THE ROLE OF LEAF ANATOMY AND MORPHOLOGY IN DETERMINING OZONE SUSCEPTIBILITY IN CUT-LEAF CONEFLOWER

A Thesis
by
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ABSTRACT

THE ROLE OF LEAF ANATOMY AND MORPHOLOGY IN
DETERMINING OZONE SUSCEPTIBILITY IN CUTLEAF CONEFLOWER
(August 2011)

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Tropospheric ozone is one of the most important air pollutants globally and has deleterious impacts on both animal and plant health. The phytotoxic effects of ozone result in foliar injury known as stipple, decreases in photosynthesis and can reduce yield. Cutleaf coneflower (*Rudbeckia laciniata* var. *digitata*) is an ozone sensitive native wildflower growing within Great Smoky Mountains National Park (GRSM) where ozone pollution is often a problem. Individual coneflowers exhibit substantial variation in ozone sensitivity, yet the causes for this are not yet known.

The purpose of my study was to evaluate whether differences in leaf anatomy and morphology between sensitive and tolerant individuals of coneflower were responsible for this variation in ozone susceptibility. I hypothesized that sensitive individuals would have thinner leaf and mesophyll layers, greater internal airspace, greater exposed cell surface, and thinner cell walls.

In 2004, leaf samples were collected in June, July and August and from both sun and shade sites to account for seasonal and micro-habitat influences. This was a near record low ozone year with a SUM00 (total of hourly ozone values) of 202 ppm*hrs and a SUM60 (total of hourly values ≥ 60ppb) of 40.5 ppm*hrs. However, I was still able to obtain both sensitive and tolerant individuals for analysis. Micrographic measurements were made on thin prepared sections using light microscopy and included cuticle thickness, leaf and mesophyll thickness, internal airspace, exposed cell surface, cell area, live and dead cell number and cell wall thickness.

There were few effects on any parameters related to sensitivity and the majority of differences found were related to season and habitat effects. Adaxial cuticle thickness in August was greater for sensitive than tolerant plants and may have been a response to ozone exposure rather than a factor influencing sensitivity. Spongy mesophyll cell area was greater in sensitive plants but this did not correspond with greater exposed cell surface area and as such should not affect sensitivity. June and July cell death was significantly higher in tolerant plants which may be the result of programmed cell death induction, a containment strategy to limit the spread of an attack. In general, leaf anatomy and morphology did not differ between sensitive and tolerant plants, and therefore, these attributes do not appear to be the cause of the sensitivity differences in this species. Since previous research has not shown any physiological or biochemical differences between individuals of varying sensitivity, the causes may be reside at the molecular level.

ACKNOWLEDGEMENTS

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Introduction

Tropospheric ozone (O_3) is one of the most widespread and well documented air pollutants across the United States and globally (Krupa and Manning 1988, Jones et al. 2007, Ahlfors 2008). It is a secondary pollutant produced by photochemical reactions of nitrogen oxides (NO_x), carbon monoxide (CO) and volatile organic compounds (VOCs). Although it is a naturally occurring molecule in the troposphere resulting from stratospheric incursions, lightning and fires (Edwards et al. 2003), excessive anthropogenic emissions of NO_x and VOCs have led to substantial ambient concentration increases (Horowitz 2006).

Ozone's phytotoxic nature has been well documented for agricultural plants and forests trees (Mills et al. 2007, Karnosky et al. 2007). Exposure of vegetation to O_3 results in decreased growth and productivity, foliar injury and increased sensitivity to other biotic and/or abiotic stressors (US EPA 1996, Chappelka and Samuelson 1998). O_3 effects on agricultural crops, singularly or in combination with other air pollutants, have been reported to cause up to 90% yield loss in some instances (Heck et al. 1982), although such yield losses tend to be rare. It has been estimated that a 25% reduction in O_3 would increase agricultural yields by 1 to 2 billion dollars a year (Heck et al. 1982, Adams et al. 1989, Murphy et al. 1999).

Such economic losses are likely to increase in newly expanding industrial nations because of weak air pollution regulation (Fuhrer 2009, Marshall 2002) and could contribute to global food shortages (Chameides et al. 1989, 1994). In comparison to agriculture research, much less attention has been paid to the effect of O_3 on natural ecosystems and wild plants (Davison and Barnes 1998). While natural ecosystems bordering urban areas are certainly at risk for O_3 exposure and injury, long range transport processes can move O_3 and/or its precursors great distances and can therefore affect remote natural areas (Gregg et al. 2003). In fact, some rural ecosystems, such as mountainous habitats, may be more susceptible to increased air pollution as a result (Gregg et al. 2003, Mehlhorn and Wellburn 1987) because of a lack of O_3 scavengers in these areas.

Great Smoky Mountains National Park (GRSM) is one such area that is largely unpopulated and contains large expanses of natural vegetation, including one of the largest blocks of old-growth temperate deciduous forest in North America (NPS 2010a) . It is also an area that has seen dramatic increases of ambient O_3 levels (Chappelka et al. 2003, NPS 2010b) while most other parts of the country have experienced decreases, the result of the more stringent air quality standards promulgated by the Clean Air Act Amendments (US EPA 2001). Weather patterns transport air pollutant precursors from highly populated industrial cities to GRSM, forming O_3 along the way (Mueller 1994). This O_3 is also less likely to be readily broken down due to a lack of O_3-depleting atmospheric chemicals (Musselman and Minnick 2000). In order to protect

important ecosystems, GRSM has been designated as a Class I area by the Clean Air Act, which mandates that the area be monitored for any detrimental impacts from air pollution (US Department of Interior 1982).

GRSM is an ideal area to study O_3 pollution because of its geographic location which results in high exposures, large numbers of O_3-sensitive species, and because the results of such studies can be generalized over a large portion of the southern Appalachians. Neufeld et al. (1992) used open-top chambers to show that more than 30 native plant species in GRSM are O_3-sensitive. A plant is considered O_3 sensitive when it exhibits foliar injury at or near ambient O_3 concentrations in experimental chambers or in the field under ambient conditions (NPS 2003). A subset of the O_3 sensitive species has been proposed for use as bioindicator species because they allow for easy field identification of O_3 effects (Chappelka et al. 2003). For a plant to be considered a bioindicator it should have a wide regional distribution, be easy to identify and have easily recognizable O_3-induced foliar injury that occurs at or near ambient O_3 levels (NPS 2003).

One possible bioindicator species is cutleaf coneflower (*Rudbeckia laciniata)* referred to hereafter as simply coneflower. This species is a forest edge plant (Finkelstein et al. 2004), but also grows widely throughout 46 of the conterminous states (USDA Plants Database). At lower elevations, it is confined to habitats near streams, since it appears to be sensitive to water stress (Neufeld, unpubl. data). There are two varieties in GRSM: var. *laciniata* grows on and around Clingmans Dome, while var. *digitata* is found at Purchase Knob

(Cox and Urbatsch 1994). Variety *digitata* is similar to var. *laciniata*, but it is slightly smaller in stature. From personal observations (Neufeld) there seem to be no differences in the appearance of O_3-induced stipple between these two closely related varieties. Stipple consists of numerous small areas of the leaf blade that become pigmented after exposure to O3. My study, as reported in this thesis, investigated only the var. *digitata* growing at Purchase Knob.

Coneflower is considered to be highly sensitive to O_3 as determined from open-top chamber (Neufeld et al. 1992) and field studies in GRSM (Chappelka et al. 2003). However, considerable individual variation in O_3 sensitivity is apparent (Chappelka et al. 2003, Davison et al. 2003, Burkey et al. 2006) and plants can be divided into either sensitive or tolerant genotypes. While variation in O_3-sensitivity may detract from a plant's ability to be utilized as a bioindicator species (Kline et al. 2008, Martin et al. 2001) it also provides an opportunity to investigate the bases for this sensitivity difference. With respect to the coneflower, the results of a variety of studies have yet to determine any causal mechanisms that could account for the sensitivity differences noted in the field.

There are numerous mechanisms by which differential O_3-sensitivity may arise in plants. Differences in diffusion of O_3 into the leaf from the bulk air constitute the first mechanism in a pathway that ultimately ends up at the cell wall and plasma membrane. The movement of O_3 from the surrounding atmosphere to the stomata and finally into the plant cell can be understood by examining the diffusional resistances it encounters during the route (Gaastra 1959, Chameides 1989, Plöchl et al. 2000). Aerodynamic resistances are affected primarily by the

boundary layer over the habitat which itself is a function of wind speed, uniformity of canopy architecture and turbulence (Selldén and Pleijel 1995). However, unless sensitive and tolerant plants grow in very different areas, this aspect of the pathway will not contribute to sensitivity differences.

The leaf or laminar boundary layer is considered the next resistance in series which O_3 must traverse (Chameides 1989, Plöchl et al. 2000) and is controlled by some of the same factors that influence boundary layer depth at the canopy surface. The thickness of the leaf boundary layer varies according to leaf size, shape, pubescence and wind speed (Gates 1980, Aphalo and Jarvis 1993). However, at typical wind speeds this resistance is relatively small (Heath 1980, Chameides 1989) compared with stomatal resistances. Cuticular resistance to the diffusion of O_3 is quite high (Kerstiens and Lendzian 1989) and unless there were significant differences in cuticle structure between sensitive and tolerant plants, this portion of the deposition pathway will not be important for determining whole plant sensitivity to O_3.

Some researchers have linked O_3 sensitivity to a larger stomatal aperture, higher density, and stomatal conductance (Elkiey and Ormrod 1979, Barnes et al.1988, Pääkkönen et al. 1993, Pääkkönen et al. 1997, Ferdinand et al. 2000, Kollist et al. 2000, Paoletti and Grulke 2005, 2010, Lin et al. 2001, Alves et al. 2007, Guidi et al. 2010). Higher stomatal conductances (g_s) may be achieved in a variety of ways, including higher stomatal densities, large stomata, prolonged stomatal opening over the course of a day, and reduced sensitivity to stresses that cause stomatal closure (Grulke et al. 2007). Stomatal conductance is

species specific, influenced by leaf/plant age and mediated by a variety of concomitant environmental stimuli such as CO_2 concentration, soil moisture, vapor pressure deficit (VPD), gaseous pollutants, leaf temperature and irradiation (Schulze 1987, Chaves et al. 2003). Greater g_s leads to increased O_3 deposition and injury because there is a higher internal dose to the leaf (Heath 1980, Gerosa et al. 2003, Pleijel et al. 2006, Crous et al. 2006, Brosché et al. 2010).

Stomatal conductance has also been shown to have contradictory results with respect to O_3 sensitivity (Barnes et al. 1999). For example, increased O_3 sensitivity has been attributed in part to higher rates of g_s in new introductions of Greek wheat cultivars (*Triticum aestivum*). (Barnes et al. 1990, Velissarou et al. 1992, Pleijel et al. 2006). Studies of white clover clones (*Trifolium repens* L.cv. Regal) of known O_3 sensitivity showed that they had comparable physiological responses in clean air but had drastically different ones during O_3 exposure (Crous et al. 2006). The O_3 sensitive clone had a 30% decline in g_s while the tolerant clone had none (Crous et al. 2006). During O_3 exposure, these clones also differed significantly in photosynthetic capacity, carboxylation and electron transport rates which may make the sensitive clone more vulnerable to excess reactive oxygen species (ROS) generation, membrane damage and visible injury (Crous et al. 2006). With respect to coneflower, researchers found O_3-induced changes only after exposure but not before (Peoples 2005, Grulke et al. 2007).

Mesophyll and apoplastic resistances complete the series of diffusional resistances that may impact the entry of O_3 into the cell. The internal leaf architecture is the end result of both physiological adaptations to the local

physical environment (sun vs. shade, for example) as well as long-term evolutionary adaptations that maximize carbon uptake with respect to water loss (Cowan 1977). Palisade mesophyll cells are tightly packed columnar cells located in the upper portions of the leaf blade, and are the site of most photosynthesis that occurs while spongy mesophyll cells are more loosely spaced, irregularly shaped, and receive less light of varying quality as a result of incident light having to first pass through the palisade mesophyll cells located above these cells (Smith and Hughes 2009).

Palisade and spongy mesophyll layer thicknesses, thickness ratios and cell densities may play important roles in determining the sensitivity of plants to O_3 (Evans and von Caemmerer 1996, Ferdinand et al. 2000). Evans and Ting (1974) and Evans et al. (2009) found that the spongy mesophyll layer offers little resistance to gas exchange in comparison to the palisade layer. As a result, individuals with lower ratios of palisade to spongy mesophyll thickness would be expected to have higher diffusion of O_3 internally throughout the leaf, which could be postulated to lead to higher O_3-sensitivity. In fact, in a study involving black cherry (*Prunus serotina*), sensitive genotypes were found to have lower ratios of palisade to spongy mesophyll layer thickness, as well as lower total leaf thickness (Bennett et al. 1992, Fredericksen et al. 1995). Oksanen et al. (2001) found that palisade and spongy mesophyll thicknesses as well as their ratios were lower in O_3-sensitive aspen clones (*Populus tremuloides*) than in O_3-tolerant clones.

Mesophyll thickness and cellular density and distribution directly affect the amount of internal air space and intercellular exposed cell surface area which can be exposed to O_3. Individuals with decreased palisade and spongy mesophyll cell density as well as increased spongy mesophyll layer thickness would be expected to have greater internal air space and greater intercellular exposed surface area (Bennett et al. 1992).

Finally, O_3 must diffuse through the cell wall (apoplastic space) in order to reach the plasma membrane. Cell wall thickness can influence the residence time of O_3 by increasing the tortuosity of the path while also allowing increased opportunities for antioxidant scavenging, especially with extracellular ascorbic acid (ASC) (Chameides 1989, Moldau 1998, Plöchl et al. 2000) and other low molecular weight antioxidants. Plants with decreased cell wall thickness may be more impacted by O_3 due to decreased time for detoxification and lower active pools of reduced apoplastic ASC. Oksanen et al. (2001), for example, found that O_3 sensitive aspen clones (*Populus tremuloides*) had 8-16% thinner cell walls in comparison to O_3 tolerant clones.

If O_3 is able to pass through the cell wall without being detoxified, it encounters the plasma membrane, where it can oxidize the lipid bilayer, as well as form toxic byproducts, such as malondialdehyde (Heath 1978). After interacting with the membrane, it may or may not enter the cell, since it is so reactive, but it will activate the production of ROS such as superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), peroxyl radicals as well as other active O_2 species, all of which have high oxidative characteristics (Kangasjärvi et al. 1994,

Chernikova et al. 2000). A number of studies have focused on the correlation between ASC and O_3 sensitivity (Luwe and Heber 1995, Kollist et al. 2000). ASC is considered to be the "*first line of defense*" against O_3 (Moldau 1998, Plöchl et al. 2000, Turcsányi et al. 2000). Correlations with cellular ascorbic acid are not as pronounced as those with ASC (Burkey and Eason 2002).

ASC is manufactured within the cell and found within all sub-cellular compartments (Smirnoff 2000) but 1-10% of the total leaf ascorbate is transported to the apoplast (Noctor and Foyer 1998, Plöchl et al. 2000, Apel and Hirt 2004). Ascorbate functions in other cellular processes aside from its role as an antioxidant, such as in cell elongation, cross-linking of cell wall proteins and redox balance shift notification (Horemans et al. 2000, Pastori et al. 2003, Pignocchi and Foyer 2003). ASC can act as an immediate scavenger of oxidants external to the cell and has been shown in a variety of plant species to offer protection from O_3 injury (Burkey 1999, Turcsányi et al. 2000, Zheng et al. 2000, Burkey and Eason 2002, Burkey et al. 2003). However, ASC levels are essentially undetectable in *R. laciniata* and it does not function as an effective apoplastic antioxidant (Burkey et al. 2006). Burkey et al. (2006) also found that both sensitive and tolerant cutleaf coneflower leaves had a low ability to reduce dehydroascorbic acid (DHA) to ASC, meaning that much of the pool of ascorbic acid was in the oxidized state, and unavailable to detoxify O_3. This suggests that the basis for the difference in O_3 sensitivity in this species is most likely unrelated to characteristics of the cell wall.

