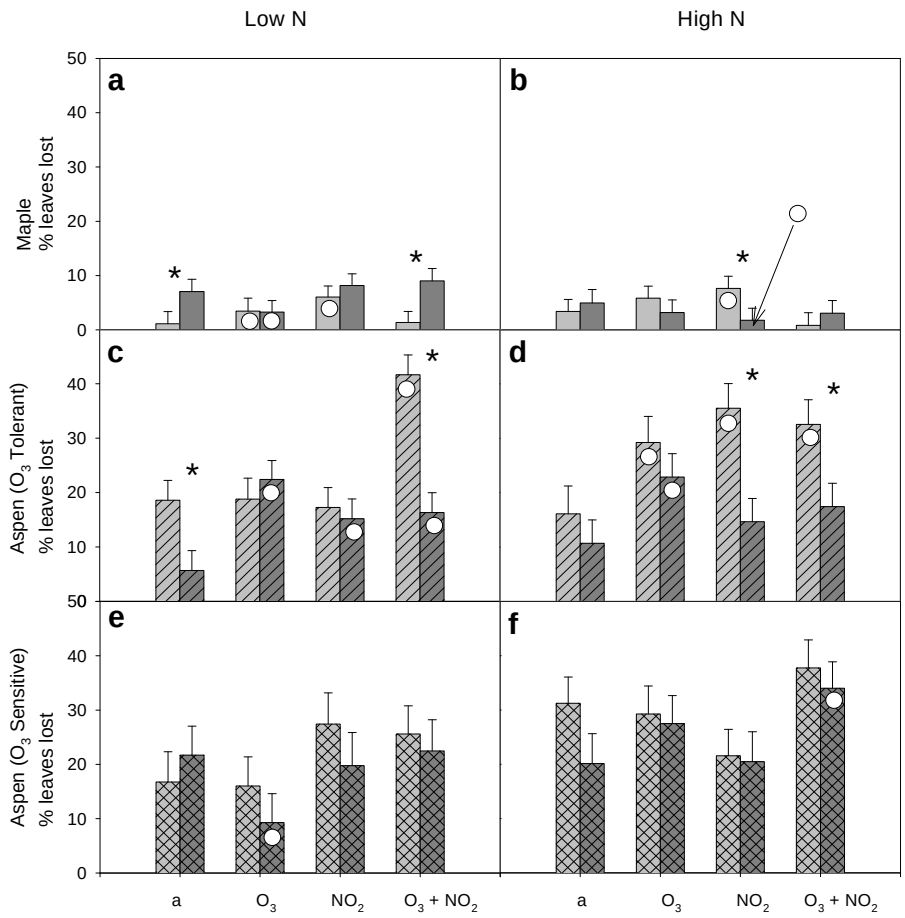

Figure 4.5: Mean % of the total number of leaves produced that were lost during the growing season. Light gray bars indicate ambient (380ppm) CO_2 and black bars indicate elevated (560ppm) CO_2. The left-hand panels are the seedlings grown in low soil NO_3^- and the right-hand panels are those grown in 30 kg NO_3^- ha^{-1} yr^{-1}. On the x-axis, a = ambient NO_2 + ambient O_3, O_3 = ambient NO_2 + elevated O_3, NO_2 = elevated NO_2 + ambient O_3, and $O_3 + NO_2$ = elevated NO_2 + elevated O_3. Asterisks indicate a significant difference between the ambient and elevated CO_2 treatments. Open circles indicate a significant difference between that treatment and ambient NO_2 + ambient O_3 treatment in the same CO_2 group.

fumigation alone did not affect SLA, but seedlings in the $CO_2 + NO_2 + O_3$ treatment had lower SLA than in the $NO_2 + O_3$ or ambient treatments. Under low soil NO_3^-, CO_2 also decreased SLA under elevated O_3. Unlike the O_3 tolerant aspens, the O_3 sensitive aspens exhibited decreased SLA under elevated CO_2 alone in both low and high soil NO_3^-. Under high soil NO_3^-, CO_2 also decreased SLA under elevated O_3 (regardless of NO_2) and seedlings in the $CO_2 + NO_2 + O_3$ treatment had lower SLA

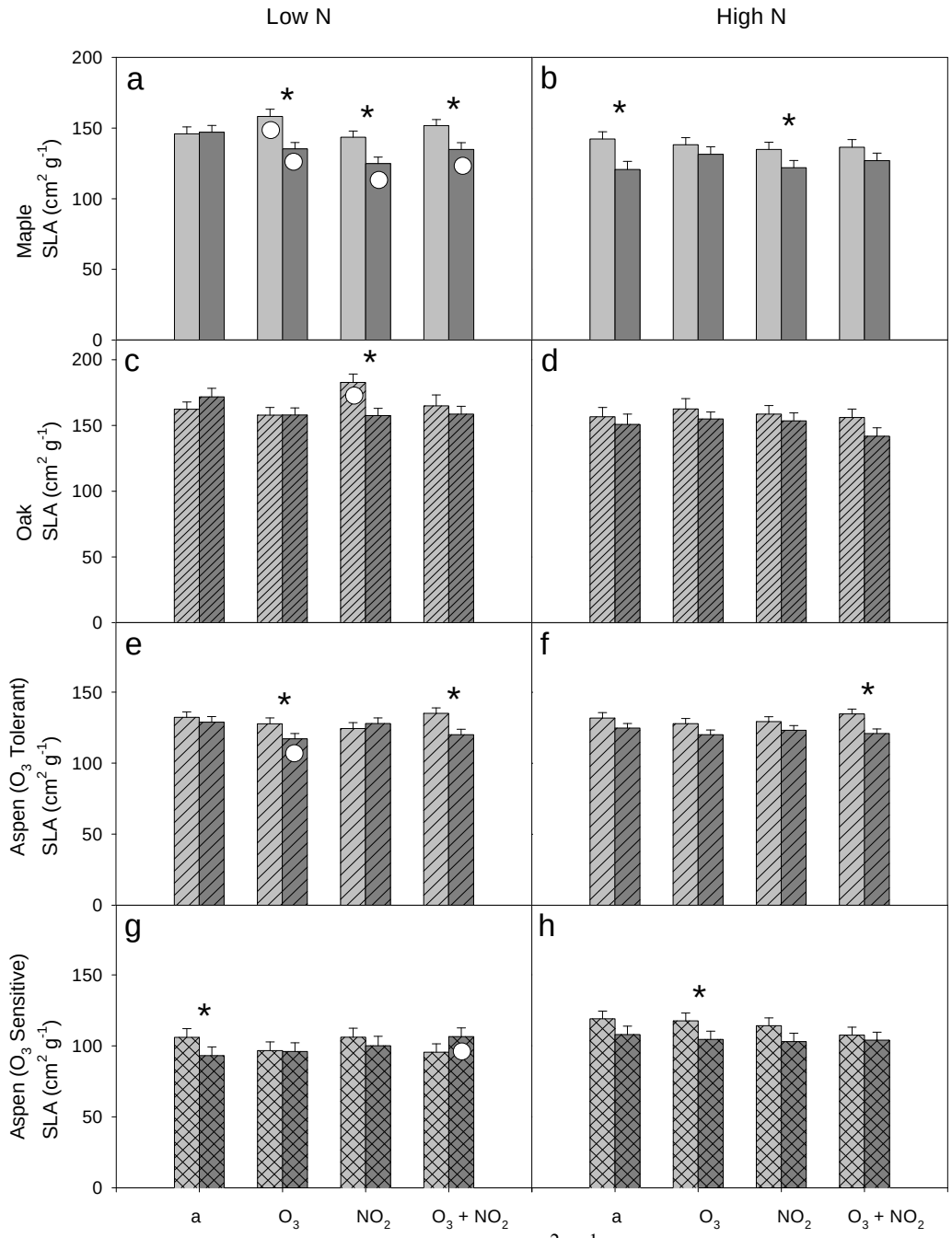

Figure 4.6: Mean specific leaf area (SLA) (cm^2 g^{-1} dry wt). Light gray bars indicate ambient (380ppm) CO_2 and black bars indicate elevated (560ppm) CO_2. The left-hand panels are the seedlings grown in low soil NO_3^- and the right-hand panels are those grown in 30 kg NO_3^- ha^{-1} yr^{-1}. On the x-axis, a = ambient NO_2 + ambient O_3, O_3 = ambient NO_2 + elevated O_3, NO_2 = elevated NO_2 + ambient O_3, and $O_3 + NO_2$ = elevated NO_2 + elevated O_3. Asterisks indicate a significant difference between the ambient and elevated CO_2 treatments. Open circles indicate a significant difference between that treatment and ambient NO_2 + ambient O_3 treatment in the same CO_2 group.

than those in the ambient treatment. When soil NO_3^- was low, $NO_2 + O_3$ caused lower SLA in O_3 sensitive seedlings, but adding CO_2 eliminated the effect causing seedlings in the $CO_2 + NO_2 + O_3$ treatment to have SLA values similar to the control.

Discussion

In this study, fumigations using single treatments rarely caused changes in the total biomass of seedlings. In contrast to what is reported for many plant species (e.g. Curtis and Wang 1998, Norby et al. 1999, and Ainsworth and Long 2005), we did not find a single example of CO_2 alone enhancing total seedling biomass. This response in sugar maples is consistent with the findings from the Rhinelander $CO_2 + O_3$ FACE experiment where it took several years for sugar maples to respond to the treatments (Karnosky *et al.* 2005). Only the O_3 sensitive aspens growing in low soil NO_3^- had decreased biomass when O_3 alone was elevated, which concurs with many studies cited in the review by Chappelka and Samuelson (1998). The O_3 sensitive aspens in low soil NO_3^- were also the only species to exhibit decreased biomass when fumigated with NO_2 alone.

