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Research

Stomatal behavior of ozone-sensitive and -insensitive coneflowers (*Rudbeckia laciniata* var. *digitata*) in Great Smoky Mountains National Park

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Summary

• Morphological and physiological attributes were assessed to elucidate the underlying mechanisms of ozone (O_3) sensitivity in a highly sensitive species, cutleaf coneflower (*Rudbeckia laciniata* var. *digitata*).

• Foliage at the same height in the canopy on paired O_3-sensitive and -insensitive cutleaf coneflowers was assessed for level of foliar symptoms, stomatal density, stomatal responsiveness to dynamic changes in light and leaf-to-air vapor pressure deficit (VPD), steady-state responses to light and CO_2, intrinsic transpirational efficiency, and plant water balance.

• There were no morphological differences between the sensitivity types that might have contributed to greater O_3 uptake in sensitive individuals. Stomata of sensitive plants were less responsive than those of insensitive plants to experimentally increased and decreased light intensities, and to increased VPD. O_3-insensitive plants had greater intrinsic transpirational efficiencies, greater maximum assimilation rates under saturating CO_2 and light, and greater carboxylation rates.

• Different physiological attributes vary independently within an individual plant, which collectively confer sensitivity or insensitivity to O_3 injury.

Key words: dynamic stomatal response, Great Smoky Mountains, ozone exposure, *Rudbeckia laciniata*, stomatal conductance, sunfleck, vapor pressure deficit.

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Introduction

The southern Appalachian Mountain region experiences some of the highest ozone (O_3) concentrations of any natural area in the eastern USA (Mueller, 1994; Hildebrand *et al.*, 1996; Chappelka *et al.*, 1997; Samuelson & Kelly, 1997). Both the Shenandoah and Great Smoky Mountains (GRSM) National Parks in the region have been designated as Class I wilderness areas, and are protected by the 1977 Clean Air Act. A considerable effort has been made to understand O_3 effects on the dominant species in these wilderness areas. Foliar symptoms have been found on both tree and native wildflower species (Duchelle & Skelly, 1981; Duchelle *et al.*, 1983; Neufeld *et al.*, 1992; Hildebrand *et al.*, 1996; Chappelka *et al.*, 1997, 1999a,b, 2003; Samuelson & Kelly, 1997; Davison *et al.*, 2003; Hughes *et al.*, 2005; Souza *et al.*, 2006). Visible foliar symptoms in the field have been found on over 90 species occurring in the GRSM, 30 of which were verified in open-top chambers (Neufeld *et al.*, 1992). Long-term foliar injury symptoms in southern Appalachian trees have also been correlated with decreased growth (*Liriodendron tulipifera*; Somers *et al.*, 1998) and seedling biomass in open-top chambers (*Prunus serotina*; Neufeld *et al.*, 1995). Ozone exposure also affects the understory vegetation (Duchelle & Skelly, 1981;

Duchelle *et al.*, 1983; Chappelka *et al.*, 1997; Chappelka *et al.*, 2003; Davison *et al.*, 2003; Souza *et al.*, 2006) and community composition (Barbo *et al.*, 1998) through impacts on both growth and reproduction (Chappelka, 2002).

In GRSM, one of the most sensitive species to O_3 exposure is cutleaf coneflower (*Rudbeckia Laciniata* var. *digitata*), which commonly occurs in thick swards along forest margins. The development of foliar injury is proportional to O_3 exposure in the field (Chappelka *et al.*, 2003; M. Roberts, unpublished field data from 2004) and in controlled chamber exposures (Neufeld *et al.*, 1992). In the field, roughly 50% of the plants have been found to exhibit foliar injury. For the overall population, the upper half of the leaves are asymptomatic (Chappelka *et al.*, 2003). The foliar injury consists of a purplish-brownish bronzing, which is restricted to the adaxial epidermal and upper palisade cells. These water-soluble brown pigments have detectable effects on leaf spectral properties (H. S. Neufeld, unpublished data), although their exact function is not well understood.

Sensitivity to O_3 can vary greatly among adjacent individuals within a single population (Chappelka *et al.*, 2003). With increasing O_3 exposure, first sensitive individuals and then insensitive individuals develop visible foliar symptoms. By the end of the growing season, all plants show some visible foliar symptoms, but sensitive individuals usually have much more injury. The causes of this difference in sensitivity are not understood in this species. They could include differences in stomatal uptake of O_3, through variations in stomatal density, size, or conductance, all of which would affect uptake into the leaf. Alternatively, differences in sensitivity could arise from internal leaf anatomical features, such as cell sizes and densities, which would affect the tortuosity of O_3 diffusional paths within the leaf (Plöchl *et al.*, 2000). Insensitive plants could have thicker cell walls, with larger pools of antioxidants, such as ascorbic acid, which would protect the leaf against reactive oxygen species (ROS). However, preliminary studies of the internal leaf anatomy of sensitive and insensitive individuals have so far revealed no observable differences (T. Dolan, manuscript in preparation). Additional work by our group (Burkey *et al.*, 2006) has shown that both sensitive and insensitive coneflowers have almost no apoplastic ascorbate. Other antioxidant defense capacities, and differences at the molecular level are still a possibility, but no such work has yet been carried out on this species.