O_3 exposure itself has been reported to induce changes in anatomical characteristics in leaves. Some studies have shown that ozone can cause changes in stomata density (Matyssek et al. 1991, Günthardt-Goerg et al. 1993) and function (Murata et al. 2001, Schroeder et al. 2001, Zhang and Outlaw 2001), as well as leaf and mesophyll layer thickness (Oksanen et al. 2001, 2005, Bussotti et al. 2005, Prozherina et al. 2003, Borowiak et al. 2010, Hartikainen et al. 2009). Most changes are thought to result from effects on leaf maturation (i.e., expansion, Bohler et al. 2010) which can change the ratio of cells that differentiate into guard cells (and hence stomata) and those that become normal epidermal cells. It can also decrease photosynthetic capacity and subsequent carbon allocation (Barnes 1972, Coleman et al. 1995), which can reduce leaf size and again alter the ratio of guard cell to epidermal cell densities. Therefore it was important for me to investigate the influence of seasonal exposure and age-related interactions by doing multiple sampling as the season and ozone exposures progressed.

The objective of this study was to investigate whether leaf anatomy and morphology influence O_3 sensitivity in the two sensitivity classes of coneflower. This study was approached from a histological perspective by measuring the anatomical and morphological characteristics of O_3 sensitive and tolerant plants. Figure 1 shows diagrammatically how leaf anatomy and morphology may affect the sensitivity of a leaf to O_3. The following hypotheses were made regarding the mechanisms by which individual coneflowers differ in their sensitivity to O_3: O_3-sensitive plants would be expected to have thinner leaves; a lower palisade to

spongy mesophyll thickness ratio; relatively more leaf volume occupied by spongy mesophyll; lower cell density and higher intercellular airspace volume; greater exposed cell surface area; and finally, thinner cell walls. These hypotheses were tested by examining micrographs of leaf cross-sections from plants growing at Purchase Knob in GRSM, and by comparing leaves through the season, and by comparing young (symptomless) and old (with O_3-induced stipple) leaves late in the season. To account for any possible changes resulting from growing conditions (specifically light), plants growing beneath a forest canopy were compared to those growing in full sun in an adjacent field.

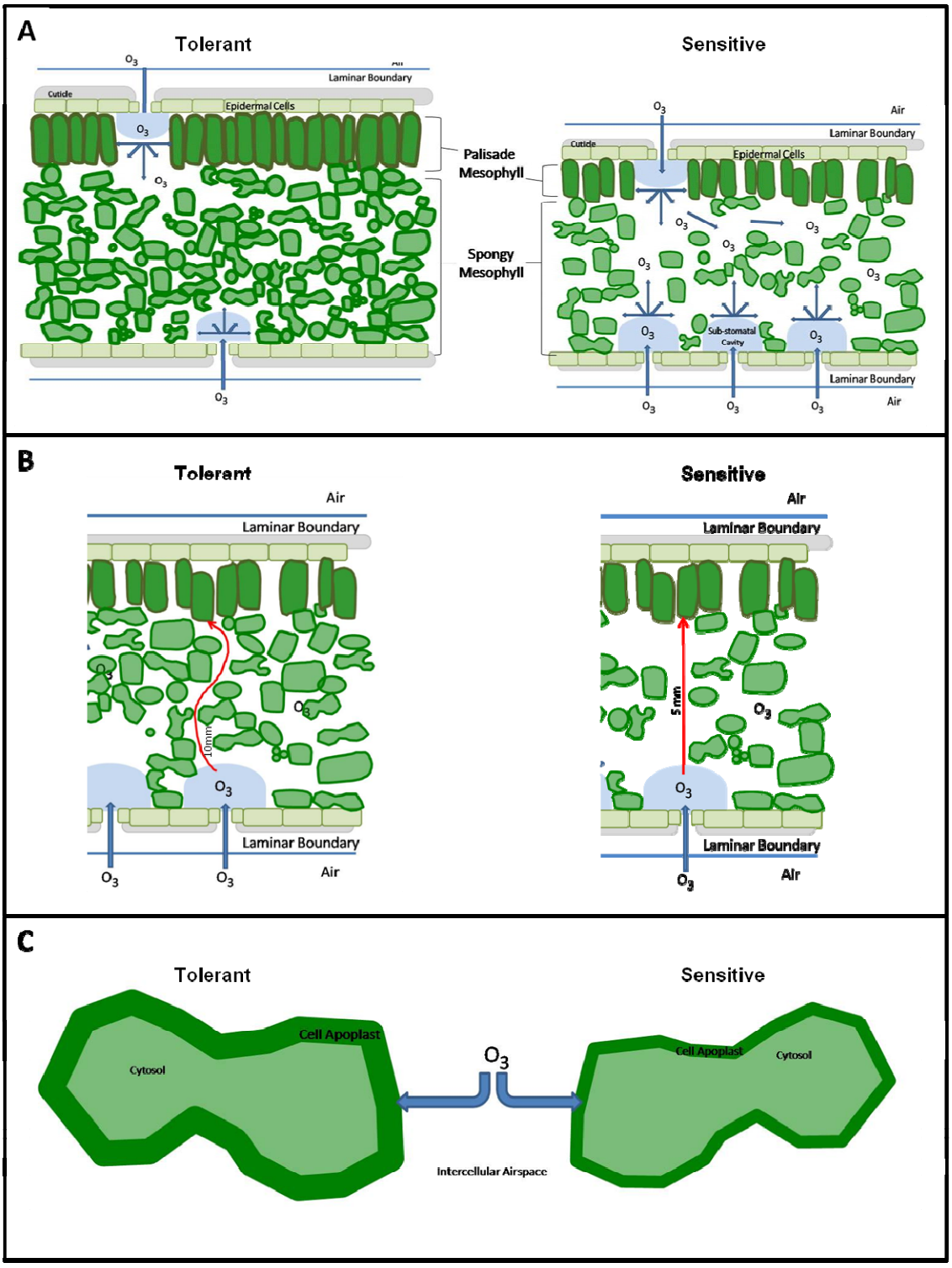

Fig 1. Hypothetical diagram of a leaf cross-section. A. Comparison of leaf cross-sections depicting postulated anatomical and morphological differences in sensitive and tolerant plants. B. Comparison of leaf cross-sections depicting the differences in mesophyll tortuosity through which a gas must traverse a leaf (tortuous path represented by the arrows crossing the spongy mesophyll). C. Comparison of cell wall thickness differences.

Materials and Methods

The field site was in Great Smoky Mountains National Park (GRSM) at the Appalachian Highlands Science Learning Center at Purchase Knob, near Waynesville, NC (35.588N, 83.074W, 1515 m asl). Sample collection occurred on June 15th, July 12th and August 4th of 2004 where two subplots were selected; one for shade plants growing under a forest canopy and one for sun plants growing in the field at the forest edge. Ten plants were selected for leaf collection and flagged per site for a total of 20 plants. Plants could not be chosen based on their sensitivity to O_3 early in the season (June or July) because of a lack of any visible foliar injury. The leaves collected in June and July were the fourth or fifth leaf, respectively, from the bottom of the plant to ensure that the leaves were of comparable ages and fully matured.

In August, foliar injury was visible and sensitivity of sample plants was determined. Within the field site, five of the sample plants were determined to be O_3-sensitive and five O_3-insensitive. Some plants were lost within the forest site due to trampling, resulting in only six plants remaining from the original ten selected. Of these, four were O_3-sensitive and two were O_3-insensitive. Leaves were rated for foliar stipple according to a modified classification scale of foliar injury (Chappelka et al. 2003).

Three leaf samples of each sensitive plant were collected from both sites, for a total of 27 samples. In O_3-sensitive plants, three leaves were chosen based on their rating of injury; injury class 1 (0% injury), injury class 3 (7-25%) and injury class 6 (76-100%). Leaves of class 1 were the youngest while leaves of class 6 were the oldest. In this case, age acted as a surrogate for O_3 exposure. To ensure analogous comparisons with sensitive plants two leaf samples (upper and lower) were taken from the insensitive plants in both sites, totaling 14 samples. Upper leaves on insensitive plants would be compared with injury class 1 leaves on sensitive plants, while lower leaves on insensitive plants would be compared with injury class 6 leaves on sensitive plants. Such comparisons make the assumption that each set of leaves on the two classes of plants had comparable O_3 exposures. All leaf samples were placed immediately on ice and transported back to Appalachian State University in Boone, NC, for histological preparation. Histological preparation methodologies were modified from Oksanen et al. (2001).

Leaf tissue samples, approximately 5 x 10 cm, were cut using a razor blade from the middle of the leaf next to the mid-vein but avoiding major veins. The samples were placed in test tubes and fixed overnight in a solution of 2.5% glutaraldehyde in 0.1 M Millonig's phosphate buffer under vacuum in order to remove any air that may have been within the leaf. The samples were washed in a 0.1 M phosphate buffer three times for 10 minutes each. All samples were dehydrated for 20 minutes in a serial dilution of acetone and water beginning with 30% acetone and ending with three rinses of 100% acetone. Following

dehydration, leaves were placed in an acetone and Spurr's epoxy resin (Spurr 1969) and serially diluted for 1-8 hours beginning with 25% epoxy resin and ending with three iterations of 100% epoxy resin. All leaves were spun in a centrifuge for 1 hour to ensure complete removal of all acetone and finally cured in a drying oven at 60°C overnight.

Leaves were prepared for light microscopy by making ~1 μm thick sections using glass knives with a Reichert-Jung Ultracut E Ultramicrotome. Sections were placed in individual water droplets on a glass slide and adhered to the slide by placement on a hot plate for five minutes. Sections were stained using Toluidine blue O for 15-30 seconds on the hot plate. Light micrographs were taken using a Jena-lumar microscope with bright field illumination at 12.5X magnification. Figure 2 includes representative micrographs of coneflower cross-sections. One micrograph of each leaf sample was analyzed using Image J software provided by the National Institutes of Health. Since Image J software measures numbers of pixels, it was necessary to convert pixels to micrometers using a stage micrometer scale. All micrographs analyzed with Image J were cropped to 200 μm wide prior to measurements. Additional micrographs were taken of August samples using an Olympus IX81 inverted microscope at 40x magnification and measurements of cell wall and cuticle thickness were determined using Olympus MicroSuite Biological Suite software (Melville, NY).

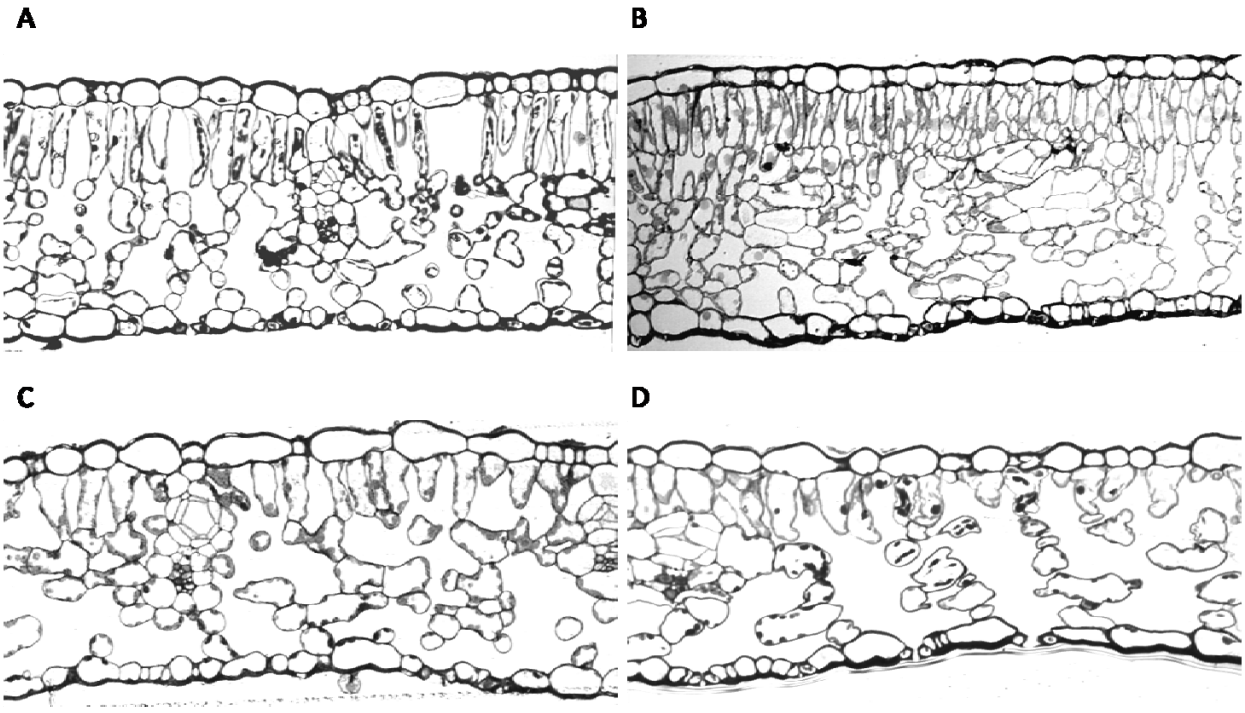

Fig 2. Representative micrographs of coneflower cross-sections (x12.5). A. Field Sensitive. B. Field Tolerant. C. Forest Sensitive. D. Forest Tolerant.

From the micrographs, measurements were made for each leaf sample which included measurements of total leaf cross-sectional thickness, palisade and spongy mesophyll cell layer thicknesses and ratios of palisade, spongy and total leaf layer thicknesses. Area measurements were taken of total cross-sectional airspace, palisade and spongy mesophyll layer airspaces as well as palisade and spongy mesophyll cell areas. Mesophyll tortuosity is a function of how internal leaf anatomy (cell density, area, and internal airspace) influences gas diffusion through the leaf interior and was determined by dividing cell area (μm^2) by mesophyll area (μm^2). This

definition serves as a surrogate for more traditional measures of tortuosity (citations) and assumes that the more cell area per unit leaf area, the more cell surfaces O_3 will encounter while diffusing through the leaf. If there were no cells, then O_3 could diffuse along a straight line path from one point in the leaf to another (low tortuosity), whereas if there is a large amount of cell surface area, the pathway across a given leaf section will be longer (i.e., higher tortuosity). The numbers of palisade, spongy and total cells (palisade + spongy), dead palisade, spongy and total mesophyll cells were tallied for each cross-section. Cell death was determined by visible collapse of a cell within a cross-section. Percent total cell number to dead cells was also determined. Leaf measurement details can be found in Table 1. Stomatal density measurements were performed on additional plants collected from the same populations, but only from the plants grown in the field (see Grulke et al. 2007).

Statistical Analysis

Stomatal density was analyzed using separate two-sample two-way *t*-tests for the adaxial and abaxial leaf surfaces. June and July samples were analyzed for site and month effects using one way ANOVA and Tukey's multiple range test. Main effects were analyzed in August using two and three-way factorial ANOVAs and Tukey's multiple range test. Differences were considered significant at $p \leq 0.05$. In most instances, data did not require transformation prior to analysis**.**

Table 1. Morphological measurements including labels and measurement methodology.