If we assume that the single-gas effects are additive, we would predict that maples, hemlock, oaks, and O_3 tolerant aspens would have no changes in total biomass as a result of any treatment combination. We would also predict that O_3 sensitive aspens being grown in low soil NO_3^- would have decreased total biomass in the $NO_2 + O_3$, $CO_2 + O_3$, $CO_2 + NO_2$, and $CO_2 + NO_2 + O_3$ treatments. Instead, we often observed combinations of treatments that produced changes in total biomass greater than those we would have predicted from singular treatments. For example, when soil NO_3^- was low, O_3 sensitive aspens had reduced biomass in the $NO_2 + O_3$ treatment as expected, but elevated CO_2 had the surprising effect of eliminating the effects of the NO_2 and $NO_2 + O_3$ treatments. When soil NO_3^- was high, both maples and hemlocks

had increased biomass in the $CO_2 + NO_2 + O_3$ treatment, even though none of the treatments singly had an effect. These responses suggest that in the future when CO_2, NO_2, and O_3 are higher, maple, hemlock, oak, and aspen seedlings in low NO_3^- will produce biomass at a rate similar to what they produce in unpolluted areas today. In high soil NO_3^- environments, aspens and oaks will not change biomass production; however, maples and hemlocks may have greater rates of biomass accumulation than they do today.

The ability of trees to remove CO_2 from the atmosphere and store it as wood has many politicians and environmentalists promoting the planting of trees to help reduce the concentration of CO_2 in the atmosphere. Many hope that faster growth of young trees under elevated CO_2 will result in faster carbon storage, which can be achieved if the extra carbon being fixed goes into the production of wood (stem) biomass. The only case where we saw an increase in stem biomass under elevated CO_2 was in hemlocks when CO_2 was applied in combination with NO_2 and O_3 in a high soil NO_3^- environment. In all other species, no treatment combination that included CO_2 produced higher stem biomass than the ambient treatment. These results imply that regional models including an assumption of greater carbon storage in the future may be overestimating the carbon storage potential of forests, particularly when soil fertility is low, a finding that supports modeling efforts by Albani *et al.* (2006) who found that carbon storage in the Harvard Forest was overestimated when the model assumed increasing carbon storage with rising CO_2.

In addition to changing the way that total seedling biomass responded to the gaseous treatments, soil NO_3^- availability also changed the way that biomass was allocated within seedlings. Although many theoretical treatments have predicted that plants will increase their root production under elevated CO_2, most studies do not see this response (e.g., Curtis and Wang 1998, Ainsworth and Long 2005). In low soil

NO_3^- environments, neither hemlock, oak, nor O_3 sensitive aspens had any change in root:shoot, similar to the responses of other studies. Maple and O_3 tolerant aspens exhibited opposite results, suggesting different N requirements of the two species. In maples, elevated CO_2 reduced root:shoot, but only when NO_2 was also elevated. This may indicate that maples were using NO_2 as a source of nitrogen and increased leaf production to increase acquisition of both C and N. Conversely, O_3 tolerant aspens had the more commonly predicted response of increased root:shoot under elevated CO_2 driven by an increase in root production. This supports the theory that plants with more C available should increase their allocation to tissues that acquire nutrients (e.g. Curtis and Wang 1998). Interestingly, the O_3 tolerant aspens may also be using NO_2 as a source of N. When NO_2 was elevated it eliminated the effects of elevated CO_2 restoring % root, % leaf, and root:shoot values to those seen in the ambient treatment. In low fertility soil, there was decreased allocation to root biomass in the $CO_2 + NO_2 + O_3$ treatment in maple, but this was the only case where root:shoot was different in the $CO_2 + NO_2 + O_3$ treatment than in the ambient treatment. In high fertility soil, one might expect that plants would be less likely to increase allocation to roots because of a reduced need to explore the soil volume to acquire nutrients. The responses of maple, hemlock, and oak seedlings under high soil NO_3^- supported this hypothesis, showing no response of root:shoot to elevated CO_2. Conversly, both trembling aspen clones increased their root:shoot under elevated CO_2. In both cases it was allocation to the > 2 mm root fraction that was increased, which may indicate carbon storage in these woody, structural roots rather. Despite the changes to aspen root:shoot caused by elevated CO_2, the other treatments had cancelling effects and we found that none of our species had altered root:shoot in the future $CO_2 + NO_2 + O_3$ treatment in areas with high soil fertility.

There are several processes that likely play important roles in explaining the responses of plant growth to CO_2, NO_2, and O_3 fumigation. The first process is from the interaction of plant leaves with oxidative compounds like O_3 and NO_2. Plants generally protect themselves from gaseous oxidants by either closing their stomata and decreasing the fluxes of all gases in and out of the leaves (Eller and Sparks 2007) or by removal of gases from the internal leaf space through reactions with antioxidant compounds (Noctor and Foyer 1998). If oxidants are not removed before they reach sensitive parts of the leaves they can damage leaf tissue which must then be repaired or replaced by the plant. Repairing damaged tissue is likely to increase respiration rates and requires using photosynthetic products that could otherwise be allocated to growth, which means that more photosynthetic products will be required in order to maintain the same level of growth as plants that are not experiencing oxidative stress. To generate more photosynthate seedlings must either increase the amount of carbon fixed per unit leaf area or increase the total leaf area per seedling.

In this study, we examined both early (O_3 tolerant and sensitive aspen) and late successional (maple, hemlock, and oak) tree species. These two functional groups often behave differently physiologically and it is not surprising these groups have differential responses to fumigation by CO_2, O_3, and NO_2. For example, the response of stomatal conductance was strongly related to functional type, with the majority of responses to CO_2 appearing in the late successional species. Maples and oak both frequently exhibited decreased stomatal conductance under elevated CO_2. The reduction in stomatal conductance when both CO_2 and O_3 were elevated implies that less O_3 entered the leaves of these seedlings and may explain why maples were not damaged by elevated O_3.

In the early successional species, elevated CO_2 did not tend to decrease stomatal conductance, which may have be due in part to leaf age. The early-

successional species had many more young leaves than the late-successional species as they continuously dropped older leaves and built new ones throughout the growing season. Young aspen leaves have lower conductance and photosynthesis (Noormets *et al.* 2001), and may be less responsive than older leaves, which would explain why trends in photosynthesis and stomatal conductance did not appear to be as closely linked to the trends in biomass as seen in the late-successional species. The one exception to the general trend was that O_3 sensitive aspens had lower conductance in the $CO_2 + O_3$ treatment than under elevated CO_2 alone, which helps explain why elevated O_3 did not reduce biomass when CO_2 was elevated

Photosynthetic acclimation to elevated CO_2 only appeared to happen in the late successional species. Many species exhibit lower photosynthesis after long exposure to elevated CO_2 than would be expected based on the instantaneous responses of individuals grown under ambient CO_2 and briefly exposed to elevated CO_2 (e.g. Kubiske et al 1997, Curtis et al. 2000, Ainsworth and Long 2004, Sefcik et al. 2007), but we observed this phenomenon only in maples. When gas exchange was measured on the maple seedlings at the CO_2 concentration under which they were growing, the net carbon assimilation was the same for individuals in the elevated and ambient CO_2 treatments. This indicates that maples did not increase their rate of carbon fixation (per unit leaf area) under elevated CO_2 and explains why there was no increase in biomass caused by elevated CO_2.

There was little evidence of photosynthetic acclimation in aspens, especially when soil NO_3^- was low. Young aspen leaves tend to have lower A and stomatal conductance than older ones (Noormets *et al.* 2001) and young leaves may be less likely to exhibit photosynthetic acclimation if leaves must be exposed for a certain amount of time before they acclimate. Others have found that acclimation of photosynthesis to elevated CO_2 varies by species and other environmental conditions

(e.g. Curtis and Wang 1998, Norby et al 1999, Ainsworth and Long 2005). It is possible that had we measured gas exchange on older leaves we would have seen acclimation of photosynthesis or declines in stomatal conductance as we did in sugar maples, but that would not necessarily have given us a more accurate picture of total seedling gas exchange. The aspens put on new leaves continuously and lost anywhere from 5 – 40% of their leaves through the course of the growing season, so at any given time a large amount of their total leaf area is made up of young leaves.

Maples grown under high soil NO_3^- exhibited the greatest biomass in the CO_2 + NO_2 + O_3 treatment even though net photosynthesis was low; however, seedlings in this treatment also had high respiration rates and total leaf area. This suggests the lower net carbon assimilation per unit leaf area was caused by higher respiration rather than lower photosynthesis and that the gross carbon assimilation in these seedlings may be equivalent to individuals in other treatments. Because the CO_2 + NO_2 + O_3 treatment caused in increase in total leaf area concurrent with the decrease in net carbon assimilation per unit leaf area, it is possible on the whole-plant level these seedlings had more photosynthate available for building additional biomass. The treatment effects on the biomass of hemlock seedlings were similar to that of the sugar maples and although we did not collect gas exchange data on the hemlock seedlings, we hypothesize that a similar mechanism was acting.