The purpose of this study was to more fully characterize the physiological differences between cutleaf coneflowers of different sensitivities, and to determine any potential causal mechanisms for those differences. We hypothesized that differences in stomatal density or behavior in response to changing environmental conditions may be responsible for the differences between plants of different sensitivities. We tested our hypotheses by quantifying stomatal densities on both leaf surfaces, and by measuring gas exchange responses to changes in light and vapor pressure deficit (VPD) on paired samples of both sensitive and insensitive plants. Because drought stress has been known to alter the expression of foliar O_3 injury, we tested whether there were any significant differences in the water relations between the two sensitivity types. In addition, we tested the effects of O_3 sensitivity on carbon acquisition attributes, which could potentially affect resources and energy available for antioxidant defense or reparation activities.

Materials and Methods

Research site

Research was conducted during the period 28 July to 4 August 2004 at the Appalachian Highlands Science Learning Center, Purchase Knob (35.588N, 83.074W; elevation 1515 m above sea level), GRSM, in the southern Appalachian Mountains of western North Carolina, USA. O_3 exposures at the Learning Center are monitored by the State of North Carolina, and archived by the National Park Service Air Quality database. O_3 concentrations were lower (42 ppb, 12 h d^{-1}, from 15 May through 15 September) than a previous 5-year average of 49 ppb over the growing seasons 1990–1994 (Fig. 1). O_3 concentrations exceeded 60 ppb on 14 days during the 2004 growing season. AOT40 (accumulated dose of O_3 over a threshold of 40 ppb) was calculated from the initiation of leaf growth (1 June) until the sampling dates for foliar injury and physiological measurements (28 July through 4 August 2004) for a 12-h day.

Plant and tissue selection

Cutleaf coneflower (*Rudbeckia laciniata* L. var. *digitata*) with no foliar symptoms of O_3 injury was designated as 'insensitive'. Plants with significant foliar injury (50–75% foliar surface area affected) in 'mid-canopy' on the flowering stem were selected within 2 m of insensitive plants, and designated as 'sensitive'. All plants selected for this study were within 3 m of the forest–meadow margin. A count of the total number of leaves produced per plant, the number of live vs dead leaves, the leaf position used for gas exchange measurements, and a score for leaf injury were recorded for each plant used for physiological measurements. A simple scoring system was used to quantify foliar injury where 0 = 0%, 1 = 1–6%, 2 = 7–25%, 3 = 26–50%, 4 = 51–75%, and 5 = 76–100%.

Stomatal density

Section of area 20 × 20 mm from the center lobe of leaves from five O_3-sensitive and five O_3-insensitive plants were preserved in a solution of formalin:acetic acid:water (FAA; 10 : 7 : 1 ratio by volume) and then impressions were made using silicon sealant. Abaxial and adaxial surfaces were viewed with a FEI Quanta 200 Environmental Scanning Electron Microscope (FEI Co., Hillsboro, OR, USA) in low