Anatomical Measurement	Anatomical Label	Measurement Method
Adaxial Cuticle Thickness incl. Epidermal Cell Wall Thickness (µm)	Adaxial Cuticle Thickness (µm)	Average of 5 measurements of 5 cells
Abaxial Cuticle Thickness incl. Epidermal Cell Wall Thickness (µm)	Abaxial Cuticle Thickness (µm)	Average of 5 measurements of 5 cells
Palisade Mesophyll Layer Width (µm)	Palisade Thickness (µm)	Average of 5 measurements
Spongy Mesophyll Layer Width (µm)	Spongy Thickness (µm)	Average of 5 measurements
Total Leaf Width (µm)	Leaf Thickness (µm)	Average of 5 measurements
Palisade Mesophyll Layer Width Relative to Total Leaf Thickness (%)	% Palisade Mesophyll	Ratio
Spongy Mesophyll Layer Width Relative to Total Leaf Thickness (%)	% Spongy Mesophyll	Ratio
Palisade Mesophyll Layer Width Relative to Spongy Mesophyll Layer Thickness (%)	% Palisade to Spongy Mesophyll	Ratio
Palisade Mesophyll Layer Airspace (μm^2)	Leaf Airspace (mm^2)	Sum of all air space
Spongy Mesophyll Layer Airspace (μm^2)	Palisade Airspace (mm^2)	Sum of all air space
Total Leaf Airspace (mm^2)	Spongy Airspace (mm2)	Sum of all air space
Palisade Mesophyll Layer Exposed Cell Surface (µm)	Palisade Exposed Cell Surface (µm)	Sum of all exposed cell surface
Spongy Mesophyll Layer Exposed Cell Surface (µm)	Spongy Exposed Cell Surface (µm)	Sum of all exposed cell surface
Total Leaf Exposed Cell Surface (µm)	Total Leaf Exposed Cell Surface (µm)	Palisade + Spongy Exposed Cell Surface
Palisade Cell Wall Thickness (µm)	Palisade Cell Wall (µm)	Average of 5 measurements of 5 cells
Spongy Cell Wall Thickness (µm)	Spongy Cell Wall (µm)	Average of 5 measurements of 5 cells
Palisade Mesophyll Layer Cell Area (μm^2)	Palisade Cell Area (μm^2)	Average of 5 cells
Spongy Mesophyll Layer Cell Area (μm^2)	Spongy Cell Area (μm^2)	Average of 5 cells
Total Leaf Cell Area (μm^2)	Total Leaf Cell Area (μm^2)	Average of 5 cells
Palisade Mesophyll Area (μm^2)	Palisade Mesophyll Area (μm^2)	Palisade Layer Width * 200µm
Spongy Mesophyll Area (μm^2)	Spongy Mesophyll Area (μm^2)	Spongy Layer Width * 200µm
Palisade Layer Tortuosity	Palisade Layer Tortuosity	Palisade Cell Area/Palisade Mesophyll Area
Spongy Layer Tortuosity	Spongy Layer Tortuosity	Spongy Cell Area/Spongy Mesophyll Area
Palisade Cell Number	Palisade Cells	Tally of all palisade cells
Spongy Cell Number	Spongy Cells	Tally of all spongy cells
Total Cell Number	Total Cells	Palisade + Spongy Cells
Palisade Mesophyll Dead Cell Number	Dead Palisade Cells	Tally of all dead palisade cells
Spongy Mesophyll Dead Cell Number	Dead Spongy Cells	Tally of all dead spongy cells
Total Lead Dead Cell Number	Total Dead Cells	Palisade + Spongy Dead Cells
Palisade Mesophyll Dead Cell Number Relative to Total Leaf Dead Cell Number (%)	% Palisade Death	Ratio
Spongy Mesophyll Dead Cell Number Relative to Total Leaf Dead Cell Number (%)	% Spongy Death	Ratio
Palisade Mesophyll Dead Cell Number Relative to Spongy Mesophyll Dead Cell Number (%)	% Palisade to Spongy Death	Ratio

Results

Cuticle Thickness and Stomatal Density

Adaxial cuticle thickness was greater ($p = 0.0276$) in sensitive than insensitive plants in August but no differences were found for abaxial cuticle thickness (Figure 3, Table 2). For all August analyses, leaf age never was significant for any parameter, and hence will not be discussed further. As previously determined by Grulke et al. (2007) during that same growing season, stomatal density was not significantly different between O_3 sensitive and insensitive genotypes for either adaxial ($p = 0.2562$) or abaxial surfaces ($p = 0.9357$). More than 90% of the stomata are located on the abaxial surface (Grulke et al. 2007).

Leaf Thickness (Mesophyll Layers and Total Leaf Thickness)

For each sampling month, field plants generally had thicker leaves. In June, all leaf thicknesses were greater in the field site than the forest site: palisade mesophyll ($p = 0.0122$), spongy mesophyll ($p = 0.0024$), and total leaf ($p = 0.0009$, Figure 4A, Table 3A). In July, only palisade mesophyll was greater in field plants than forest plants ($p = 0.0150$, Figure 4A, Table 3A). For June and July, there were no significant differences for either month or sensitivity (Figure 4B, Table 3B,).

In August, palisade, spongy and total leaf had thicknesses that were again greater in field plants than forest plants ($p > 0.0001$, Figure 4C, Table 3C). Percent palisade, % spongy, and their ratio were not significant for any site, month or sensitivity (Figure 5A-5C, Table 3A-3D).

Internal Leaf Airspace

Spongy mesophyll was affected the most with respect to internal airspace. In June, it was greater in field plants than forest plants, ($p = 0.0461$, Figure 6A, Table 4A). Spongy mesophyll ($p = 0.0085$) and total leaf ($p = 0.0401$) airspace were both greater in June than July in field plants (Figure 6B, Table 4B). In August, there were no significant differences for either site or sensitivity (Figure 6C, Table 4C). When all months were included (June-August) I found that again spongy mesophyll airspace in June was the largest ($p = 0.0159$, Table 4D).

Cell Area

There were no monthly or site differences in cell area in June and July (Figure 7A, Table 5A) nor were there sensitivity differences (Figure 7B, Table 5B). Sensitive plants had greater spongy cell area than those of tolerant plants (p 0.0416, Figure 7C, Table 5C). In August, forest plants had greater spongy cell area then field plants ($p = 0.0383$, Figure 7D, Table 5C). Full seasonal (June-August) analysis found no significant differences for either cell area (Table 5D).

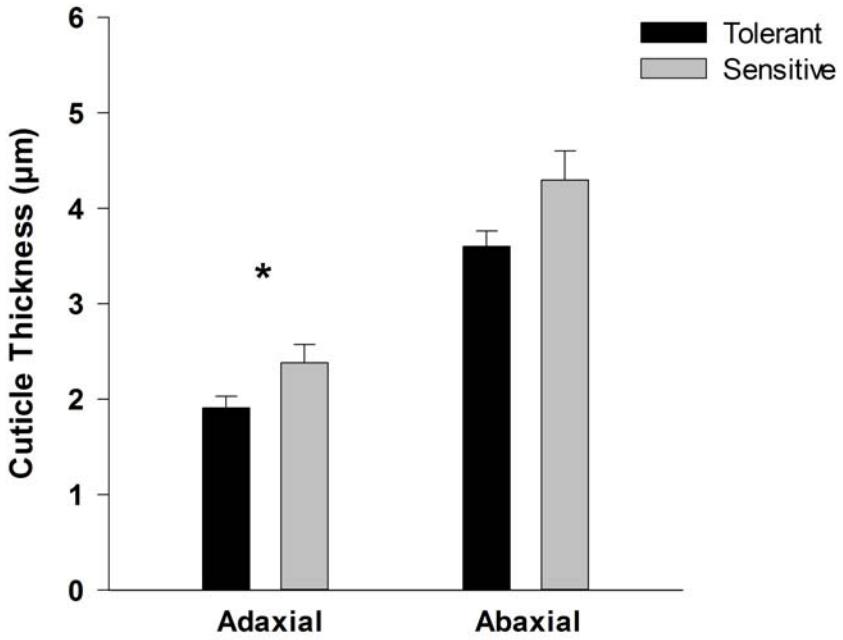

Figure 3. Late season cuticle thickness. Bars represent means ± se, N = 24. Asterisks indicate differences between sites.

Table 2. Late season (August only) Analysis of cuticle thickness.

		Adaxial		Abaxial	
Source	df	F	p	F	p
Site	1	3.03	0.1010	0.24	0.6338
Sens	1	5.88	**0.0276**	3.10	0.0975
Site*Sens	1	2.10	0.1670	0.14	0.7111
Leaf	1	3.83	0.0680	0.50	0.4899
Site*Leaf	1	1.03	0.3249	0.07	0.7942
Sens*Leaf	1	2.28	0.1508	0.72	0.4080
Site*Sens*Leaf	1	1.21	0.2876	0.79	0.3874

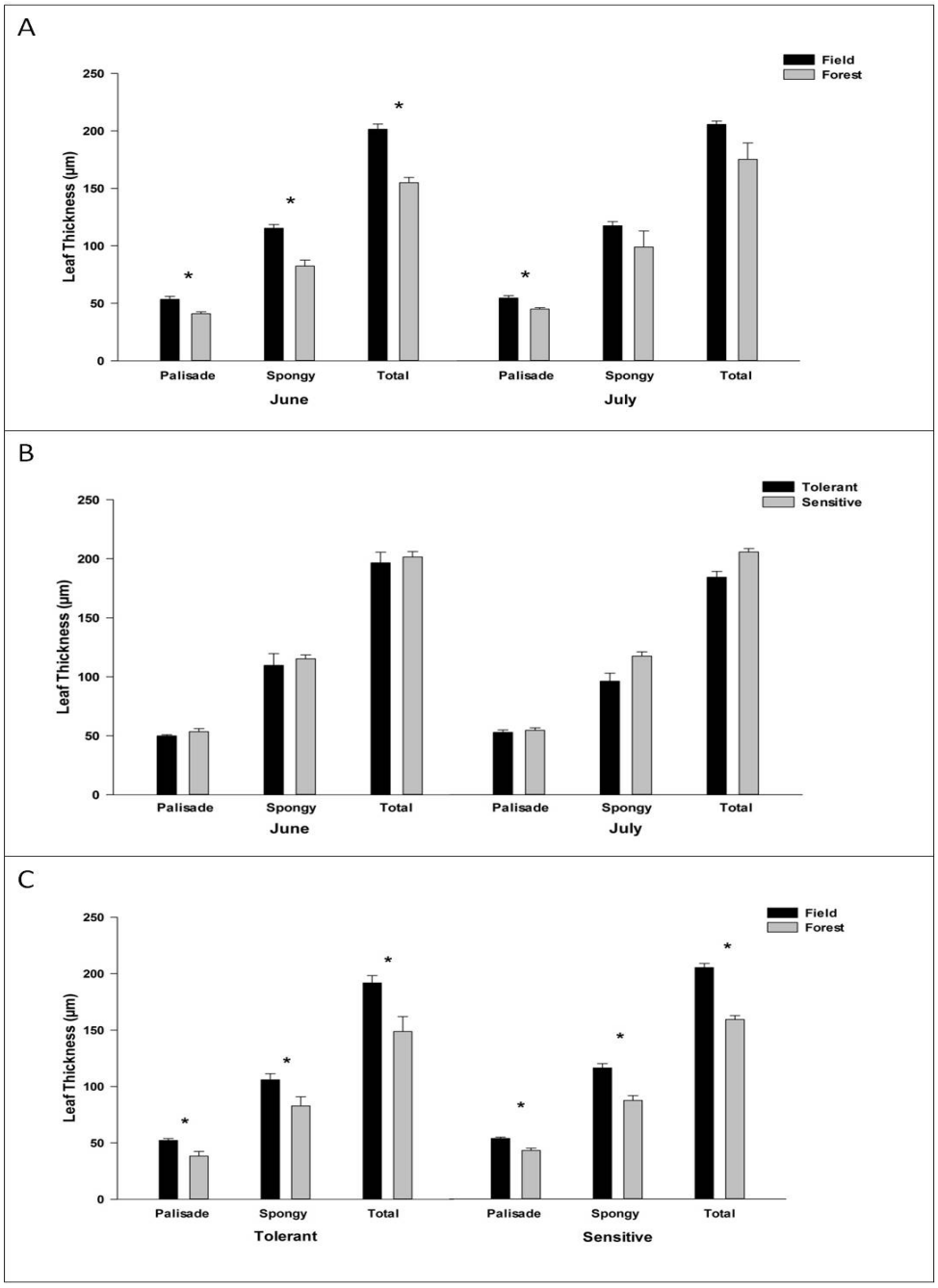

Figure 4. Leaf Thickness. A. Early and Mid-season (June and July) comparison of site and month for sensitive plants. B. Early and Mid-season (June and July) comparison of sensitivity in field plants only. C. Late season (August) comparison of sensitivity. Bars represent means $\pm$ se, N = 30 for A, 19 for B and 41 for (C). Asterisks indicate differences between sites.

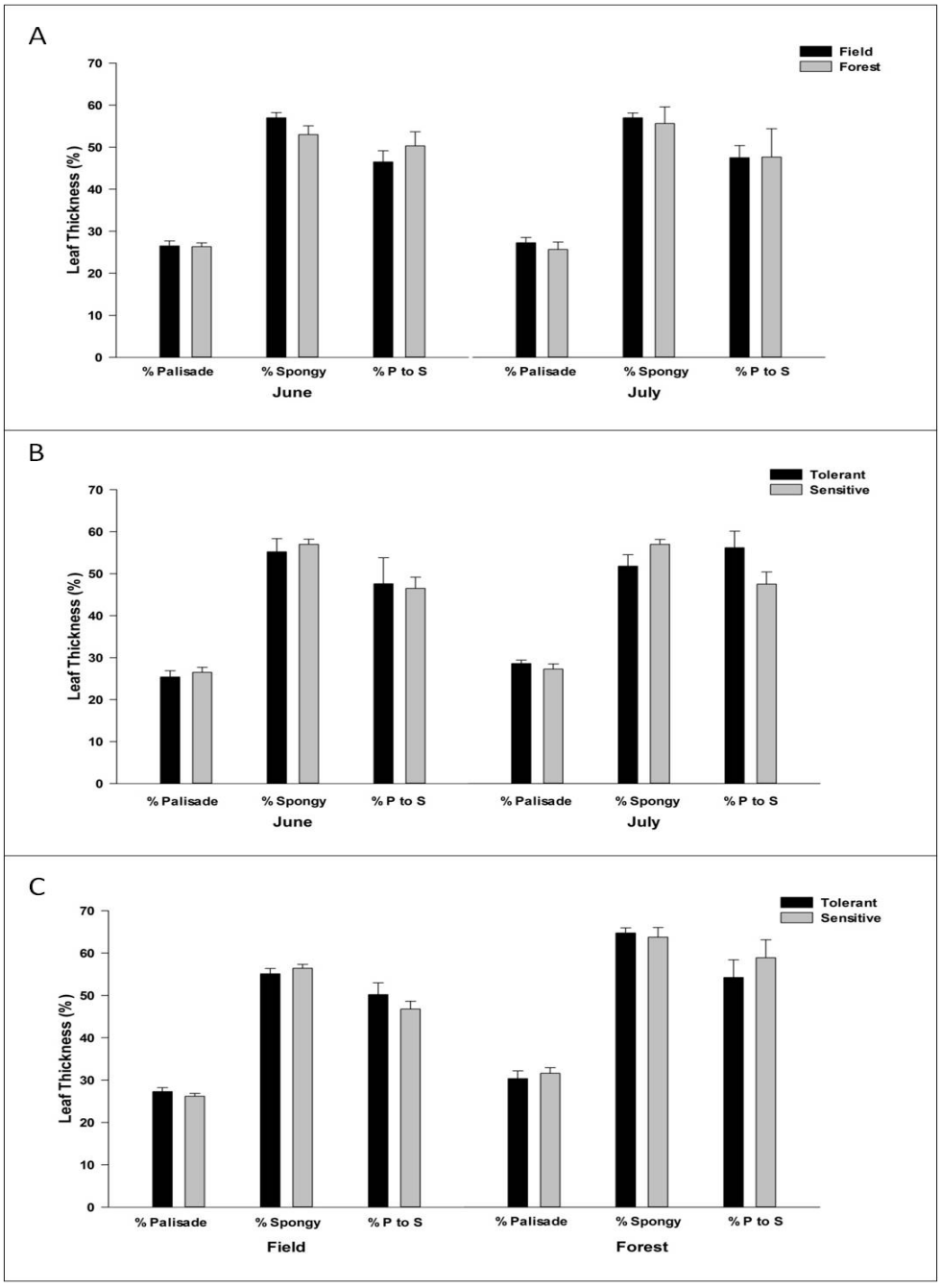

Figure 5. Proportional Leaf Thickness. A. Early and Mid-season (June and July) comparison of site and month for sensitive plants. B. Early and Mid-season (June and July) comparison of sensitivity in field plants only. C. Late season (August) comparison of sensitivity. Bars represent means $\pm$ se, N = 30 for A, 19 for B and 41 for C.

Table 3. Early, Mid and Late Season Analysis of Leaf and Mesophyll Thickness.