In O_3 tolerant aspens, there were no changes in biomass or conductance even though elevated CO_2 tended to increase net carbon assimilation. When soil NO_3^- was high, the increased respiration in the treatments where CO_2 was combined with NO_2 or O_3 suggests that seedlings in these treatments were acquiring more carbon and using it to offset the damage caused by NO_2 and O_3 rather than increasing total biomass. In O_3 sensitive aspens, elevated CO_2 eliminated the detrimental effects of elevated NO_2 and/or O_3 concentrations, but different mechanisms appear to account for the same

effect caused by the different treatments. Elevated O_3 alone clearly decreased total biomass, but adding CO_2 restored it. The decreased stomatal conductance under elevated $CO_2 + O_3$ likely decreased the amount of O_3 entering the leaves and therefore limited the plants' actual exposure to the oxidant. Under elevated NO_2, there were no differences in conductance between CO_2 alone and $CO_2 + NO_2$, but A was higher in $CO_2 + NO_2$ than NO_2 alone. The greater A indicates more carbon being assimilated per unit leaf area and would give the individuals in the $CO_2 + NO_2$ treatment more photosynthate that could be used to repair the damage caused by elevated NO_2. The combination of $CO_2 + NO_2 + O_3$ had A and conductance rates similar to CO_2 alone, but also had higher respiration. The conductance values suggest that the individuals in the $CO_2 + NO_2 + O_3$ treatment received similar doses of O_3 and NO_2 as those in the O_3 alone and NO_2 alone treatment and probably suffered a similar level of damage. The fact that A was the same in the control and the $CO_2 + NO_2 + O_3$ treatment while respiration was higher in the $CO_2 + NO_2 + O_3$ treatment shows that the individuals in the $CO_2 + NO_2 + O_3$ probably had higher rates of carbon fixation, but were respiring the fixed carbon faster as they repaired the damage being caused by the O_3 and NO_2. The combination of greater carbon fixation and higher respiration left the seedlings in the $CO_2 + NO_2 + O_3$ with a similar amount of photosynthate available for new growth as the individual in the control group, which explains why the total biomass production in the two treatments was similar.

Despite the lack of changes in biomass, the alteration of SLA shows that elevated CO_2 did alter tissue (at least in some treatment combinations) for all the species. The general production of thicker leaves under elevated CO_2 shows that seedlings were adding leaf biomass that was not being used to increase photosynthetic surface area. Since a decline in % N is a nearly universal finding of elevated CO_2 studies (e.g. Curtis and Wang 1998, Norby *et al.* 1999, and Ainsworth and Long 2005)

it is likely that these plants were increasing their storage of carbon-rich molecules (like cellulose) in their leaves which could have impacts for entire ecosystems when these leave become the substrate for herbivory (Coviella and Trumble 1999) and soil microbial activities (King *et al.* 2001).

Overall we saw surprisingly few changes as a result of the treatments we applied. We saw no reason to suspect that carbon storage in tree seedlings will increase under future CO_2 conditions, nor that O_3 will decrease it. Gas exchange was generally a good predictor of total seedling growth when net carbon assimilation, respiration, and stomatal conductance were all considered. Soil fertility and plant functional type altered the responses of total biomass to the gaseous treatments; a reminder of the importance of including multiple species and ecological conditions in global change studies. There were many cases where looking at single or 2-factor treatments would have led us to different conclusions about biomass and allocation responses to the combination of $CO_2 + NO_2 + O_3$. As this and other studies have shown, in order to predict how plants will respond to the many simultaneous changes occurring in Earth's atmosphere we must design experiments that expose plants to simultaneous treatments and not rely so heavily on single-factor studies.

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REFERENCES

Ainsworth EA, Long SP (2005) What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO_2. New phytologist 165: 351–372

Albani M, Medvigy D, Hurtt GC, Moorcroft PR (2006) The contributions of land-use change, CO2 fertilization, and climate variability to the Eastern US carbon sink. Global Change Biology 12(12):2370-2390

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

Coleman M.D., R.E. Dickerson, J.G. Isebrands, and D.F. Karnosky. (1995). Photosynthetic productivity of aspen clones varying in sensitivity to topospheric ozone. *Tree Physiology* 15:585-592

Coviella, C. E. and J. T. Trumble (1999). Effects of elevated atmospheric carbon dioxide on insect-plant interactions. *Conservation Biology* 13(4): 700-712.

Curtis PS, Vogel CS, Wang XZ, Pregitzer KS, Zak DR, Lussenhop J, Kubiske M, Teeri JA (2000) Gas exchange, leaf nitrogen, and growth efficiency of Populus tremuloides in a CO2-enriched atmosphere. Ecological Applications 10(1):3-17

Curtis PS, Wang X (1998) A meta-analysis of elevated CO_2 effects on woody plant mass, form, and physiology. Oecologia 113:299-313

EEA, Copenhagen, (2008) http://themes.eea.europa.eu/IMS/IMS/ISpecs/I Specification 20080701123452/IAssessment1219309276318/view_content (5/17/2009)

Eller ASD, Sparks JP (2006) Predicting leaf-level fluxes of O-3 and NO2: the relative roles of diffusion and biochemical processes. Plant Cell and Environment 29:1742-1750

Gaucher C, Costanzo C, Afif D, Mauffette Y, Chevrier N, Dizengremel P (2003) The impact of elevated ozone and carbon dioxide on young *Acer saccharum* seedlings. Physiologia Plantarum 117:392-402

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

IPCC (2007). Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change [Solomon, S., D. Qin, M. Manning, Z. Chen, M. Marquis, K.B. Averyt, M.Tignor and H.L. Miller (eds.)]. Cambridge

Isebrands JG, McDonald EP, Kruger E, Hendrey G, Pregitzer K, Percy K, Sôber J, Karnosky DF. 2001. Growth responses of *Populus tremuloides* clones to interacting carbon dioxide and tropospheric ozone. *Environmental Pollution* 115: 359–371

Karnosky DF, Pregitzer KS, Zak DR, Kubiske ME, Hendrey GR, Weinstein D, Nosal M, Percy KE (2005) Scaling ozone responses of forest trees to the ecosystem level in a changing climate. Plant Cell and Environment 28:965-981

King JS, Pregitzer KS, Zak DR, Kubiske ME, Holmes WE (2001) Correlation of foliage and litter chemistry of sugar maple, *Acer saccharum*, as affected by elevated CO2 and varying N availability, and effects on decomposition. Oikos 94:403-416.

Kubiske ME, Pregitzer KS, Mikan CJ, Zak DR, Maziasz JL, Teeri JA (1997) *Populus tremuloides* photosynthesis and crown architecture in response to elevated CO2 and soil N availability. Oecologia 110(3):328-336

NADP http://nadp.sws.uiuc.edu (05/17/2009)

NASA Visible Earth http://visibleearth. nasa.gov/view_rec.php?id=15000 (05/17/2009)

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

Noormets A., A. Sober, E.J. Pell, R.E. Dickson, G.K. Podila, J. Sober, J.G. Isebrands, D.F. Karnosky. (2001). Stomatal and non-stomatal limitation to photosynthesis in two trembling aspen (Populus tremuloides Michx.) clones exposed to elevated CO_2 and/or O_3. *Plant, Cell and Environment*. 24(3):327-336

Norby RJ, Wullschleger SD, Gunderson CA, Johnson DW, Ceulemans R (1999) Tree responses to rising CO_2 in field experiments: implications for the future forest. Plant, Cell and Environment 22:683-714

Ollinger S.V., J.D. Aber, P.B. Reich. (1997). Simulating ozone effects on forest productivity: Interactions among leaf-, canopy-, and stand-level processes. *Ecological Applications* 7(4):1237-1251

Oren R, Ellsworth D, Johnson KH, Phillips N, Ewers BE, Maler C, Schafer KVR, McCarthy H, Hendrey G, McNulty S, Katul G (2001) Soil fertility limits carbon sequestration by forest ecosystems in a CO2-enriched atmosphere. Nature 411: 469-472.

Qiao Z, Murray F (1997) The effects of root nitrogen supplies on the absorption of atmospheric NO2 by soybean leaves. New Phytologist 136:239-243

Rennenberg H, Gessler A (1999) Consequences of N deposition to forest ecosystems - Recent results and future research needs. Water Air and Soil Pollution 116:47-64

Rowland AJ, Drew MC, Wellburn AR (1987) Foliar Entry and Incorporation of Atmospheric Nitrogen-Dioxide into Barley Plants of Different Nitrogen Status. New Phytologist 107: 357-371.

Sefcik LT, Zak DR, Ellsworth DS (2007) Seedling survival in a northern temperate forest understory is increased by elevated atmospheric carbon dioxide and atmospheric nitrogen deposition. Global Change Biology 13:132-146

Siegwolf RTW, Matyssek R, Saurer M, Maurer S, Gunthardt-Goerg MS, Schmutz P, Bucher JB (2001) Stable isotope analysis reveals differential effects of soil nitrogen and nitrogen dioxide on the water use efficiency in hybrid poplar leaves. *New Phytologist* 149:233-246

Skärby L., H. Ro-Poulsen, F.A.M. Wellburn, and L.J. Sheppard. (1998). Impacts of ozone on forests: a European perspective. *New Phytologist*, 139:109-122

Sparks JP (2009) Ecological ramifications of the direct foliar uptake of nitrogen. Oecologia 159:1-13

Srivastava HS, Ormrod DP (1989) Nitrogen-Dioxide and Nitrate Nutrition Effects on Nitrate Reductase-Activity and Nitrate Content of Bean-Leaves. Environmental and Experimental Botany 29: 433-438.

Vallano DM, Sparks JP (2007) Quantifying foliar uptake of gaseous nitrogen dioxide using enriched foliar $\delta^{15}N$ values. New Phytologist 177:946-955

Zaehle S, Bondeau A, Carter TR, Cramer W, Erhard M, Prentice IC, Reginster I, Rounsevell MDA, Sitch S, Smith B, Smith PC, Sykes M (2007) Projected changes in terrestrial carbon storage in Europe under climate and land-use change, 1990-2100. Ecosystems 10:380-401

Chapter 5

Ramifications of a changing atmosphere on plant growth, phenology, and reproduction: single and combined effects of rising carbon dioxide, nitrogen dioxide, and ozone

Abstract

The rising concentrations of atmospheric carbon dioxide (CO_2), nitrogen dioxide (NO_2), and ozone (O_3) are likely to change the growth, reproduction, and phenology of plants in the future. We know that elevated CO_2 tends to cause an often transient increase in growth, decreased stomatal conductance, and increased reproductive output, but its effects on phenology are highly variable. Even within the study species used here, *Arabidopsis thaliana*, elevated CO_2 has been shown to accelerate, delay, or cause no change in flowering time. Elevated O_3 is detrimental to plants and can decrease growth, lower reproductive output, and typically delays flowering. Nitrogen dioxide can cause oxidative damage to plants, but when it enters plant leaves it reacts to form nitrate and nitrite, providing a potentially beneficial extra source of nitrogen. In this study, we grew *A. thaliana* in open-top chambers with ambient or 560 ppm CO_2, ambient or 40 ppb NO_2, ambient or 100 ppb (5 days/week) O_3, and grown in ambient or 30 kg ha^{-1} yr^{-1} soil NO_3^-. We monitored the time required for plants to bolt, flower, and produce pods. Further, we measured total biomass throughout the life-cycle and total reproductive output. In general, we found that elevated CO_2 accelerated reproductive stages by 1-3 days. However, if plants were simultaneously exposed to O_3, reproductive stages were delayed by 2-4 days, suggesting that plants grown in polluted areas in the future will have delayed phenology compared with those growing today. Plants exposed to elevated CO_2 exhibited increased total biomass and reproductive output, but the addition of O_3 or NO_2 reduced or eliminated

this effect. These responses could not have been predicted by the responses of *A. thaliana* to single-gas treatments. The non-additive and often unexpected influences of multifactor treatments are particularly necessary if we are to generate accurate predictions of future plant performance.

Introduction

The composition and chemistry of the atmosphere is changing and these changes are largely driven by human activities. The future atmosphere is likely to have higher concentrations of carbon dioxide (CO_2), nitrogen dioxide (NO_2), and ozone (O_3) (IPCC 2007). Therefore, in the future plants will persist in an environment richer in compounds known to have large effects on the growth, phenology, and reproductive output of plants. Most global change studies examine the effects of gases individually. However, there is no *a priori* reason to suggest the effects of single gas exposures will be additive or that we can predict the influence of multiple simultaneous gas exposures based on single-factor studies. In this study, we exposed plants simultaneously to multiple gas species with the explicit goal to better understand plant performance under likely future atmospheric conditions.

Single-gas fumigation studies have found that elevated CO_2 typically, at least transiently, increases growth (e.g., Curtis and Wang 1998, Norby *et al.* 1999, Ainsworth and Long 2005), decreases stomatal conductance (Ainsworth and Long 2005), increases reproductive output in crop plants, but not consistently in plants form natural populations (see review by Jablonski *et al.* 2002), reduces the %N of seeds (Jablonski *et al.* 2002) and alters phenology (see review by Springer and Ward 2007). Interestingly, it has been reported that phenology can be accelerated or delayed in response to elevated CO_2. In fact, both responses have been observed in the focal

species used in the study described here: *Arabidopsis thaliana* (Springer and Ward 2007).

Elevated O_3 is a strong oxidant and is detrimental to both plants and animals. In many plants it has been shown to cause visible leaf damage (e.g., Greitner *et al.* 1994, Coleman *et al.* 1995), decrease photosynthesis (e.g., reviews by Chappelka and Samuelson 1998, Skärby *et al.* 1998), decrease growth (see review by Fuhrer 2009), and reproductive output (see review by Black *et al.* 2000). In annual plants, the timing of reproduction is often linked to some minimum plant size. The reduction in total biomass coupled with direct damage to inflorescences and other reproductive structures can lead to delayed flowering, decreased number of seeds produced, and decreased individual seed mass (Black *et al.* 2000). The effects of elevated O_3 on growth are often tempered by the addition of elevated CO_2 (*e.g.* Isebrands *et al.* 2001, Karnosky *et al.* 2003, King *et al.* 2005) which suggests that the effects on phenology and reproductive output may be similarly balanced when both gases are elevated.

When NO_2 enters plant leaves it disproportionates into nitrate and nitrite (Rennenberg and Geβler 1999, Siegwolf *et al.* 2001) and can potentially act as a source of nitrogen and have a beneficial effect on plants (see review by Sparks 2009), but NO_2 has also been found to decrease or have no effect on growth (*e.g.* Zeevart 1976, Rowland *et al.* 1985, Rajagopal and Saxe 1988, Vallano and Sparks 2007). Although little is known about the effects of NO_2 on plant phenology it is likely its effects will be largely dependent on whether it increases or decreases total plant biomass.

There are thought to be two general mechanisms of plant protection from phytotoxic gases: prevention of the gases from entering the leaves through decreases in stomatal conductance and elimination of gases within the leaves before they come in contact with living cells. Reduction of stomatal conductance has been shown to

decrease the total flux of reactive gases into the leaf and explain 56% and 84% of the movement of O_3 and NO_2 into leaves, respectively (Eller and Sparks 2006). This physical mechanism can be effective, but also necessitates a decrease in photosynthesis as the diffusion of CO_2 into the leaf is also reduced. Once an oxidative gas is inside the leaf, it will either oxidize some internal component or be quenched by an antioxidant compound in the leaf cell wall (Noctor and Foyer 1998). Ascorbate and glutathione are the two most commonly described antioxidant compounds in plants (Foyer and Noctor 2005).

Changes in reproductive timing may have larger ramifications for annual compared to perennial plants because annuals must reproduce within a single growing season and lack of reproduction in any given year leads to a removal of a genotype from the population. In contrast, a perennial plant may simply delay reproduction to a subsequent year. Changes in seed size and number can alter the number of offspring each individual is likely to contribute to the next generation and changes in the timing of phenology, particularly flower and seed production, can decrease total reproductive output and potential fitness (Schemske 1977, Waser 1978, O'Neil 1997). When flowering time is altered it can disrupt pollinator/flowering cycles that are crucial to the survival of both the pollinator and the plants. Delays in the production of seeds can result in plants failing to reproduce before the end of the growing season (Ward and Kelly 2004) and make them more susceptible to reproductive failure as a result of late-season stochastic events.

The overarching objective of this study was to determine if the application of multiple gas species would change the response of plant phenology and reproduction in ways that were not merely the summation of responses to single gas fumigations. In particular, we expected that enhanced CO_2, a treatment likely to alter stomatal conductance will modify the effects other treatments by changing their ability to enter

leaves or to be eliminated from within the leaf (Eller and Sparks 2006). Elevated CO_2 typically decreases stomatal conductance, so we expect that the effects of O_3 and NO_2 will be lessened under future CO_2 conditions, but we also expect that the detrimental effect of O_3 will offset the positive effect of CO_2. This is the first study to examine how plant phenology, reproduction, and growth respond to the effects of these pollutants under current and future atmospheric CO_2 conditions and will provide better predictions of future plant performance than can be obtained using single treatment studies.

Methods

Site description:

This study was conducted in an open field at the University of Michigan Biological Station in Pellston, MI USA (45°33′14″N 84°47′4″W). The average high and low June-August temperatures at the site were 23 and 13^{o} C, respectively. In order to reduce the ambient light level, a shade cloth was erected over the entire site, reducing the incoming light by 50%. An additional plastic shield was placed over the plants within each chamber blocking natural precipitation and causing a 75% reduction in the total incoming light.

Plant material and chambers:

On June 24th 2007, wild-type *A. thaliana* seeds were planted in a 2:1 mixture of sand and topsoil. The seeds were all germinated under ambient conditions and thinned to one plant per pot 2-3 days following germination. After thinning, each pot was randomly assigned to a treatment.

One flow-through open-top chamber (0.8m x 0.8m x 1m) was placed over 60-80 *A. thaliana* individuals and two 0.16 m^3 rootboxes containing tree seedlings from a

companion study. The chamber frames were made of 1/2 inch PVC pipe and the frames were wrapped with transparent 0.8 mm PVC film. Fans encased in metal blower boxes were connected to a perforated ring of PVC that was placed at the bottom of the chamber. The bulk flow from the blower box through the chamber was 600-700 lpm resulting in a turnover time of less than 2 minutes. A smoke test showed that the chambers became fully mixed in less than 10 seconds.

The chambers were assigned to a block based on their location in the field in order to minimize the effects of possible light, wind, and moisture differences across the field. Within each block, each chamber was randomly assigned one of the eight possible treatment combinations.

Treatments:

Carbon dioxide was purchased from Airgas (Charlevoix, MI) as a liquid, NO_2 was purchased in 10,000ppm tanks from Scott-Marin Specialty Gas (Riverside, CA), and O_3 was generated by pumping ambient air through a LG-7 CD Laboratory O_3 generator (Ozone Engineering, El Sobrente CA). The gas from each tank was delivered to a manifold block where it was split into 40 lines, each to a needle valve with a flowmeter. Each needle valve controlled the flow of CO_2, NO_2, or O_3 to a single chamber. Opaque black PTFE tubing (Forberg Scientific, MI) was used for all NO_2 and O_3 lines and Poly Flo tubing (J.F. Good Company, OH) was used for CO_2 lines. Return lines placed in each chamber were used to bring air from the chamber to a solenoid system that automatically sampled each chamber every 4 hours for CO_2 and NO_2. O_3 in each chamber was monitored manually every other day with a ThermoEnvironmental Model 49 Chemiluminescence O_3 monitor. A LiCor 6252 IRGA was used for analysis of CO_2 concentration and an EcoPhysics CLD 760 with a

PLC 660 NO_x converter for analysis of NO_2 and NO concentrations. Both analyzers were calibrated weekly.

Elevated CO_2 chambers were set to 560ppm CO_2 and elevated NO_2 chambers received 40ppb NO_2 and the treatments were applied between 7am and 7pm. The elevated O_3 treatment was 100 ppb and was applied 5 days/week from 10am until 4pm. The NO concentration in elevated NO_2 chambers was typically 3-7ppb. Ambient CO_2 concentration was 365ppm, ambient O_3 was typically 30-40 ppb, and ambient NO_2 and NO concentrations were both <1ppb.