A. Early and mid-season (June and July only) comparison of site and month for sensitive plants.

			PL		SL		TL		%PL		%SL		%PL to SL	
Source	Site or Month	df	F	p	F	p	F	p	F	p	F	p	F	p
Site	June	1	14.72	**0.0122**	31.87	**0.0024**	49.96	**0.0009**	0.01	0.9206	3.12	0.1377	0.83	0.4039
	July	1	11.37	**0.0150**	2.24	0.1949	6.06	0.0572	0.57	0.4827	0.14	0.7234	0.00	0.9809
Month	Field	1	0.12	0.7418	0.22	0.6577	0.62	0.4611	0.19	0.6791	0.00	1.000	0.06	0.8080
	Forest	1	4.36	0.1050	1.26	0.3245	1.86	0.2439	0.11	0.7523	0.36	0.5811	0.13	0.7415

B. Early and mid-season (June and July) comparison of sensitivity for field plants.

		PL		SL		TL		%PL		%SL		%PL to SL	
Source	df	F	p	F	p	F	p	F	p	F	p	F	p
Sens	1	1.75	0.2019	3.53	0.0766	3.98	0.0614	0.01	0.9256	2.30	0.1471	1.26	0.2755
Month	1	1.49	0.2507	0.36	0.7055	0.25	0.7790	2.15	0.1457	0.28	0.7581	0.87	0.4358
Sens*Month	1	0.21	0.6531	1.19	0.2897	1.56	0.2273	0.86	0.3656	0.54	0.4712	0.76	0.3946

C. Late season (August only) comparison of sensitivity for sites and all leaf ages.

		PL		SL		TL		%PL		%SL		%PL to SL	
Source	df	F	p	F	p	F	p	F	p	F	p	F	p
Site	1	30.30	**<0.0001**	27.95	**<0.0001**	58.39	**<0.0001**	0.05	0.8183	0.78	0.3829	0.27	0.6045
Sens	1	1.92	0.1757	2.21	0.1473	3.22	0.0823	0.00	0.9837	0.40	0.5303	0.06	0.8030
Site*Sens	1	0.00	0.9561	0.01	0.9440	0.04	0.8493	0.05	0.8225	0.01	0.9184	0.03	0.8585
Leaf	2	1.12	0.3391	1.83	0.1174	1.67	0.2050	0.00	0.9984	1.84	0.1765	0.59	0.5587
Site*Leaf	2	1.88	0.1692	0.89	0.4223	0.11	0.8961	2.04	0.1475	2.15	0.1285	3.20	0.0547
Sens*Leaf	1	0.15	0.7006	1.41	0.2448	0.08	0.7831	0.20	0.6540	2.45	0.1278	0.73	0.3988
Site*Sens*Leaf	1	0.00	0.9561	0.43	0.5175	0.30	0.5852	0.20	0.6540	0.15	0.7038	0.25	0.6233

D. Entire season comparison (June to August) for plants (leaf 4 only) in the field only.

		PL		SL		TL		%PL		%SL		%PL to SL	
Source	df	F	p	F	p	F	p	F	p	F	p	F	p
Sens	1	1.75	0.2019	3.53	0.0766	3.98	0.0614	0.01	0.9256	2.30	.01471	1.26	0.2755
Month	2	1.49	0.2507	0.36	0.7055	0.25	0.7790	2.15	0.1457	0.28	0.7581	0.87	0.4358
Sens*Month	1	0.21	0.6531	1.19	0.2897	1.56	0.2273	0.86	0.3656	0.54	0.4712	0.76	0.3946

Mesophyll Layer Tortuosity

In August, forest plants had greater mesophyll tortuosity than field plants ($p = 0.0098$, Figure 8, Table 6A). When all months were included, there were no other significant differences found in June or July for mesophyll tortuosity (Table 6B).

Exposed Cell Surface

Exposed cell surfaces had no significant differences in June or July for site (Figure 9A, Table 7A) or sensitivity (Figure 9B, Table 7B). In August, spongy mesophyll exposed cell surface was greater in forest pants than in field plants ($p = 0.0295$, Figure 9C, Table 7C). Full seasonal (June-August) analysis found no significant differences (Table 7D).

Cell Wall Thickness

For palisade cell wall thickness in August (Figure 10A, Table 8A), there was a significant site x sensitivity interaction ($p = 0.0356$). Tolerant plants in the field had thicker palisade cell walls than tolerant plants in the forest, but there was no difference for the sensitive plants between sites. There was also a leaf x sensitivity interaction ($p = 0.0155$). For sensitive plants in the field, older leaves (leaf 1) had thicker palisade cell walls than younger leaves (leaf 7). Spongy cell wall thickness did not show any significant site, sensitivity or leaf age effects (Figure 10B, Table 8A).

Cell Number and Dead Cells

Field plants tended to have greater cell numbers per unit area measured than those of forest plants. June field plants had greater cell numbers in spongy mesophyll ($p = 0.0086$, Figure 11A, Table 9A) and for total leaf cell number ($p = 0.0008$, Figure 11A, Table 9A). There were no significant differences for cell numbers between sensitivity classes in either June or July (Figure 11B, Table 9B). In August, both the spongy mesophyll cell number ($p = 0.0005$) and total leaf cell number ($p = 0.0007$) were greater in field plants than forest (Figure 10C, Table 9C). Full seasonal (June-August) analysis found no significant differences (Table 9D).

Variation in number of dead cells was relatively high in June and July (Figure 12A), and partially as a consequence of this there were no significant differences detected between sites for either month. Tolerant plants in June and July had a greater number of dead cells in the palisade mesophyll ($p = 0.0368$), the total leaf ($p = 0.0319$), and % total cell death ($p = 0.0392$, Figure 12B, 13B, Table 10B). Late season analysis found no significant differences for site, sensitivity or leaf age (Figure 12C, 13C, Table 10C). When comparing sensitivity of just field plants, tolerant plants had a greater amount of cell death in palisade cell death ($p = 0.0214$), % palisade cell death ($p = 0.0417$), total leaf cell death ($p = 0.0268$), and % total leaf cell death ($p = 0.0271$, Table 10D).

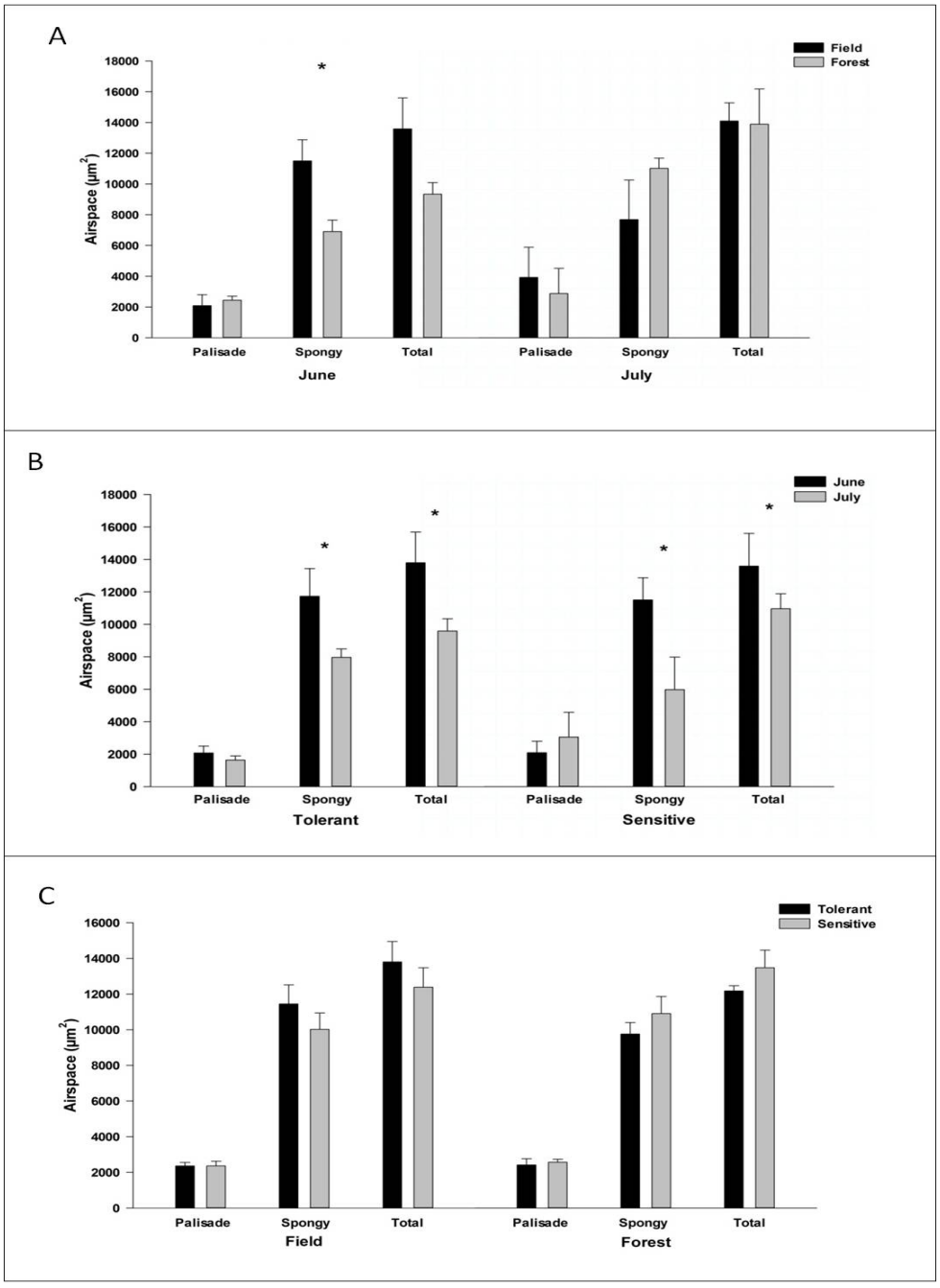

Figure 6. Internal Leaf Airspace. A. Early and Mid-season (June and July) comparison of site and month for sensitive plants. B. Early and Mid-season (June and July) comparison of sensitivity in field plants only. C. Late season (August) comparison of sensitivity. Bars represent means $\pm$ se, N = 15 for A, 56 for B and 41 for C. Asterisks indicate differences between sites (A) and months (B).

Table 4. Early, Mid and Late Season Analysis of Internal Airspace.

A. Early and mid-season (June and July only) comparison of site and month for sensitive plants.

			PA		SA		TA	
Source	Site or Month	df	F	p	F	p	F	p
Site	June	1	0.17	0.6996	6.96	**0.0461**	2.96	0.1459
	July	1	0.16	0.7067	0.84	0.3938	0.0.1	0.9338
Month	Field	1	0.28	0.6155	4.63	0.0685	1.40	0.2821
	Forest	1	0.13	0.7406	1.28	0.3216	0.57	0.4912

B. Early and Mid-season (June and July) comparison of Sensitivity for Field plants.

		PA		SA		TA	
Source	df	F	p	F	p	F	p
Sens	1	0.63	0.4384	0.52	0.4832	0.15	0.7056
Month	1	0.09	0.7741	9.16	**0.0085**	5.12	**0.0401**
Sens*Month	1	0.61	0.4482	0.33	0.5765	0.28	0.6072

C. Late Season (August only) comparison of sensitivity for sites and all leaf ages.

		PA		SA		TA	
Source	df	F	p	F	p	F	p
Site	1	0.41	0.5278	0.00	0.9782	0.05	0.8330
Sens	1	0.04	0.8514	0.29	0.5946	0.03	0.8545
Site*Sens	1	0.05	0.7919	1.33	0.2583	1.38	0.2484
Leaf	2	0.01	0.9896	2.99	0.0650	0.65	0.2093
Site*Leaf	2	0.29	0.7486	0.62	0.5463	0.34	0.7127
Sens*Leaf	1	0.51	0.4784	0.47	0.4995	0.20	0.6580
Site*Sens*Leaf	1	0.59	0.4474	1.57	0.2200	0.82	0.3717

D. Entire season comparison (June to August) for plants (leaf 4 only) in the field only.

		PA		SA		TA	
Source	df	F	p	F	p	F	p
Sens	1	0.71	0.4107	0.54	0.4701	0.13	0.7212
Month	2	0.10	0.9018	5.19	**0.0159**	2.26	0.1329
Sens*Month	1	0.68	0.4206	0.34	0.5652	0.24	0.6270

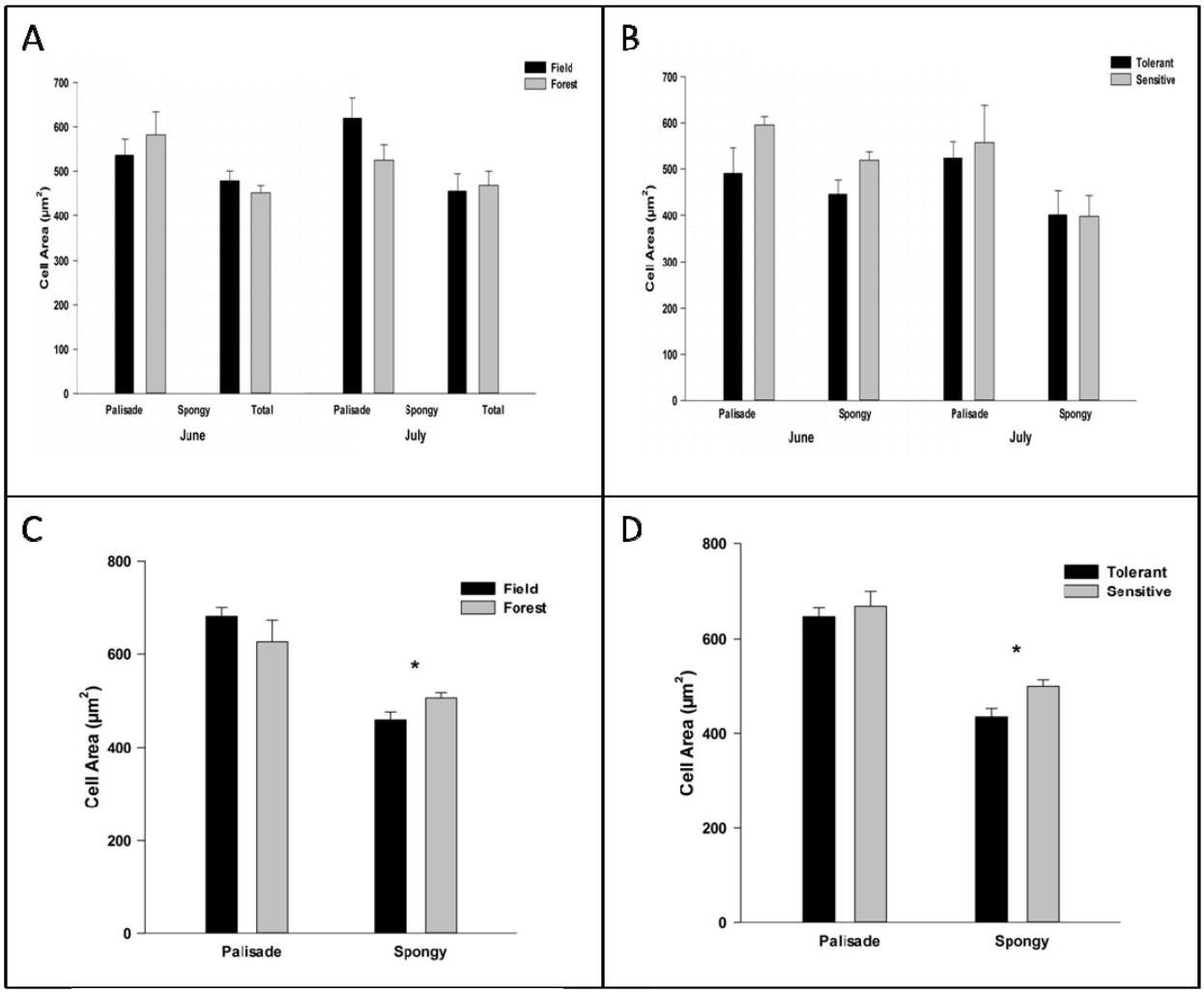

Figure 7. Mesophyll Cell Area. A. Early and Mid-season (June and July) comparison of site and month for sensitive plants. B. Early and Mid-season (June and July) comparison of sensitivity in field plants only. C. Late season (August) comparison of sensitivity. D. Late season (August) comparison of sensitivity for site and all leaf ages. Bars represent means $\pm$ se, N = 27 for A, 19 for B and 41 for C and D. Asterisks indicate differences between (C) sites and (D) sensitivity.