Once a week, all the plants received a ¼ strength Hoagland solution that was modified to include no nitrogen. Half the chambers were given additional soil nitrogen in the form of $NaNO_3$ at a rate of 30 kg N ha^{-1} yr^{-1}. The $NaNO_3$ was added to the Hoagland solution and applied every two weeks.

Ascorbate Assay

Leaf tissue was collected from 10 individuals in each treatment after 33 days of treatment. The tissue was immediately frozen using liquid nitrogen and then stored at -80^{o}C. Leaf tissue was ground in 1 mL of 2% metaphosphoric acid 2mM EDTA buffer and centrifuged at 13,000 RPM for 10 minutes. The supernatant (symplastic extract) was collected and frozen at -80 ˚C.

The assay to determine ascorbate concentration followed the procedure of Rao and Ormrod 1995 and Conklin *et al.* 1996 with the modifications outlined in Eller and Sparks 2006. Absorbance of each sample was measured at 265 nm using a spectrophotometer (Beckman, DU 640) and the samples were compared to a standard curve prepared using purified ascorbic acid (Sigma, St. Louis, MO, USA).

Growth, reproduction, and phenology:

Growth was measured throughout the lifetime of the plants by harvesting 10 individuals from each treatment each week. The number of buds, flowers, and pods on each plant were counted and added to determine the maximum total reproductive output and the roots, leaves, bolts, and pods were separated to determine the biomass of each tissue type. All the tissues were dried and weighed for final biomass determination. The seeds were separated from the pods, weighed separately and the mean seed weight from a single pod was multiplied by the total number of pods each plant produced during its lifetime to get an estimated total seed production that included the pods that released their seeds before the harvest. Then a subset of seeds from each sample were counted and weighed to determine the mean weight (size) of an individual seed. The estimated total seed production was divided by the mean seed size to estimate the total number of seeds produced by each plant.

Phenological transitions were monitored by counting the number of individuals with bolts, flowers, and pods each day.

Statistical Analysis:

Treatment means were compared using the mixed model ANOVA technique with pairwise comparisons, block as a random factor, and $\alpha = 0.1$. The data for the figures of time required to bolt, flower, and produce pods were generated using the survivorship analysis PROC LIFETEST with block as a random factor. All statistical analyses were done using the SAS statistical software (SAS Institute Inc., SAS Version 9.1.3, Cary, NC) and figures were generated in SigmaPlot (SPSS Science, Chicago, IL).

Results

Phenology:

The effects of O_3 and NO_2 on the initiation of the reproductive phase (bolting) were different under current and future CO_2 conditions. Under current CO_2 neither O_3 nor NO_2 had an effect on bolting (Fig 5.1 a + b). Despite the absence of single-gas effects, when soil N was high O_3 and NO_2 in combination delayed bolting by 3 days (median number of days until bolting). In the future CO_2 scenario, elevated O_3 delayed bolting by 2-3 days irrespective of the NO_2 and soil NO_3^- conditions (Fig 5.1 c + d). Elevated CO_2 caused a one day acceleration of bolting, but the addition of N

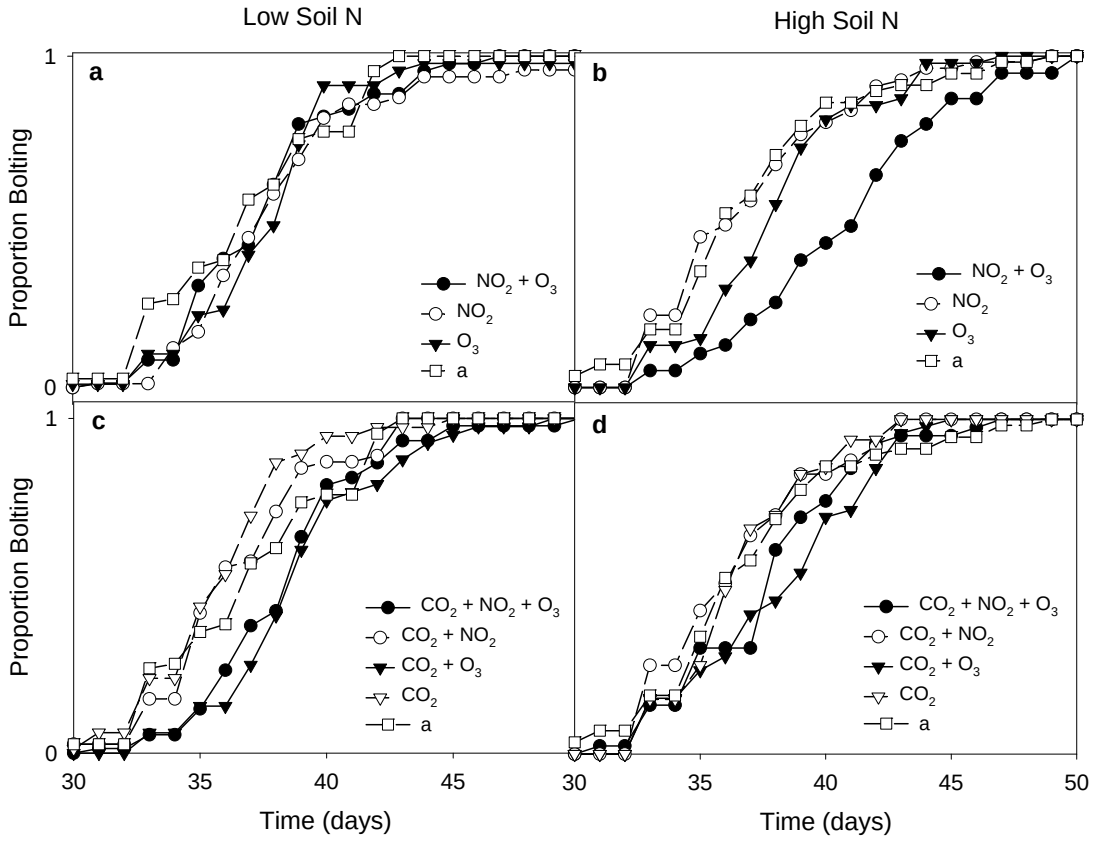

Figure 5.1: The proportion of individuals in each treatment with a bolt on each day. The top panels show the responses under current CO_2 conditions, the bottom panels show the responses under elevated CO_2 with the ambient treatment from the current CO_2 group as a reference.

(either NO_2 or soil NO_3^-) eliminated it.

Changes in the time required to initiate reproduction led to changes in flowering time. The 3-day delay in bolting caused by $NO_2 + O_3$ expanded to a 4-day delay in flowering (high soil NO_3^- only) and was the only response seen under current CO_2 conditions (Fig 5.2 a + b). Under elevated CO_2, O_3 continued to be a delaying influence (regardless of NO_2 or NO_3^-), but the gap between the ambient treatment and the treatments including $CO_2 + O_3$ was reduced to 1-2 days. In contrast, the acceleration caused by elevated CO_2 was more pronounced in flowering than bolting (Fig 5.2 c + d); causing a 2-day acceleration that was again eliminated by N.

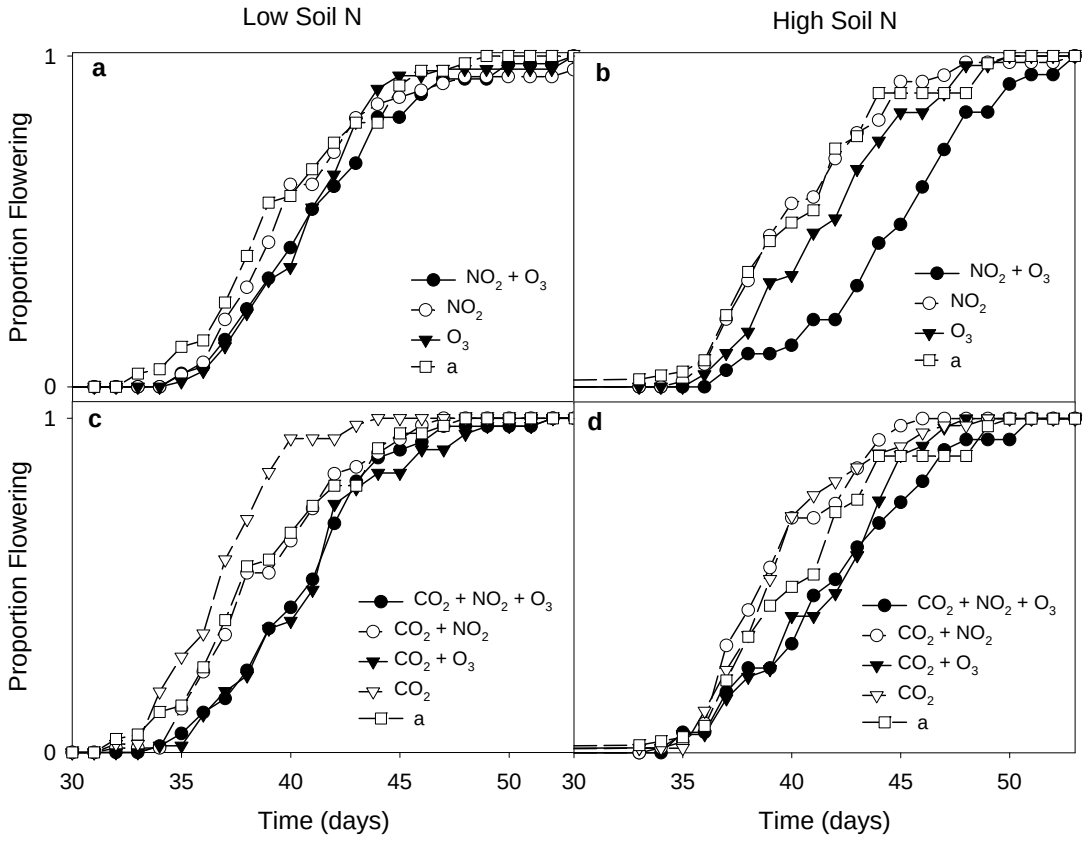

Figure 5.2: The proportion of individuals in each treatment with flowers on each day. The top panels show the responses under current CO_2 conditions, the bottom panels show the responses under elevated CO_2 with the ambient treatment from the current CO_2 group as a reference.

Under current CO_2 conditions the changes in flowering time led to greater changes in time until pod production, but the opposite was true under elevated CO_2. Under current CO_2, the delay caused by $NO_2 + O_3$ increased to 5 days (high soil NO_3^-) and there were still no effects of any other treatments (Fig 5.3 a + b). In the future CO_2 scenario, elevated O_3 still caused a 2-day delay in pod production, but high soil NO_3^- now eliminated the effect (Fig 5.3 c + d). This suggests that high soil NO_3^- accelerated the transition from flowering to pod production such that the delay in flowering time did not alter the timing of pod production. The presence of elevated CO_2 was still an accelerating force (in low soil NO_3^-), but the time until pod production was only accelerated by 1 day.

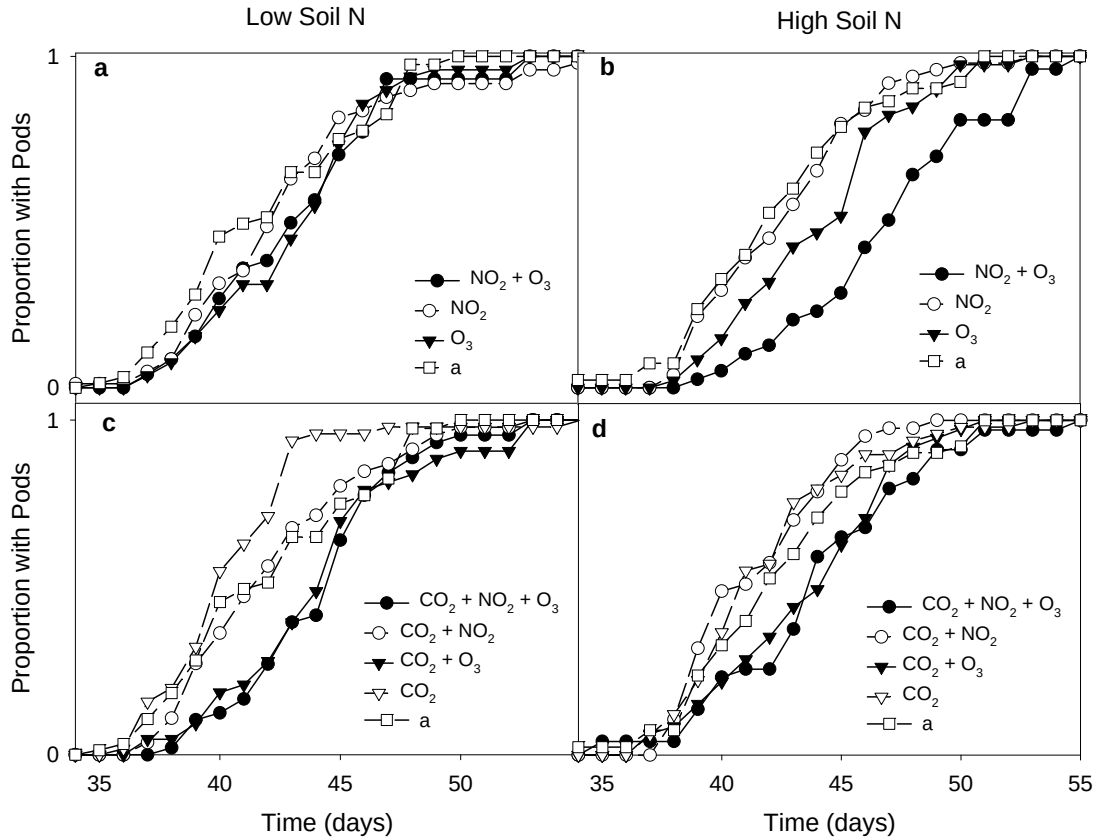

Figure 5.3: The proportion of individuals in each treatment with pods on each day. The top panels show the responses under current CO_2 conditions, the bottom panels show the responses under elevated CO_2 with the ambient treatment from the current CO_2 group as a reference.

Biomass:

The initiation of the reproductive phase of the *A. thaliana* life-cycle occurred around 35 days, so the leaf + root biomass at that time (very few individuals had bolts, but if they did the bolts were not included in the biomass) is indicative of the resources available to the plant at the start of reproduction. There were no differences in either soil N group when CO_2 was ambient (Fig 5.4 a + e). Elevated CO_2 alone increased biomass only when soil NO_3^- was low. Under the high CO_2 + low soil NO_3^- conditions, the enhancement caused by elevated CO_2 was reduced by the addition of elevated O_3, with the lowest biomass in the CO_2 + O_3 treatment (Fig 5.4b). A nearly opposite effect was seen when under high soil NO_3^-; elevated CO_2 alone did not alter biomass, but the addition of either O_3 or NO_2 alone resulted in significantly increased biomass (relative to the ambient, but not CO_2 alone) (Fig 5.4g).

Biomass measured on plants harvested at 42, 54, and 72 days is indicative of the total amount of biomass each plant was able to produce (including reproductive effort) and gives an idea of each plant's ability to acquire and use resources. The smallest plants continued to be present in the ambient CO_2 + low soil NO_3^- group and there were no differences caused by NO_2 or O_3 (Fig 5.4b). When soil NO_3^- was high, NO_2 alone increased biomass at the final harvest (72 days), but elevated O_3 eliminated the effect (Fig 5.4f). When CO_2 was elevated, treatment differences started to appear at 54 days when elevated CO_2 caused increased biomass in low soil NO_3^-; an effect eliminated by the addition of NO_2 and/or O_3, which had biomass values similar to the ambient treatment. The same trends were seen at 72 days, but they were less pronounced (low soil NO_3^-), suggesting that the differences occurred between 42 and 54 days and did not accumulate after 54 days. When soil NO_3^- was high, the differences began appearing at 54 days and became greater through time (Fig 5.4h). Elevated CO_2 increased biomass at 54 days and increased O_3 reduced the effect. By

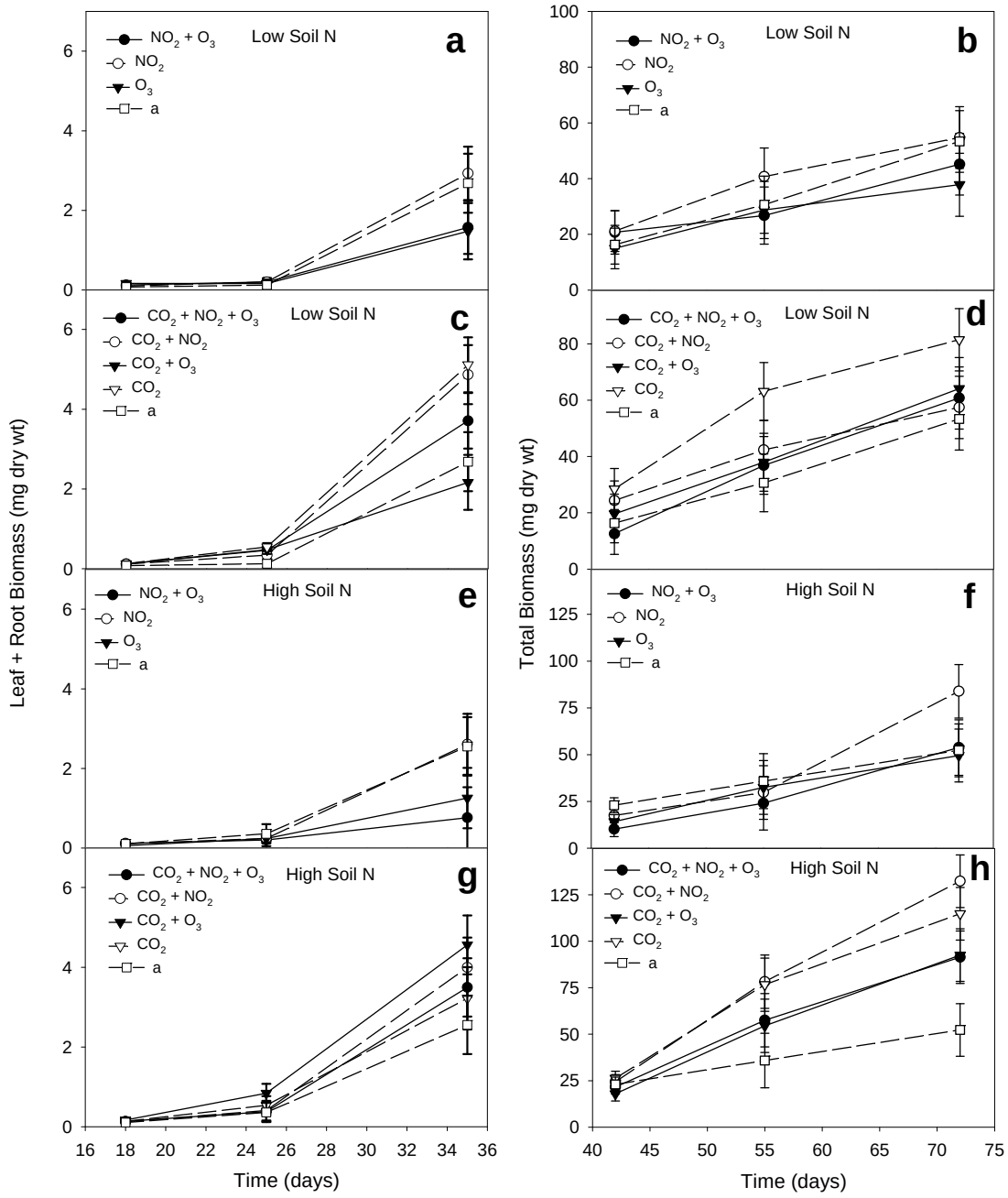

Figure 5.4: Biomass (mg dry wt) vs time following planting in the high N treatment. The left panels show the first three harvest when very few individuals had produced bolts. The weight of the bolt (if present) was subtracted to give the biomass of resource gathering tissue (leaves and roots). The right panels show the last three harvests at which point most individuals had bolts and the total biomass is shown. Panels a-d had low soil NO_3^- and e-h had high soil NO_3^-.