Table 5. Early, Mid and Late Season Analysis of Cell Area.

A. Early and mid-season (June and July only) comparison of site and month for sensitive plants.

			P Cell Area		S Cell Area	
Source	Site or Month	df	F	p	F	p
Site	June	1	0.50	0.4953	0.56	0.4712
	July	1	1.43	0.2542	0.04	0.8465
Month	Field	1	1.84	0.1921	0.25	0.6264
	Forest	1	0.86	0.3895	0.22	0.6561

B. Early and Mid-season (June and July) comparison of Sensitivity for Field plants.

		P Cell Area		S Cell Area	
Source	df	F	p	F	p
Site	1	0.00	0.9854	0.00	0.9573
Sens	1	0.12	0.7372	0.00	0.9445
Site*Sens	1	2.75	0.1108	0.68	0.4187

C. Late Season (August only) comparison of sensitivity for sites and all leaf ages.

		P Cell Area		S Cell Area	
Source	df	F	p	F	p
Site	1	1.65	0.2079	4.68	**0.0383**
Sens	1	0.44	0.5135	4.52	**0.0416**
Site*Sens	1	0.07	0.7920	1.50	0.2296
Leaf	2	0.15	0.8592	0.00	0.9604
Site*Leaf	2	0.63	0.5408	0.76	0.4749
Sens*Leaf	1	0.00	0.9632	0.71	0.4067
Site*Sens*Leaf	1	0.22	0.6412	1.49	0.2318

D. Entire season comparison (June to August) for plants (leaf 4 only) in the field only.

		P Cell Area		S Cell Area	
Source	df	F	p	F	p
Sens	1	1.52	0.2320	0.62	0.4403
Month	2	1.85	0.1848	0.24	0.7868
Sens*Month	1	0.33	0.5749	0.74	0.3997

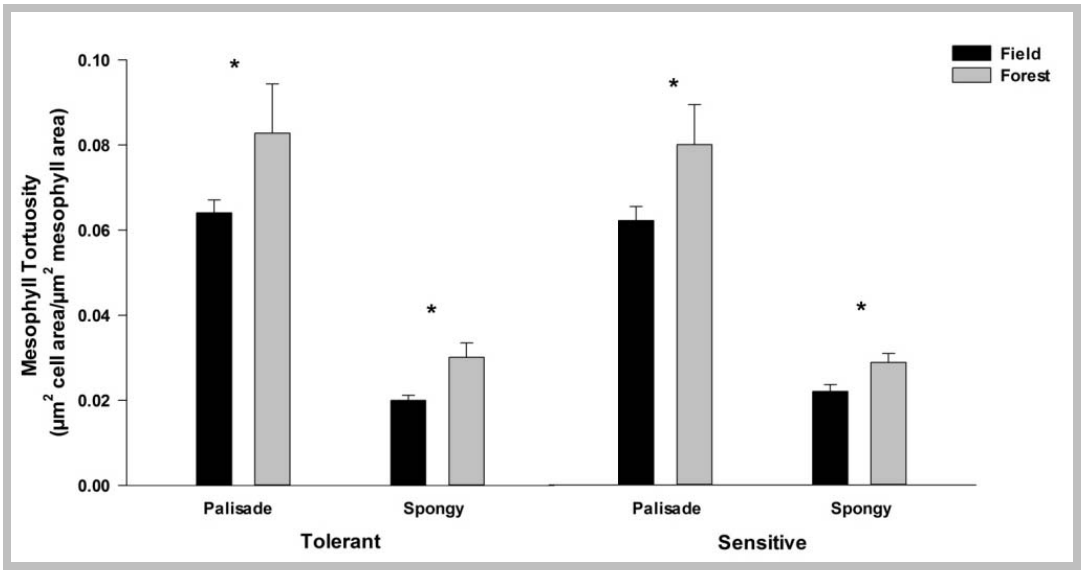

Figure 8. Mesophyll Tortuosity. Late season (August) comparison of sensitivity for site and all leaf ages. Bars represent means ± se, N = 32. Asterisks indicate differences between sites.

Table 6. Early, Mid and Late Season Analysis of Mesophyll Tortuosity

A. Late season comparison of site and sensitivity. Only those plants from August were analyzed.

		Palisade Tortuosity		Spongy Tortuosity	
Source	df	F	p	F	p
Site	1	7.69	**0.0098**	17.42	**0.0003**
Sens	1	0.13	0.7213	0.04	0.8515
Site*Sens	1	0.00	0.9507	0.66	0.4224

B. Entire season comparison (June to August) for plants (leaf 4 only) in the field only.

		Palisade Tortuosity		Spongy Tortuosity	
Source	df	F	p	F	p
Sens	1	0.01	0.9305	0.89	0.3577
Month	2	3.02	0.0726	0.89	0.4265
Sens*Month	1	0.20	0.6635	0.02	0.8942

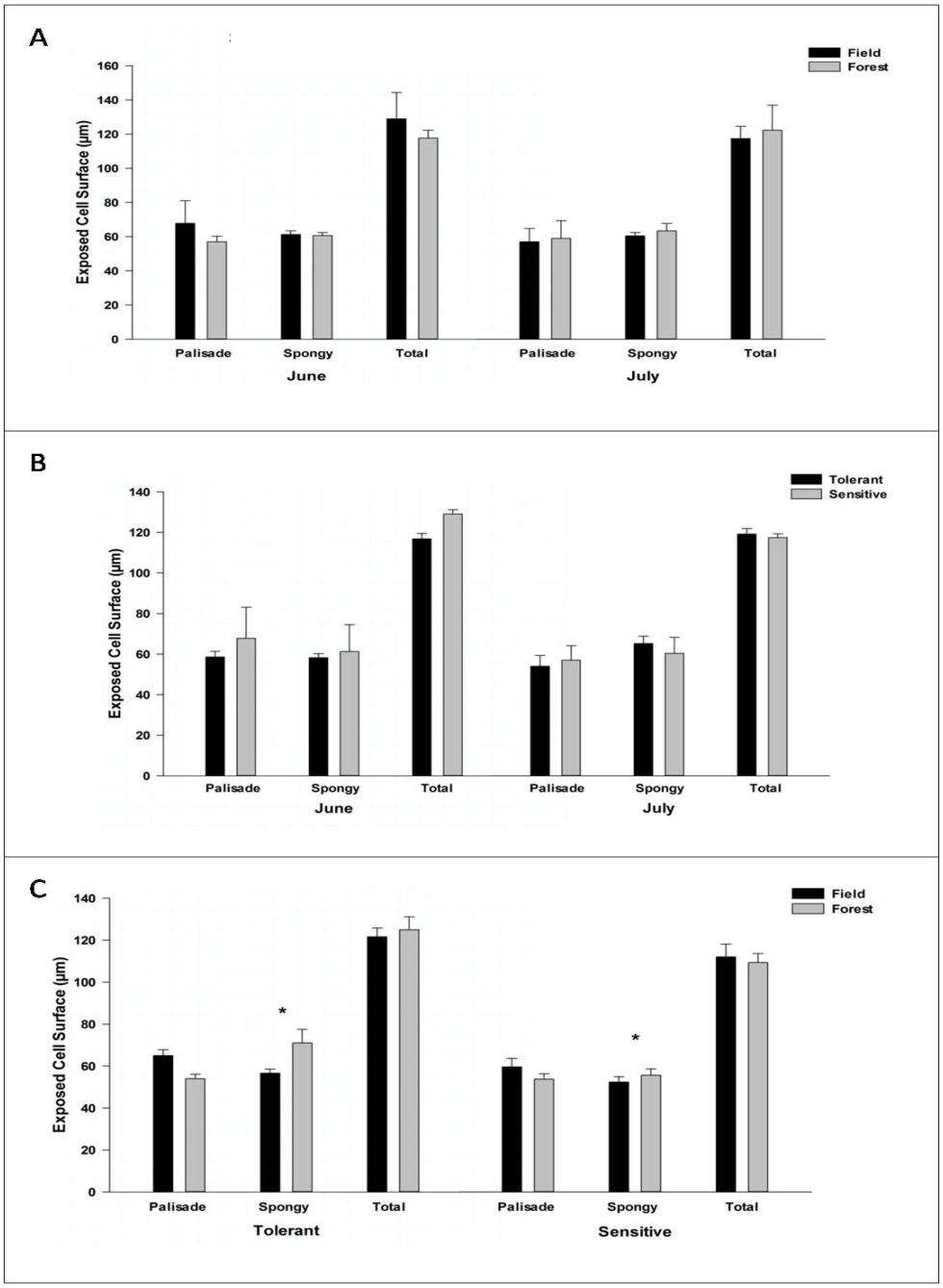

Figure 9. Exposed Cell Surface. A. Early and Mid-season (June and July) comparison of site and month for sensitive plants. B. Early and Mid-season (June and July) comparison of sensitivity in field plants only. C. Late season (August) comparison of sensitivity. Bars represent means $\pm$ se, N = 15 for A, 19 for B and 41 for C. Asterisks indicate differences between sites.

Table 7. Early, Mid and Late Season Analysis of Exposed Cell Surface.

A. Early and mid-season (June and July only) comparison of site and month for sensitive plants.

			PE		SE		TE	
Source	Site or Month	df	F	p	F	p	F	p
Site	June	1	0.02	0.8812	0.51	0.5020	0.12	0.7415
	July	1	0.45	0.5308	0.04	0.8499	0.37	0.5686
Month	Field	1	0.54	0.4873	0.09	0.7789	0.54	0.4845
	Forest	1	0.03	0.8615	0.32	0.5992	0.09	0.7761

B. Early and Mid-season (June and July) comparison of Sensitivity for Field plants.

			PE		SE		TE
Source	df	F value	F	p	F	p	F
Sens	1	0.69	0.4195	0.15	0.7029	0.42	0.5276
Month	1	1.10	0.3109	1.87	0.1920	0.33	0.5758
Sens*Month	1	0.18	0.6803	3.04	0.1016	0.76	0.3978

C. Late Season (August only) comparison of sensitivity for sites and all leaf ages.

			PE		SE		TE
Source	df	F value	F	p	F	p	F
Site	1	2.84	0.1020	5.21	**0.0295**	0.00	0.9509
Sens	1	1.95	0.1723	0.76	0.3895	0.20	0.6556
Site*Sens	1	0.04	0.8369	1.77	0.1926	0.28	0.6036
Leaf	2	0.62	0.5459	0.99	0.3820	0.12	0.8849
Site*Leaf	2	0.33	0.7249	0.04	0.9583	0.11	0.8971
Sens*Leaf	1	0.06	0.8150	1.24	0.2737	0.49	0.4910
Site*Sens*Leaf	1	0.06	0.8150	1.19	0.2840	0.15	0.7008

D. Entire season comparison (June to August) for plants (leaf 4 only) in the field only.

		PE		SE		TE	
Source	df	F	p	F	p	F	p
Sens	1	0.59	0.4507	0.06	0.8074	0.26	0.6148
Month	2	0.47	0.6300	0.45	0.6565	0.11	0.8968
Sens*Month	1	0.15	0.7010	1.23	0.2814	0.47	0.4993

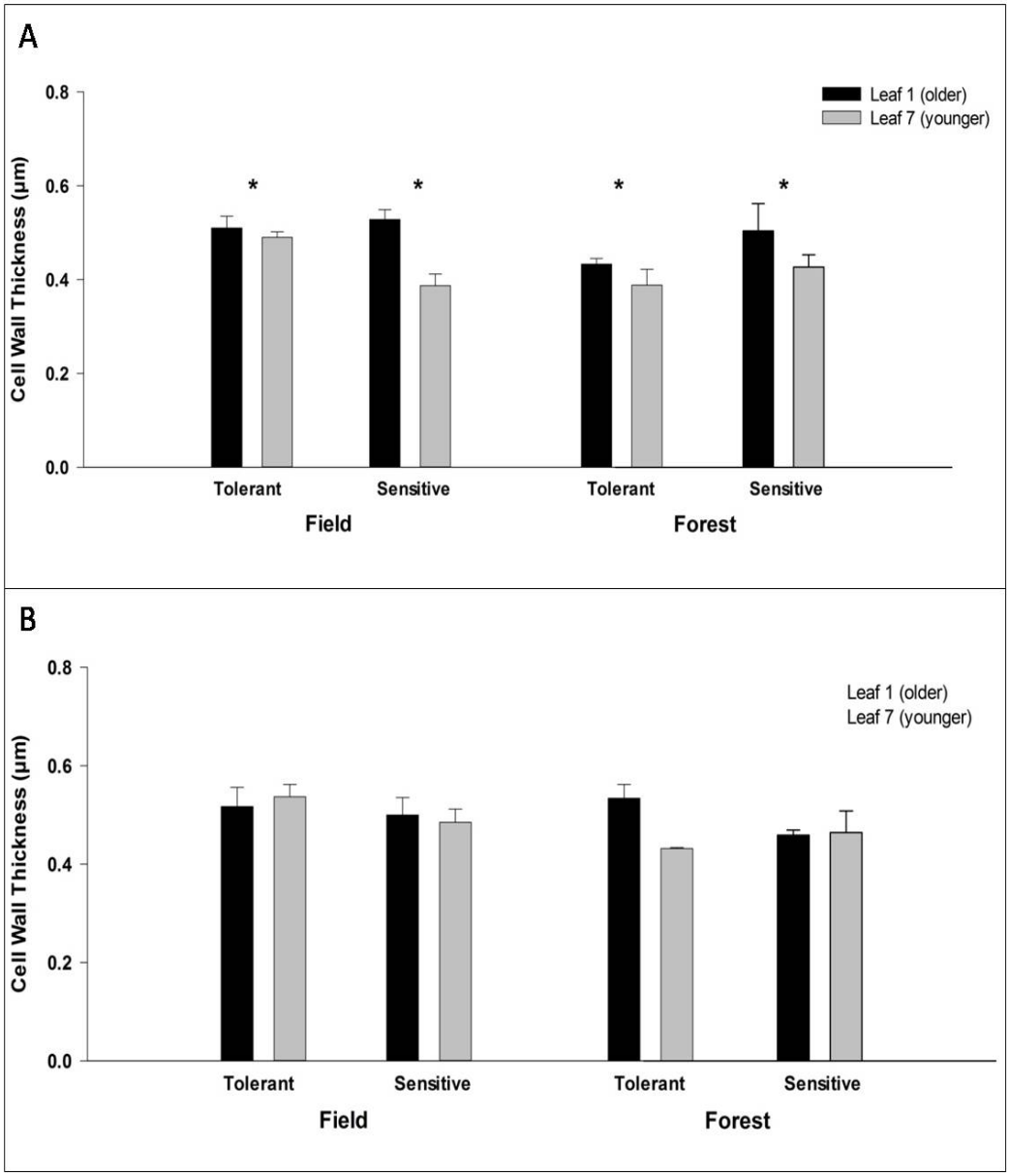

Figure 10. Late season (August) analysis of site, sensitivity and leaf age. A. Palisade cell wall thickness (µm). B. Spongy cell wall thickness (µm). Bars represent means $\pm$ se, N = 24. Asterisks indicate differences between leaf age.

Table 8. Late Season Analysis of Cell Wall Thickness.