72 days, all the treatments including elevated CO_2 had greater biomass than the biomass and elevated O_3 still reduced biomass, but the effect was no longer significant ($CO_2 + O_3$ vs CO_2 p = 0.14, $CO_2 + NO_2 + O_3$ vs CO_2 p = 0.12). These results suggest that in the future these plants will have increased biomass, but NO_2 and/or O_3 will eliminate the effect in low soil NO_3^- environments.

Seed Production:

Reproductive output can be broken into two components: reproductive effort (number of seeds produced and total seed biomass) and seed quality (in this case individual seed biomass). Our treatments had the greatest effect on reproductive output. Elevated CO_2 resulted in higher total seed biomass and a greater number of seeds produced in both soil NO_3^- groups (Fig 5.5 a, b, e, and f), however this response was tempered by the additions of other treatments. In low soil NO_3^- the addition of NO_2 and/or O_3 when CO_2 was elevated caused declines both components of reproductive effort causing the combinatorial treatments to be equivalent to the ambient treatment. When soil NO_3^- was high, elevated O_3 reduced the CO_2 enhancement although the $CO_2 + O_3$ treatments were still higher than the ambient treatment. Elevated NO_2 alone also enhanced reproductive effort when soil NO_3^- was high. The individual seed biomasses were largely unchanged by our treatments, but the two exceptions are notable (Fig 5.5 c + d). Elevated O_3 decreased individual seed biomass in both the ambient and elevated CO_2 treatments when soil NO_3^- was low, but the addition of NO_2^- eliminated the effect. When soil NO_3^- was high, the combination of $CO_2 + NO_2$ resulted in an increase in individual seed biomass.

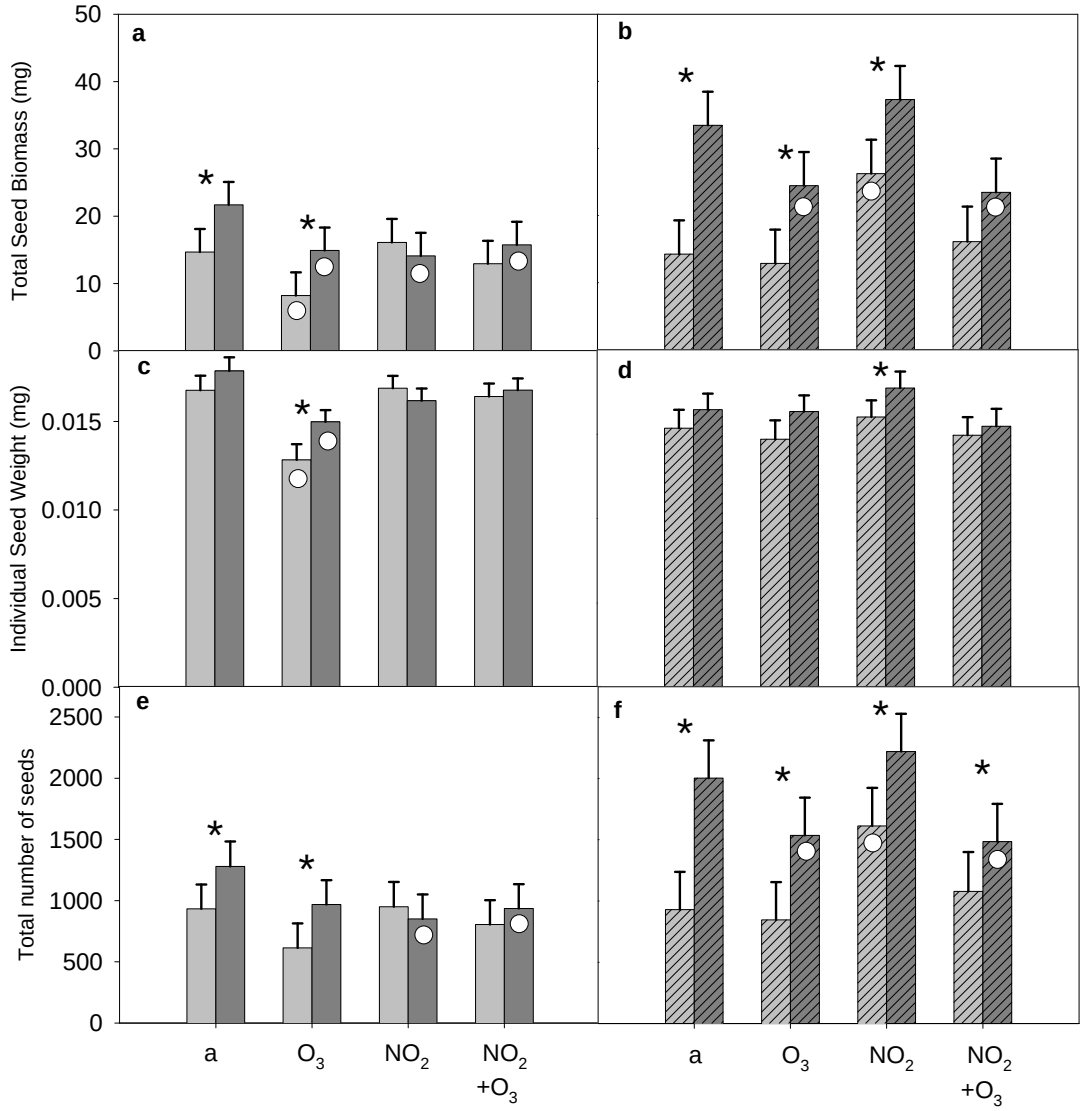

Figure 5.5: Total seed biomass (top panels), individual seed weight (middle panels), and total number of seeds produced (bottom panels). The low nitrogen treatment is shown with solid bars and the high nitrogen treatment with lined bars. Light and dark bars represent ambient and elevated CO_2, respectively. Asterisks indicate a significant difference between the ambient and elevated CO_2 groups. Open circles indicate a treatment that is significantly different from the ambient NO_2 + ambient O_3 group at the same CO_2 level (for example a difference between elevated NO_2 + elevated CO_2 and elevated CO_2 alone).

Ascorbate:

Arabidopsis thaliana individuals appear better able to increase their ascorbate production in respond to gaseous oxidants under elevated CO_2 and high soil NO_3^- (Fig

5.6 a+b). The combination $CO_2 + O_3$ caused an increase in ascorbate production regardless of soil NO_3^-, as did the application of NO_2 alone. When soil NO_3^- was high, NO_2 increased ascorbate production in both ambient and elevated CO_2 especially when O_3 was also elevated. This suggests that plant's ability to chemically respond to pollutant gases may be strongly dependant on the amount of C and N available to them.

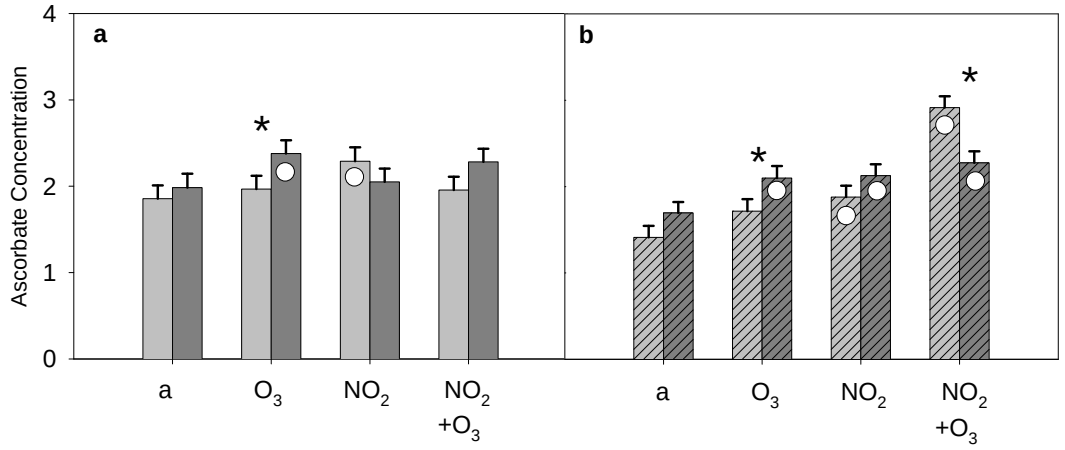

Figure 5.6: Ascorbate concentration (μmol asc g fw^{-1}) in leaves at day 33. The low nitrogen treatment is shown with solid bars and the high nitrogen treatment with lined bars. Light and dark bars represent ambient and elevated CO_2, respectively. Asterisks indicate a significant difference between the ambient and elevated CO_2 groups. Open circles indicate a treatment that is significantly different from the ambient NO_2 + ambient O_3 group at the same CO_2 level (for example a difference between elevated NO_2 + elevated CO_2 and elevated CO_2 alone).

DISCUSSION

The most important findings in this study are: 1) future concentrations of atmospheric CO_2 may cause elevated O_3 to delay bolting, flowering, and pod production even in species that have no response to O_3 under current CO_2 conditions and 2) although elevated CO_2 appears to increase reproductive output when applied as a single treatment, the addition of O_3 (both soil NO_3^- groups) and NO_2 (low soil NO_3^-) greatly reduce the effect. Studies that use only single treatments are likely to

underestimate the importance of O_3 concentration to the phenology of plants in the future and overestimate the reproductive benefit of elevated CO_2.

Flowering is a particularly important phenological stage because of the potential impacts on both the plant and on pollinators and herbivores that are dependent on the flowers (Hegland *et al.* 2009). In some systems there is close coupling between the timing of flower production and the emergence of certain insect species and an acceleration or delay of even 1-2 days (as seen here) could result in food shortages for the pollinator and reduced pollination of flowers. We found that under future CO_2 conditions, areas with clean air are likely to experience accelerated flowering while areas with O_3 pollution will experience delays. This will cause both spatial and temporal shifts in phenology which could cause even greater decoupling of plant-insect interactions.

The alterations in the timing of pod production due to elevated CO_2, and delays due to $CO_2 + O_3$ were not as great as the changes seen in flowering (and were completely eliminated when soil NO_3^- was high), which suggests that plants were able to (at least partially) recover from or compensate for their earlier accelerations and delays. Delays in pod production can be extremely detrimental if they result in plants failing to set seed before the end of the growing season (Ward and Kelly 2004). Since *A. thaliana* is largely self-fertilized, the timing of flowering may be less important to its survival than the timing of pod production, which may explain why pod production was less affected than flowering.

Accelerations in phenology due to elevated CO_2 (Springer and Ward 2007) and delays due elevated O_3 (Black *et al.* 2000) can often, though not always, be explained by changes in biomass. Changes in total biomass can also be strongly associated with changes in reproductive output (Navas *et al.* 1997). In this study we found that plant size at day 35 explained some of the patterns in phenology, size at 35 and 72 days both

contributed to total reproductive output, and ascorbate production provided some explanations for the differences in biomass.

Increased ascorbate can be indicative of both the oxidative stress that a plant is experiencing and its ability to respond to that stress (see review by Conklin and Barth 2004). Plants that are exposed to oxidative stress and do not increase their production of antioxidants are less likely to be able to remove the oxidants from the apoplast before they cause damage within the leaf and should therefore show more evidence of oxidative damage. Because O_3 is such a strong oxidant, it is likely that plants grown under elevated O_3 were experiencing oxidative stress, however, they did not increase their ascorbate production unless CO_2 (or NO_2 in the high soil NO_3^- group) was also elevated. This suggests that perhaps they did not have the extra resources required to increase ascorbate production. If the low ascorbate levels represent an inability to generate protective antioxidants, it may explain why the elevated O_3 + ambient CO_2 treatments had the lowest biomasses at day 35. In the low NO_3^- group these biomass declines may have been responsible for the delayed bolting, flowering, and pod production in the $CO_2 + O_3$ and $CO_2 + NO_2 + O_3$ treatments. Something different appears to be happening in the high soil NO_3^- groups because all the treatments under elevated CO_2 had similar biomasses at day 35 and yet there were delays in phenology caused by elevated O_3. Ascorbate was increased by exposure to O_3 and/or NO_2 in the elevated CO_2 treatment, but perhaps this was not enough to fully protect the plants from their exposure to gaseous pollutants. If these individuals were having spend extra resources to repair damaged tissue, they may have had less available to initiate reproduction even though they managed to maintain the same vegetative biomass.

Total reproductive output tracks very nicely with biomass at day 72. Elevated CO_2 increased total biomass, total seed biomass, and number of seeds regardless of soil NO_3^-, but elevated O_3 reduced the CO_2 effect (as did NO_2 when soil NO_3^- was

low), suggesting that even though ascorbate was increased, it was not enough keep up with the cumulative oxidative damage. These results reveal that even in cases where studies have shown increased reproduction under elevated CO_2, rising O_3 may reduce (high soil NO_3^-) or eliminate (low soil NO_3^-) the effect.

This is the first study to look at the effects of these components of atmospheric change and it is risky to generalize too broadly from a study using only one species. Reviews of the CO_2 (Springer and Ward 2007) and O_3 (Black *et al.* 2000) effects on plant phenology outline the wide range of responses seen in different species and we may expect to see the same variability in responses to multiple treatments. However, this study did reveal a number of multi-treatment effects that could not have been predicted from the responses to individual gases and are important for generating accurate predictions of future plant performance.

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REFERENCES

Ainsworth EA, Long SP (2005) What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO_2. New phytologist 165: 351–372

Black V.J., C.R. Black, J.A. Roberts, and C.A. Stewart. (2000). Impact of ozone on the reproductive development of plants. *New Phytologist*, 147:421-447

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

Coleman M.D., R.E. Dickerson, J.G. Isebrands, and D.F. Karnosky. (1995). Photosynthetic productivity of aspen clones varying in sensitivity to tropospheric ozone. *Tree Physiology* 15:585-592

Conklin, P. L. and C. Barth (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(8): 959-970.

Curtis PS, Vogel CS, Wang XZ, Pregitzer KS, Zak DR, Lussenhop J, Kubiske M, Teeri JA (2000) Gas exchange, leaf nitrogen, and growth efficiency of Populus tremuloides in a CO2-enriched atmosphere. Ecological Applications 10(1):3-17

Curtis PS, Wang X (1998) A meta-analysis of elevated CO_2 effects on woody plant mass, form, and physiology. Oecologia 113:299-313

Eller ASD, Sparks JP (2006) Predicting leaf-level fluxes of O-3 and NO2: the relative roles of diffusion and biochemical processes. Plant Cell and Environment 29:1742-1750

Fuhrer J. (2009). Ozone risk for crops and pastures in present and future climates. *Naturwissenschaften*, 96:173-194

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

Hegland S.J., A. Nielsen, A. Lazaro, A.L. Bjerknes, O. Totland. (2009). How does climate warming affect plant-pollinator interactions? *Ecology Letters* 12(2):184-195

IPCC (2007). Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change [Solomon, S., D. Qin, M. Manning, Z. Chen, M. Marquis, K.B. Averyt, M.Tignor and H.L. Miller (eds.)]. Cambridge

Isebrands JG, McDonald EP, Kruger E, Hendrey G, Pregitzer K, Percy K, Sôber J, Karnosky DF. 2001. Growth responses of *Populus tremuloides* clones to interacting carbon dioxide and tropospheric ozone. *Environmental Pollution* 115: 359–371

Jablonski L.M., X. Wang, P.S. Curtis. 2002. Plant reproduction under elevated CO_2 conditions: a meta-analysis of reports on 79 crop and wild species. *New Phytologist,*156: 9-26

Karnosky DF, Pregitzer KS, Zak DR, Kubiske ME, Hendrey GR, Weinstein D, Nosal M, Percy KE (2005) Scaling ozone responses of forest trees to the ecosystem level in a changing climate. Plant Cell and Environment 28:965-981

King JS, Pregitzer KS, Zak DR, Kubiske ME, Holmes WE (2001) Correlation of foliage and litter chemistry of sugar maple, *Acer saccharum*, as affected by elevated CO2 and varying N availability, and effects on decomposition. Oikos 94:403-416.

Navas, M. L., L. Sonie, J. Richarte, J. Roy (1997). The influence of elevated CO2 on species phenology, growth and reproduction in a Mediterranean old-field community. *Global Change Biology* 3(6): 523-530

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

Norby RJ, Wullschleger SD, Gunderson CA, Johnson DW, Ceulemans R (1999) Tree responses to rising CO_2 in field experiments: implications for the future forest. Plant, Cell and Environment 22:683-714

Rennenberg H, Gessler A (1999) Consequences of N deposition to forest ecosystems - Recent results and future research needs. Water Air and Soil Pollution 116:47-64

Rowland AJ, Drew MC, Wellburn AR (1987) Foliar Entry and Incorporation of Atmospheric Nitrogen-Dioxide into Barley Plants of Different Nitrogen Status. New Phytologist 107: 357-371.

Schemske D.W. (1977). Flowering phenology and seed set in claytonia-virginica (portulacaceae). *Bulletin of the Torrey Botanical Club* 104(3):254-263

Siegwolf RTW, Matyssek R, Saurer M, Maurer S, Gunthardt-Goerg MS, Schmutz P, Bucher JB (2001) Stable isotope analysis reveals differential effects of soil nitrogen and nitrogen dioxide on the water use efficiency in hybrid poplar leaves. *New Phytologist* 149:233-246

Skärby L., H. Ro-Poulsen, F.A.M. Wellburn, and L.J. Sheppard. (1998). Impacts of ozone on forests: a European perspective. *New Phytologist*, 139:109-122

Sparks JP (2009) Ecological ramifications of the direct foliar uptake of nitrogen. Oecologia 159:1-13

Springer C.J. and J.K. Ward (2007). Flowering time and elevated atmospheric CO_2. *New Phytologist,* 176: 243–255

Vallano DM, Sparks JP (2007) Quantifying foliar uptake of gaseous nitrogen dioxide using enriched foliar $\delta^{15}N$ values. New Phytologist 177:946-955

Ward, J. K. and J. K. Kelly (2004). Scaling up evolutionary responses to elevated CO2: lessons from Arabidopsis. *Ecology Letters* 7(5): 427-440.

Waser N.M. (1978). Competition for hummingbird pollination and sequential flowering in 2 colorado wildflowers. *Ecology*, 59(5):934-944

Zeevaart, A. J. (1976). Some Effects of Fumigating Plants for Short Periods with No2. *Environmental Pollution* 11(2): 97-108.