A. Comparison of late season (August) cell wall thickness.

		Palisade Cell Wall		Spongy Cell Wall	
Source	Df	F	P	F	p
Site	1	3.67	0.0734	3.01	0.1018
Sens	1	0.04	0.8357	1.44	0.2469
Site* Sens	1	5.26	**0.0356**	0.07	0.7909
Leaf	1	9.74	**0.0066**	0.28	0.6058
Site*Leaf	1	0.14	0.7124	0.78	0.3911
Sens*Leaf	1	3.31	0.0878	0.25	0.6261
Site*Sens*Leaf	1	1.12	0.3049	2.09	0.1676

B. Comparison of late season palisade cell wall thickness site and sensitivity interaction.

Source		df	F	p
	Sens	1	4.45	0.0611
Field	Leaf	1	9.95	**0.0102**
	Sens*Leaf	1	8.49	**0.0155**
	Sens	1	1.59	0.2543
Forest	Leaf	1	1.91	0.2165
	Sens*Leaf	1	0.15	0.7160
	Sens	1	15.31	0.0045
Tolerant	Leaf	1	1.46	0.2607
	Sens*Leaf	1	0.40	0.5424
	Sens	1	0.05	0.8247
Sensitive	Leaf	1	8.92	**0.0174**
	Sens*Leaf	1	0.75	0.4108

C. Comparison of late season (August) palisade cell wall thickness sensitivity and leaf interaction.

Source	Leaf or Sensitivity	df	F	p
Tolerant	Leaf 1	1	0.24	0.6429
Sensitive	Leaf 7	1	17.13	**0.0144**
Leaf 1	Tolerant	1	0.25	0.6368
Leaf 7	Sensitive	1	17.57	**0.0086**

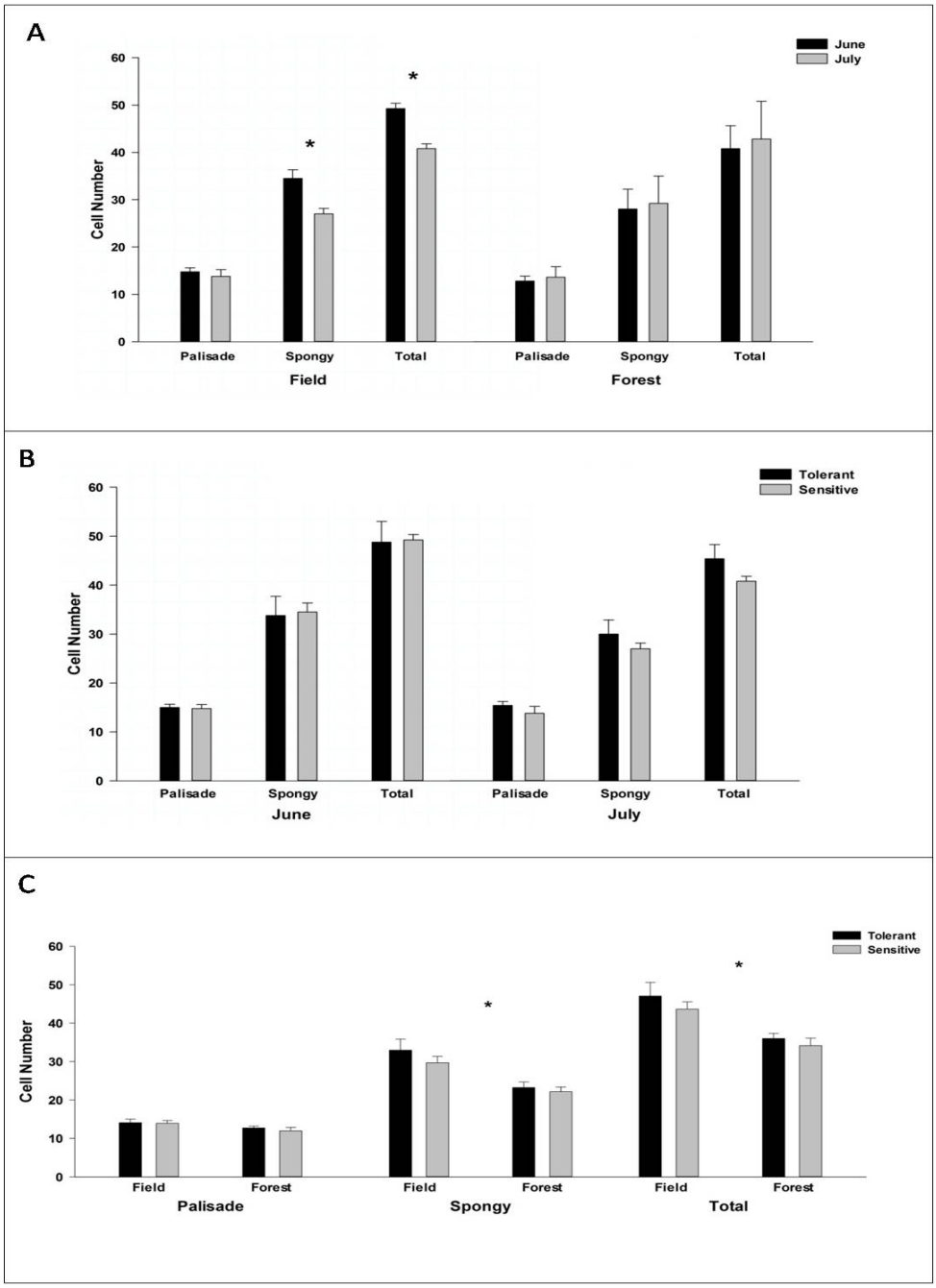

Figure 11. Cell Number. A. Early and Mid-season (June and July) comparison of site and month for sensitive plants. B. Early and Mid-season (June and July) comparison of sensitivity in field plants only. C. Late season (August) comparison of sensitivity. Bars represent means $\pm$ se, N = 15 for A, 19 for B and 41 for C. Asterisks indicate differences between (A) months and (C) sites.

Table 9. Early, Mid and Late Season Analysis of Cell Number.

A. Early and mid-season (June and July only) comparison of site and month for sensitive plants.

			P Cell #		S Cell #		T Cell #	
Source	Site or Month	df	F	p	F	p	F	p
Site	June	1	0.06	0.8165	0.02	0.8865	0.01	0.9292
	July	1	0.98	0.3501	0.92	0.3360	2.23	0.1739
Month	Field	1	0.30	0.6037	13.06	**0.0086**	31.24	**0.0008**
	Forest	1	0.11	0.7619	0.03	0.8755	0.05	0.8412

B. Early and Mid-season (June and July) comparison of sensitivity for field plants.

		P Cell #		S Cell #		T Cell #	
Source	df	F	p	F	p	F	p
Sens	1	0.88	0.3635	0.17	0.6845	0.54	0.4723
Month	1	0.08	0.7843	4.14	0.0599	4.41	0.0530
Sens*Month	1	0.47	0.5044	0.44	0.5152	0.80	0.3848

C. Late Season (August only) comparison of sensitivity for sites and all leaf ages.

		P Cell #		S Cell #		T Cell #	
Source	df	F	p	F	p	F	p
Site	1	3.45	0.0727	15.12	**0.0005**	14.04	**0.0007**
Sens	1	0.02	0.8919	1.15	0.2911	0.82	0.3726
Site*Sens	1	0.02	0.8919	0.68	0.4148	0.50	0.4848
Leaf	2	0.62	0.5428	0.22	0.8044	0.02	0.9795
Site*Leaf	2	0.88	0.3565	0.68	0.4148	0.97	0.3329
Sens*Leaf	1	0.68	0.5153	0.81	0.4557	0.99	0.3818
Site*Sens*Leaf	1	0.44	0.5128	0.26	0.6138	0.40	0.5294

D. Entire season comparison (June to August) for plants (leaf 4 only) in the field only.

		P Cell #		S Cell #		T Cell #	
Source	df	F	p	F	p	F	p
Sens	1	0.72	0.4052	0.14	0.7108	0.44	0.5152
Month	2	0.03	0.9676	1.92	0.1744	1.98	0.1655
Sens*Month	1	0.39	0.5418	0.37	0.5520	0.65	0.4297

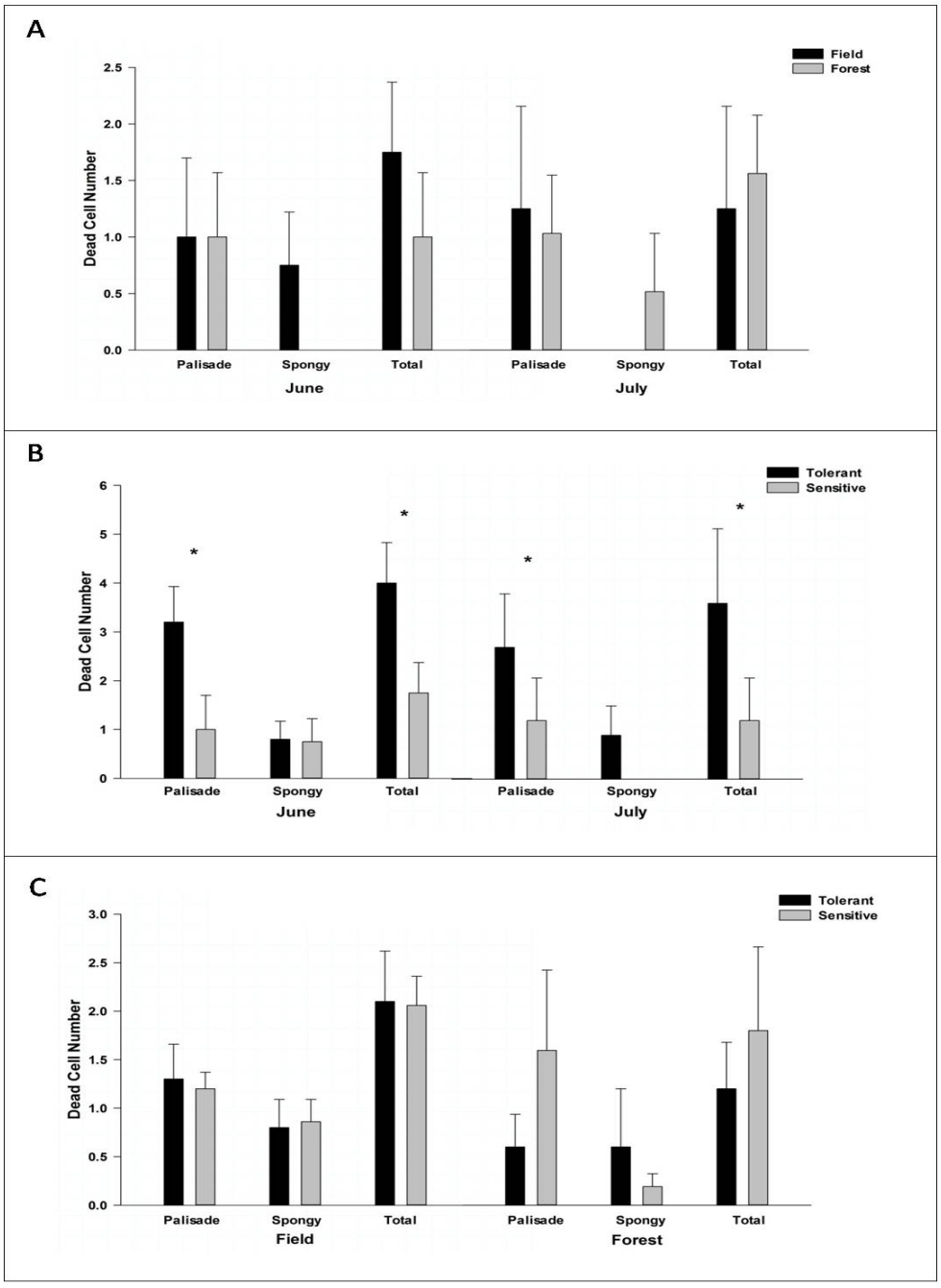

Figure 12. Cell Death. A. Early and Mid-season (June and July) comparison of site and month for sensitive plants. B. Early and Mid-season (June and July) comparison of sensitivity in field plants only. C. Late season (August) comparison of sensitivity. Bars represent means $\pm$ se, N = 15 for A, 19 for B and 41 for C. Asterisks indicate differences between sensitivity.

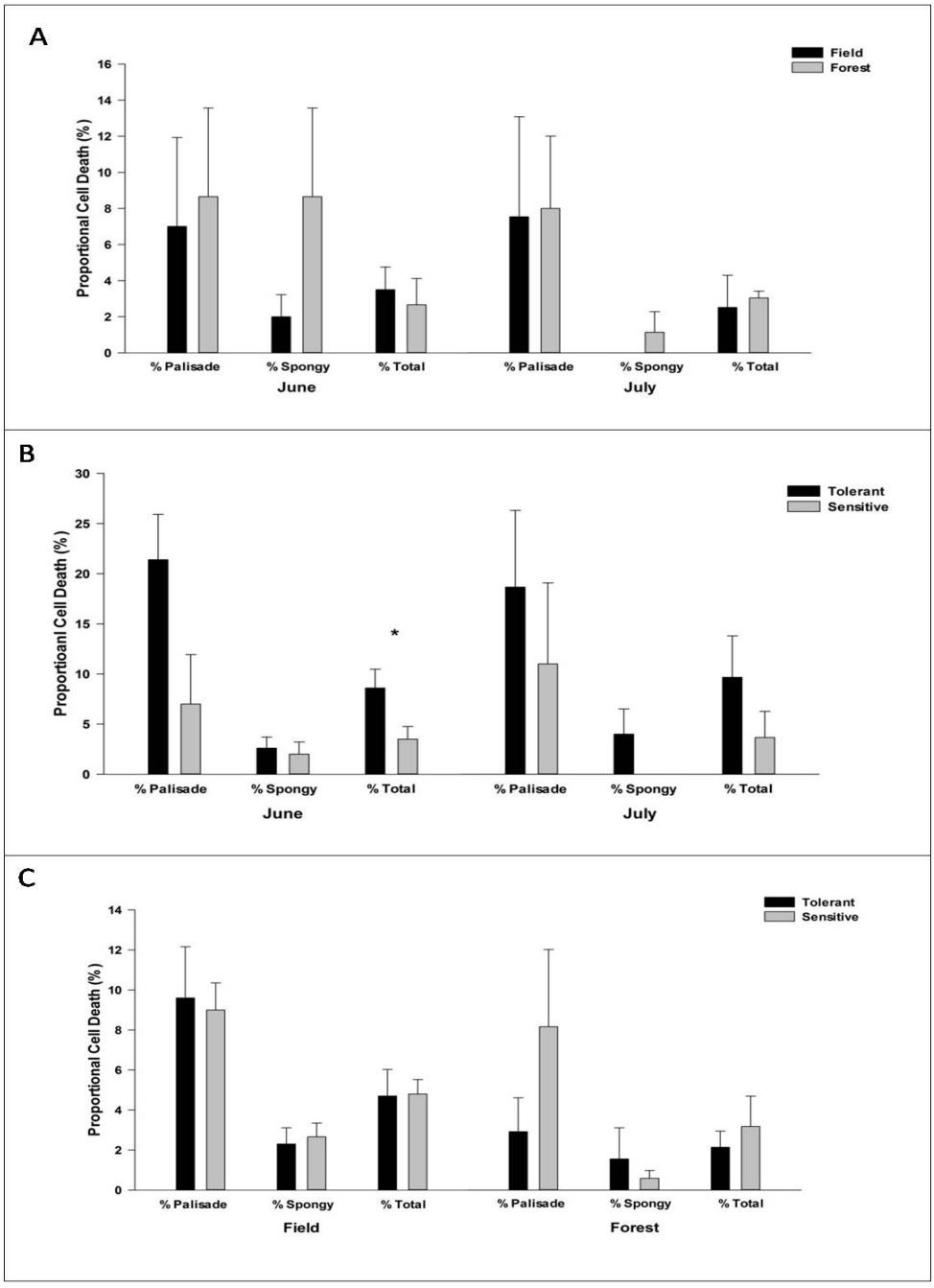

Figure 13. Proportional Cell Death. A. Early and Mid-season (June and July) comparison of site and month for sensitive plants. B. Early and Mid-season (June and July) comparison of sensitivity in field plants only. C. Late season (August) comparison of sensitivity. Bars represent means $\pm$ se, N = 15 for A, 19 for B and 41 for C. Asterisks indicate differences between sensitivity.

Table 10. Early, Mid and Late Season Analysis of Cell Death

A. Early and mid-season (June and July only) comparison of site and month for sensitive plants.

			P Death		% P Death		S Death		% S Death		Total Death		% Total Death	
Source	Site or Month	df	F	p	F	p	F	p	F	p	F	p	F	p
Site	June	1	0.00	1.0000	0.05	0.8250	1.75	0.2428	1.90	0.2261	0.71	0.4366	0.19	0.6827
	July	1	0.03	0.8754	0.00	0.9560	1.88	0.2199	1.88	0.2199	0.07	0.8056	0.05	0.8313
Month	Field	1	0.05	0.8318	0.00	0.9561	3.18	0.1176	3.46	0.1053	1.22	0.3067	0.39	0.5532
	Forest	1	0.25	0.6433	0.08	0.7962	1.00	0.3739	1.00	0.3739	0.00	1.000	0.00	1.000

B. Early and Mid-season (June and July) comparison of sensitivity for field plants.

		P Death		% P Death		S Death		% S Death		Total Death		% Total Death	
Source	df	F	P	F	p	F	p	F	p	F	p	F	p
Sens	1	5.25	**0.0368**	3.99	0.0643	0.85	0.3706	2.09	0.1692	5.60	**0.0319**	5.10	**0.0392**
Month	1	1.31	0.2698	1.24	0.2827	1.82	0.1973	0.88	0.3635	2.45	0.1380	1.13	0.3040
Sens*Month	1	0.74	0.4036	1.06	0.3193	0.61	0.4469	0.65	0.4338	0.16	0.6953	0.15	0.7024

C. Late Season (August only) comparison of sensitivity for sites and all leaf ages.

		P Death		% P Death		S Death		% S Death		Total Death		% Total Death	
Source	df	F	p	F	p	F	p	F	p	F	p	F	p
Site	1	0.17	0.6865	0.07	0.7887	1.25	0.2715	1.25	0.2729	0.30	0.5848	2.87	0.1005
Sens	1	0.28	0.6035	0.02	0.6226	0.12	0.7269	0.06	0.8056	0.09	0.7700	0.18	0.6719
Site*Sens	1	0.28	0.6035	0.02	0.6560	1.34	0.2558	0.01	0.9160	0.01	0.9295	0.55	0.4657
Leaf	2	0.19	0.8261	0.36	0.7035	0.49	0.6173	0.06	0.9447	0.04	0.9599	0.13	0.8800
Site*Leaf	1	0.66	0.5219	0.92	0.4104	1.67	0.2045	0.29	0.2510	0.73	0.4888	1.09	0.3499
Sens*Leaf	1	0.54	0.4681	0.37	0.5466	0.57	0.4559	0.67	0.4210	0.69	0.4130	0.18	0.6719
Site*Sens*Leaf	1	0.54	0.4681	0.57	0.4579	0.57	0.4559	0.92	0.3458	0.78	0.3831	0.55	0.4657

D. Entire season comparison (June to August) for plants (leaf 4 only) in the field only.

		P Death		% P Death		S Death		% S Death		Total Death		% Total Death	
Source	df	F	p	F	p	F	p	F	p	F	p	F	p
Sens	1	6.29	**0.0214**	4.77	**0.0417**	0.61	0.4454	1.72	0.2058	5.76	**0.0268**	5.74	0.0271
Month	2	0.80	0.4621	0.76	0.4827	1.05	0.3692	0.66	0.5303	1.45	0.2586	0.72	0.5018
Sens*Month	1	0.88	0.3588	1.27	0.2741	0.43	0.5175	0.62	0.4415	0.16	0.6899	0.17	0.6843

Discussion

This study found no significant anatomical or morphological differences between sensitive and tolerant genotypes of cutleaf coneflower that could account for the sensitivity differences seen in the field. The majority of significant differences that were found were related to habitat (sun vs. shade), season (June, July or August) and/or leaf age, but these do not coincide with sensitivity seen in the field.

Several mechanistic models have been proposed to account for differences in ozone sensitivity, and the most comprehensive one is that developed by Plöchl et al. (2000). This model looks at both the anatomical and physiological factors that determine the fate of O_3 when it encounters a leaf as well as chemical changes that can occur in the apoplast and symplast of the leaf. My study was based in part on this model, and I incorporated a combination of anatomical and morphological attributes of coneflower leaves to see if any of these were correlated with the differences in sensitivity observed in the field (Davison et al. 2003, Chappelka et al. 2003, Grulke et al. 2007). Previous work with this species has shown that the biochemical antioxidant defenses most likely do not play a major role, if any, in the resistance to O_3 in this species (Burkey et al. 2006). Similarly, differences in gas exchange prior to visible foliar injury, which could alter the uptake of O_3 and hence the effective dose within the leaves, also do not appear important (Peoples 2005, Grulke et al. 2007).

Because there is no evidence for either a physiological or biochemical basis for the sensitivity differences in this species, I focused on whether various aspects of the anatomy or morphology could contribute to O_3 sensitivity differences in this species. My analysis follows the path that an O_3 molecule would take as it encounters a leaf, from striking the cuticle, to penetrating into the cell wall, to the diffusional and physical barriers within the leaf, and then the interaction of this pollutant (or its byproducts) with cells of the mesophyll layers. For this analysis, I compared two sets of plants; those growing in shade, where changes in leaf structure affected by light could interact with changes in sensitivity to O_3, and those growing in full sun, where similar changes might be at work (Boardman 1977, Neufeld and Young 2003). I also attempted to compare plants earlier in the season, when O_3 impacts on leaf development would likely be smaller, so that any differences I found later could be separated from inherent ontological effects. One difficulty with this approach was that early in the season, it was not possible to classify the individuals into O_3 sensitive and O_3 tolerant individuals until symptom development late in the season. As a result, sample sizes varied according to how successful I was in selecting both sensitivity classes. A final difficulty that was encountered was the fact that 2004 was a near record low ozone year, which made it slightly more difficult (although not impossible) to classify species as sensitive or tolerant. The SUM00 and SUM06 for that year were 202 ppm*hrs and 40.5 ppm*hrs, respectively, and the number of hours above 60 and 80 were 125 and 6, respectively. Compare this to the

same values in 2002, a high ozone year, which were, in the same previous order: 234.9 ppm*hrs, 123.8 ppm*hrs, 1728 and 318 hrs (Roberts 2007).

Beginning where O_3 first encounters a leaf, O_3 uptake may be reduced if the cuticle is thicker and contains fewer cracks that would allow penetration to the epidermal layer below. If an O_3 molecule instead passes into a stomatal pore, then characteristics of the guard cells themselves, such as the thin cuticle that is often found on the inner cell walls (Sack 1987) could affect the leaf's sensitivity. If O_3 tolerant individuals have more cuticle development on the inner guard cell walls, they might be less sensitive to ozone. However, I was not able to analyze this anatomical attribute, so it remains unstudied at this time.

Once the ozone enters the stomatal pore it can diffuse throughout the leaf to cells in the spongy and palisade layers, as well as to either epidermal layer. The ability to diffuse freely would be a function of the density of the cells, their size, and hence cellular surface area exposed to the O_3, and the resultant tortuosity of the diffusional pathway. All of these factors could affect how O_3 is scavenged and detoxified. If there are abundant airspaces within the leaf, then the tortuosity (directness of the diffusional pathway) would be smaller, and O_3 could more easily get to a cell. If the cell density is high, then O_3 will be more likely to rapidly interact with a smaller subset of cells, and possibly be detoxified, thus limiting the spatial extent of damage within the leaf. Of course, as cells die from interacting with the O_3, they leave other cells at higher risk for damage from subsequent exposures and so continual exposure could lead to widespread damage anyway.

If cells are relatively large, then they will also have large surface areas to interact with the O_3 molecules. This could actually result in less injury by spreading the O_3 impact out over a larger cell surface area, allowing whatever potential antioxidant defenses are present to more satisfactorily detoxify the O_3. Finally, if cells have thicker cell walls, they may contain more soluble low molecular weight antioxidant compounds that could detoxify the O_3 before it penetrates to the plasma membrane. Current research indicates that cell walls may contain freely soluble antioxidant compounds (such as ascorbic acid), as well as cell wall bound antioxidants, whose capacity may equal that of the freely soluble compounds (Weise and Burkey 2010).

Cuticle Resistance

Deposition of O_3 to the cuticle has been studied and essentially dismissed as a factor in O_3-plant interactions (Kerstiens and Lendzian 1989). Studies of dry and wet O_3 deposition have shown that cuticle permeability does occur and can be relatively high, particularly when leaves are wet by dew or rain (Musselman and Massman 1999, Zhang et al. 2002). If there has been previous O_3 exposure, there is also the possibility that this has resulted in the degradation of the cuticular waxes (Barnes et al. 1988, Percy et al. 1994, 2002, Karnosky et al. 2002) but even this does not seem to be enough to cause any increase in O_3 sensitivity by the leaf (Kerstiens and Lendzian 1989).

The results of my study show no significant differences in cuticular thickness between coneflowers of differing sensitivity and thus, this factor is unlikely to be important. The late season (August) increases of adaxial cuticle thickness that were observed in sensitive plants could possibly be explained as a seasonal acclimatory defensive response to ozone exposure, as found in *Arbutus unedo* (Bussotti et al. 2005).

Stomatal Resistance

As stomata are considered the gate keepers of the leaf interior, their density and aperture size have been the subject of much investigation with respect to O_3 sensitivity studies (Elkiey and Ormrod 1979, Barnes et al.1988, Pääkkönen et al. 1993, Pääkkönen et al. 1997, Ferdinand et al. 2000, Kollist et al, 2000, Paoletti and Grulke 2005; 2010, Lin et al. 2001, Alves et al. 2007, Guidi et al. 2010). The influx of O_3 and other gasses into the leaf via stomata can be affected by both anatomical and physiological characteristics of the stomata. O_3 sensitivity may be a function of anatomical attributes such as a large stomatal aperture (i.e., pore size, which can be a function of guard cell length) and stomatal density (Evans and Ting 1974). High stomatal densities are generally correlated with small stomatal size and these stomata may have quicker responses to external stimuli (Hetherington and Woodward 2003). Paoletti and Grulke (2005) theorize that this ability of smaller stomata to respond faster may translate into relatively rapid reductions in $g_{s,}$ which then reduce the O_3 flux into the leaf. However, evaluations of the relationship between O_3 sensitivity and

stomatal structure and density have produced mixed results with regards to their role in determining the sensitivity of a species to O_3. Some studies have cited a positive correlation between stomatal density and injury, such as in ponderosa pine (*Pinus ponderosa*), tobacco (*Nicotiana tabacum*) and black cherry (*Prunus serotina*) (Evans and Miller 1972, Dean 1972, Ferdinand et al. 2000), whereas in other species, such as alfalfa (*Medicago sativa* L.), bean (*Phaseolus vulgaris*) and petunia cultivars (*Petunia hybrid* Vilm.), a negative correlation has been found (Turrell 1942, Evans and Ting 1974, Elkiey et al. 1979). These conflicting results may simply indicate that for these species, the flux of O_3 into the leaf is not the dominating factor determining sensitivity. Instead, either anatomical or biochemical/molecular differences may be relatively more important.

Grulke et al. (2007) found that stomatal density was not a factor influencing O_3 sensitivity in cutleaf coneflower, since both sets of plants had nearly identical densities on both the abaxial and adaxial surfaces. Peoples (2005) found that uninjured leaves of both sensitive and tolerant plants had similar photosynthetic rates and g_s, suggesting that uptake and hence dose, do not differ between the two classes of plants. The physiological differences that were found by Grulke et al. (2007) only showed up *after* injury appeared, suggesting that they are the consequence of, but not the cause of, differential O_3 uptake.

Grulke et al. (2007) also found that stomata of sensitive plants were less responsive to environmental cues, such as VPD and light. This could result in greater losses of water in sensitive individuals than in tolerant ones, because

stomata would not close under conditions when they normally should. Such a loss of stomatal control could contribute to greater O_3 doses for sensitive plants, and exacerbate their decline upon exposure to more O_3. However, as with Peoples' (2005) results, these are after-the-fact responses, and hence cannot account for the differences in sensitivity among individual plants. Thus g_s alone does not explain differences in O_3 sensitivity in these coneflowers.

O_3 exposure itself may lead to alterations in leaf development that possibly could affect the sensitivity of that leaf to O_3 (Pääkkönen et al. 1993, 1995, 1998). Studies of birch (*Betula pendula*) have shown that O_3 can induce changes in leaf development and differentiation that result in greater stomatal density but have no effect on guard cell length (Matyssek et al. 1991, Günthardt-Goerg et al. 1993, Pääkkönen et al. 1993, Frey et al. 1996). O_3's lack of influence on guard cell length was also corroborated in studies of *Betula papyrifera* (Riikonen et al. 2010), *Fraxinus excelsior* (Wiltshire et al. 1996) and *Populus* x *euramericana* (Günthardt-Goerg et al. 1996), and it has been proposed that this response may limit the negative impact of O_3 (Paoletti and Grulke 2005) by reducing the inward flux. However, there is no evidence to suggest that such changes occur in the stomata of coneflowers, and hence, neither stomatal density nor g_s, which integrate both density and size effects, are considered plausible causal factors for the differences in sensitivity between individuals.

Mesophyll Factors

The elimination of cuticular and stomatal factors as determinants of the differential sensitivity to O_3 in cutleaf coneflower leaves only internal factors, such as leaf structure, which may influence the diffusional route as described by Chameides (1989) and Plöchl et al. (2000) and cell structure, which may affect the O_3 scavenging efficiency of the leaf. Once in the leaf, O_3 must traverse the intercellular spaces in order to come into contact with individual cells. How leaf cells are packed determines the lateral pathway of gas diffusion (O_3) in the leaf (Evans and von Caemmerer 1996, Smith et al. 1997, Evans et al. 2009) and hence the tortuosity of that pathway. Anatomical structure within the leaf can determine the length of time for diffusion of O_3 as well as the amount of cell surface area in potential contact with the O_3 (Chameides 1989, Plöchl et al. 2000). Thicker leaves and thicker mesophyll layers are thought to increase the diffusional path length while greater cell density and lower intercellular space should decrease the chance that O_3 will interact with many exposed cell surfaces (Chameides 1989, Plöchl et al. 2000, Evans et al. 2009).

Upon entering a leaf, O_3 first encounters the sub-stomatal cavity and then the spongy mesophyll layer, since most stomata (over 90%; Grulke et al. 2007) are on the abaxial surface. There is limited data concerning the role of the sub-stomatal cavities in scavenging O_3, but one study of the well investigated tobacco clones, Bel W3 (O_3-sensitive) and Bel B (O_3-tolerant), found that the sensitive clones had larger sub-stomatal cavities and greater intercellular spaces (Pedroso

and Alves 2008). Sub-stomatal cavities of coneflower were not easily distinguishable as in other species, due to highly irregular cell distribution (Figure 14) and were incorporated within the corresponding mesophyll layer airspace measurements.

Several analyses have found that greater total leaf thickness and greater palisade mesophyll layer thickness do correlate well with O_3 tolerance (Bennett et al. 1992, Pääkkönen et al. 1997, Oksanen et al. 2001, Gerosa et al 2003) while spongy mesophyll layer thickness seems to have little effect (Evans and Ting 1974, Bennett et al. 1992, Pääkkönen et al. 1997). I found no relationship between O_3 sensitivity and leaf mesophyll thickness. The differences I did find between sites (field vs. forest) can be attributed to the known differences in leaf morphology typically seen in sun (thicker) versus shade (thinner) leaves (Boardman 1977, Neufeld and Young 2003). As stated earlier, O_3 exposure has been shown to induce alterations in leaf development in some species, and those alterations could affect their sensitivity to O_3 (Pääkkönen et al. 1993, 1995, 1998, Lawson et al. 2002, Bussotti et al. 2005, Hartikainen et al. 2009). Studies of CO_2 and O_3 impacts on gas exchange parameters in potato (*Solanum tuberosum* L.) showed an O_3-induced increase in leaf thickness (Lawson et al. 2002). Bohler et al. (2010) suggest the increased thickness documented in younger leaves (Paoletti et al. 2009) is simply a byproduct of the leaf expansion process, which is slowed upon exposure to O_3, thereby resulting in denser leaves that then confer protection from O_3. Other studies have found that O_3 caused new leaves to become thinner (Oksanen et al. 2001, 2005, Prozherina et al. 2003, Borowiak et

al. 2010) which may be attributed to decreases in photosynthetic capacity and carbon allocation (Barnes 1972, Coleman et al. 1995).

In other cases, the O_3 flux is not high enough to induce changes in morphological development (Bohler et al. 2010). Nonetheless, in the coneflowers that I studied, there were no significant differences in either palisade or mesophyll thicknesses between the O_3 sensitive or O_3 tolerant plants, and hence these factors do not seem to determine sensitivity in this species.

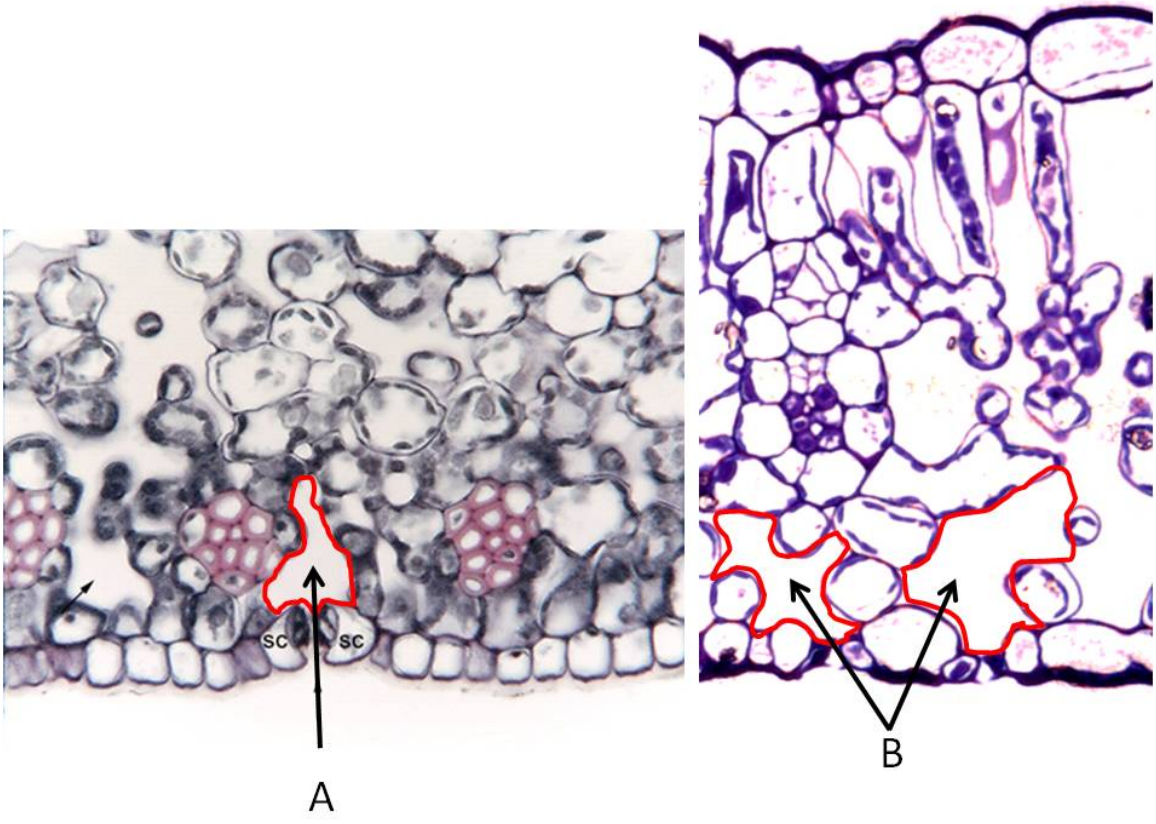

Figure 14. Leaf cross-sections depicting sub-stomatal cavities. A. *Dracaena fragrans* micrograph illustrating a more uniform sub-stomatal cavity (x60). B. Coneflower (*R. laciniata*) micrograph illustrating less distinguishable sub-stomatal cavities (x12.5).

Internal Airspace, Cell Area and Mesophyll Tortuosity

Internal airspace is a function of leaf thickness coupled with the cell area to cell density ratio. Larger amounts of internal airspace are thought to allow gasses (CO_2 or O_3) a more direct route to the palisade layer (Evans and von Caemmerer 1996, Pedroso and Alves 2008) and hence to facilitate either photosynthesis or O_3-induced damage. In general, O_3-sensitive plants have greater amounts of internal airspace (Bennett et al. 1992, Pääkkönen et al. 1997, Lee et al. 1999, Ferdinand et al. 2000, Gravano et al. 2003) than O_3-insensitive plants. Researchers have also concluded that greater spongy mesophyll airspace in conjunction with a thinner palisade mesophyll layer is common to O_3-sensitive plants (Bennett et al. 1992, Ferdinand et al. 2000). Additionally, sensitive species have more airspace within the palisade mesophyll layer, which allows O_3 access to more exposed cell surface (Evans and von Caemmerer 1996). However, the results of my study only showed that plants growing in high light had more airspace in the early season than later on, which is to be expected, considering the high photosynthetic capacity and carbon allocation of plants growing in full sun (Boardman 1977). Leaf developmental stage most likely explains the greater airspace seen in June plants compared to later season airspace.

Cell area is related to several factors impacting gas exchange within the leaf including exposed cell surface area, airspace and mesophyll tortuosity. The greater cell area seen in forest plants is most likely a shade leaf response where larger but thinner leaves are an adjustment to maximize incidence of photon

interception (Boardman 1977). Mesophyll tortuosity is essence measure of the indirectness of the path or the length of time that a gas must diffuse within the leaf (Evans et al. 2009). Greater cell density and cell area can increase the tortuosity by causing the gas to move along a longer path before encountering another cell or by slowing diffusion which would allow more time for apoplastic antioxidants to detoxify the O_3. In my study, the greater tortuosity seen for both mesophyll layers of forest plants is directly related to the greater amount of cell area also seen in forest plants. But in no case was tortuosity correlated with increased sensitivity in these coneflowers.

Cell Exposure and Cell Wall Thickness

Few studies have directly measured exposed cell surface, but it has been shown to have a positive association with photosynthesis and gas exchange capacity (Bennett et al. 1992, Pääkkönen et al. 1997, Ferdinand et al. 2000, Gerosa et al. 2003, Gravano et al. 2003) and should therefore be related to O_3 impact. However, Oksanen et al. (2001) found that sensitive aspen (*Populus tremuloides*) clones had 15-17 % smaller cell surface areas, which would negate the assumption that O_3's correlation with cell surface area is similar to that of CO_2. O_3 sensitivity may be more affected by cell wall thickness and the associated apoplastic antioxidants, i.e. ASC (Burkey and Eason 2002, Oksanen et al. 2001).

My results showed that there was more exposed cell surface within the late season palisade layers growing within the forest conditions. This agrees

with the results of greater cell area of forest plants and again shows the influence of light on morphology (Boardman 1977). However, shade coneflower plants are not necessarily more susceptible to O_3 (Roberts 2007) since O_3 concentrations are reduced in the understory, g_s are lower, and the canopy boundary layer thickness (which retards diffusion of O_3 to leaves) is greater due to lower wind speeds (Finkelstein et al. 2004). All of these effects tend to reduce the flux of O_3 to the leaf, and hence may also reduce injury to the leaf. However, the shade leaf anatomy and physiology may predispose leaves to greater sensitivity, as shown by a number of studies, including those involving sunflecks (Wei et al. 2004a, 2004b).

Cell wall thickness offers the last mechanical means of O_3 resistance before membrane oxidation. Physically, thicker cell walls can increase diffusional resistance time and therefore the opportunity for antioxidant scavenging, particularly by ASC, a process coined the "*first line of defense*" for cells (Turcsányi et al. 2000). Some theoretical computations suggest that thicker cell walls in combination with high ASC concentrations could result in full O_3 detoxification (Turcsányi et al. 2000) before encountering the plasma membrane, while others suggest that only a fraction of the O_3 would be detoxified and that elevated ASC does not always offer sufficient oxidative protection (Moldau 1998, Jakob and Heber 1998, Ranieri et al. 1999, Kollist et al. 2000, Overmyer et al. 2000, Burkey and Eason 2002, D'Haese et al. 2005). My study has determined that cell wall thickness is not a major factor for determining O_3 resistance in coneflower since there were no differences between the sensitive and tolerant

plants. That being said, the cell wall is known to harbor powerful antioxidants and coneflowers do show an increase in antioxidant capacity upon development of visible O_3-induced stippling (Burkey et al. 2006). But again, these responses become evident only after the appearance of O_3 injury; and they are not present prior to injury. As mentioned earlier, coneflowers do not contain appreciable amounts of ASC (Burkey et al. 2006), and hence this particular antioxidant, and the ones that are up-regulated after foliar injury is present, are not responsible for the sensitivity differences in this species.

Other Possible Causes of Sensitivity Differences

Differences in gene expression may be the best avenue of research left for determining the causes of differential O_3 sensitivity in cutleaf coneflowers. Studies have begun to evaluate O_3 sensitivity through the isolation of single genes and related gene families as well as through whole plant genetic expression. The O_3 sensitive *Arabidopsis* mutant, radical-induced cell death 1 (*rcd1*) was isolated based on its HR-like lesion formation (Overmyer et al. 2000) and ROS sensitivity (Belles-Boix et al. 2000). Studies have shown that the RCD1 gene is associated with the signaling pathway leading to cell death and that functional hormone responses are needed for O_3 tolerance (Ahlfors 2008). This gene has been found to alter hormone responses (ethylene [ET], jasmonic acid [JA], salicylic acid [SA] and abscisic acid [ABA]) and it is thought that decreased expression of RCD1 may result in the O_3 sensitivity seen in *Arabidopsis* ecotype Ws-0 (Li et al. 2006). Nitric oxide (NO) is another important

signaling molecule functioning in the modification of genetic expression when a plant is under O_3 stress (Ahlfors et al. 2009). These researchers have found *rcd1* to be an over producer of NO and have suggested that alterations in the ROS-NO balance can lead to O_3 hypersensitivity and cell death.

Arabidopsis thaliana ecotypes, Col-0 (O_3-tolerant) and Cvi-0 (O_3-sensitive), are currently being used in studies of O_3-induced genetic expression. These studies have illuminated a complex and balanced signaling response where the previously discussed hormones play an important role (Tamaoki et al. 2003, Li et al. 2006, Totsi et al. 2006, Mahalingam et al. 2003, 2005, 2006, Ludwikow and Sadowski 2008). Ethylene, JA, SA and ABA biosynthesis and regulation are triggered by various gene expressions resulting from O_3 exposure (Rao et al. 2002, Kangasjärvi et al. 2005, Tosti et al. 2006, Ludwikow and Sadowski 2008). Many of these studies investigating O_3 influenced genetic activation have found signaling cross-talk and equilibrium to determine the degree of O_3 sensitivity and lesion formation (Baier et al. 2005, Kangasjarvi et al. 2005). Additional transcriptomic studies involving comparisons of these mutants have revealed how hormone synthesis, regulation and interaction can confer O_3 tolerance (Li et al. 2006, Tosti et al. 2006). Using Col-0 mutants, researchers have found that ET and JA signaling induces defense gene expression which can also be suppressed by excess SA signaling (Tamaoki et al. 2003). Col-0's O_3 tolerance is suggested to be a function of a diminished degree of SA signal activation (Rao and Davis 1999, Pasqualini et al. 2002, Tamaoki et al. 2003).

O_3 concentration and sustained exposure is well known to influence the degree of plant defense response. This is seen in the more O_3 sensitive genotype (Cvi-0) where at high levels of O_3 exposure, the O_3 mediated SA induction marker (PRI) is up-regulated, high accumulation of SA occurs, and programmed cell death (PCD) may be induced (Li et al. 2006). At low O_3 levels, the SA defense signal is enough to engage the defense pathway but not great enough to engage PCD (Li et al. 2006). Cvi-0 exhibited similar decreases in JA pathway induction as reported by Rao et al. (2000) but without sensitivity consequences (Li et al. 2006). Li et al.'s (2006) study also showed more injury in Col-0 than expected while its genetic expression analysis revealed a non-hormone related PCD pathway. The reversal of sensitivity seen in Cvi-0 may be due to a novel stress-resistant pathway in which there is higher expression of stress-related genes allowing for greater adaptation to varying O_3 concentrations (Li et al. 2006).

Tosti et al. (2006) evaluated real time gene expression during and after O_3 treatment of Col-O and found differing, time coordinated responses among members within the same multigene family. Tosti et al. (2006) found O_3-induced genetic expression that resulted in ET, JA, and SA biosynthesis that was similar to that of Li et al. (2006). These researchers also determined that the biosynthesis and regulation they found was the result of genes involved with the signal transduction of the same hormones. Activation of ET and SA negative regulators (CTR1 and EDR1, respectively) possibly explains the lack of O_3 sensitivity in Col-0 plants (Tosti et al. 2006). They also found O_3 to induce the

regulation of WRKY genes, receptor-like kinases (RLK), and MAPK cascades. WRKY genes are known to be regulators of gene expression during pathogen defense, wounding and senescence (Miao et al. 2004, Journot-Catalino et al. 2006). Known as receivers and transducers, RLKs are thought to be activated by WRKYs (Tosti et al. 2006) and ROS (Kovtun et al. 2000) and thus may be associated with the maintenance and forwarding of signals induced by O_3 exposure. In particular, the O_3 up-regulated WRKY 22 is a downstream component of the MAPK signaling cascades involved in bacterial and fungal infections (Asai et al. 2002) which may explain the HR-like responses.

MAPK cascades are signaling pathways utilized to mediate communication and activation of a variety of cellular processes such as cell division, growth and environmental stimuli. Specific MAPKs are also activated very early in O_3 exposure as a result of ROS perception (Samuel et al. 2000) and thought to be connected with stress induced hormone synthesis (Kangasjärvi et al. 2005, Liu and Zhang 2004, Colcombet and Hirt 2008). Oxidative stress triggers MPK3, MPK4 and MPK6 cascades and the outright loss of control of the MPK3/MPK6 cascades can result in plants being hypersensitive to O_3 (Miles et al. 2005). For example, MKP2 is thought to be a regulator of transient MPK6 and MPK3 and evidence for this can be seen in MKP2 silenced plants where MPK6/MPK3 are unregulated, causing these plants to become O_3 hypersensitive (Lee and Ellis 2007). Tosti et al. (2006) ascribed MAPKs as a convergence point of the defense-signaling network and have shown that some WRKY genes activated by O_3 may act as upstream targets of MAPKs.

Clearly, genetic expression in plants is a complex and integrated system activated by any number of biotic and abiotic cues. The activation and biosynthesis of a variety of genes are shown to be necessary for the regulation of the hormones associated with plant defense response as well as being influenced by the accumulation and perception of said hormones. Cutleaf coneflower's O_3 response may only be fully understood through a study of genetic expression under a variety of O_3 exposures. It is quite possible that the bases for differential O_3 sensitivity in this species reside at the molecular level, where sensitive plants respond with gene up-regulation and down-regulation at lower exposures to O_3 than do tolerant plants.

Conclusion

This study has determined that leaf anatomy and morphology do not seem to be the determinants of differential O_3 sensitivity in cutleaf coneflowers. The anatomical and morphological differences that were found in this study were mostly due to micro-habitat (light), ontological (seasonal) and developmental (leaf age) differences: none were found that were compatible with the idea that they were influencing the sensitivity of this species to O_3. Future research should focus on ROS perception and signaling at the molecular level and how they in turn, influence genetic regulation. Microarrays and real-time PCR have revealed interesting time-coordinated gene expression in response to O_3 exposure (Tosti et al. 2006, Li et al. 2006) and may offer the greatest tool for understanding the causes behind O_3 sensitivity in coneflowers as well as many other species.

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VITA

Chrisha L. Dolan was born on August 17th, 1979 to Vickie and James Dolan in Fayetteville, NC. Her love of the natural world developed in the Sandhills of North Carolina, and was obvious from her collections of snail shells, bee stings and flower pressings. Chrisha attended Fayetteville Technical Community College with the intent of entering the Dental Hygienist program. A year of general studies convinced her that she was much more interested in biological studies rather than dentistry. She entered the University of North Carolina at Pembroke where she focused on botany and field biology. During this time, Chrisha had the opportunity to intern as a botanical field technician in 2000 and 2002. In 2001, she had the opportunity to spend a summer at the University of Nebraska at Lincoln researching insect herbivory preferences. She graduated in 2002 with her Bachelor's of Science in Biology concentrating on botany and environmental biology. Following graduation, Chrisha was employed as a botanical field technician and forestry research technician at Fort Bragg Army Installation. She entered graduate studies at Appalachian State University in August 2003 to work on ozone-plant interactions with Dr. Howie Neufeld and graduated May 2011. Between 2005 and 2011, Chrisha married Gary Malkin and was employed as an army contracted field technician, biologist, and GIS technician.