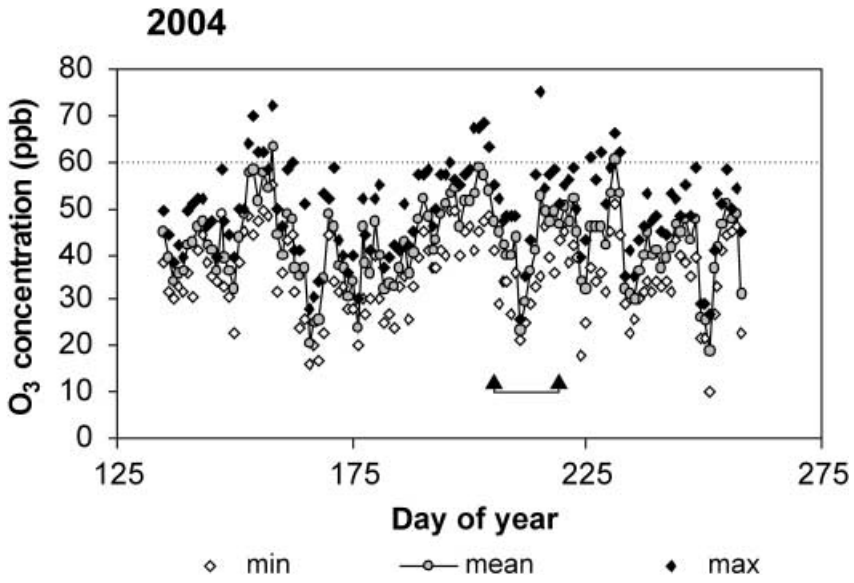

Fig. 1 Seasonal course of maximum, minimum, and mean daily ozone (O_3) concentration at Purchase Knob, Great Smoky Mountains National Park, NC, USA in 2004. Values > 60 ppb O_3 (dotted line) have been shown to experimentally induce visible injury in foliage of sensitive cutleaf coneflower (*Rudbeckia laciniata* var. *digitata*). Arrows and brackets indicate the period of 2004 field data collection. The corresponding AOT40 (accumulated dose of O_3 over a threshold of 40 ppb) for the sampling dates is 4.84–5.23 ppm h.

vacuum mode at 400×, sampling three fields per leaf, per plant. Stomatal densities were counted from digital photographs displayed on a television screen.

Gas exchange measurements

Two open gas exchange systems (Model 6400; Li-Cor, Lincoln, NE, USA) were used concurrently on one leaf each from a sensitive and an insensitive plant. The CO_2 infrared gas analyzers (IRGAs) were carefully cross-calibrated on site with a National Institute of Standards and Technology-certified secondary standard (385 ppm CO_2 in air). The H_2O IRGAs were calibrated at a similar elevation in a controlled temperature room using an air stream of known dewpoint (Model 610; Li-Cor). The electronic signals of both sample and reference IRGAs were matched after stabilization at each VPD, light intensity or CO_2 concentration before recording data. For these measurements, whole plants were either in natural shade or artificially shaded with an umbrella [external photosynthetic photon flux density (Q) ~300 μmol m^{-2} s^{-1}] for 30 min before and during measurement to minimize light, drought and temperature stress on the whole plant. Plants exposed to only 0.5 h of full sun at midday can wilt (H. S. Neufeld and A. W. Davison, personal observations). Unless an environmental variable was set to determine a specific response function, all other variables were set to constant values: reference CO_2 concentrations were set to 385 ppm, Q to 1400 μmol m^{-2} s^{-1}, leaf temperatures to between 19 and 22°C (controlling block temperature), and VPD to between 0.4 and 0.7 kPa during gas exchange measurements.

Measurements of the steady-state response to light were conducted on one leaf each on three O_3-sensitive and three O_3-insensitive plants. Light intensities were set successively at 1800, 1400, 1000, 600, 400, 120, 60, 30, and 0 μmol m^{-2} s^{-1}. Differences in light response attributes (apparent quantum efficiency, light compensation point, and maximum photosynthetic rate in saturating light) were tested with a one-way analysis of variance (S-Plus, MATHSOFT 2000). The assimilation (A) response to changes in substomatal CO_2 concentration (C_i) was effected by successively changing ambient CO_2 (C_a) concentrations from 245 to 2075 ppm CO_2 at saturating Q (1400 μmol m^{-2} s^{-1}).

The stomatal response to abrupt changes in light intensities (simulated sunflecks, from 375 to 1800 and back to 375 μmol m^{-2} s^{-1} Q) was compared between six O_3-sensitive and four O_3-insensitive plants. Stomatal conductance (g_s) was allowed to reach equilibrium on both instruments (each measuring a leaf from a sensitive or insensitive plant) before the next light intensity was imposed to minimize the effect of the external ambient conditions. The rate of change of g_s and A to both increasing and decreasing light intensities, and the equilibration value of g_s, A, and instantaneous transpirational efficiency (ITE) were tested for statistical significance. The response of g_s to changes in VPD over the range of 0.3–2.2 kPa was compared between nine O_3-sensitive and eight O_3-insensitive plants. Both total flow and flow through the desiccant were adjusted to attain the desired leaf VPD, although flow rates were held constant until g_s reached equilibrium at each VPD. A single regression was fit to the data for each O_3 sensitivity type, and tested for significant interaction between O_3 sensitivity type and VPD (S-Plus, MATHSOFT 2000).

Foliar water relations

In 2004, predawn (05:30 h) and noon foliar water relations measurements were made on leaves in the mid-canopy of 16–20 plants, half of which were O_3-sensitive and half O_3-insensitive. Total leaf water potential (Ψ_T) was measured on

three to five leaf discs (0.63 cm diameter) placed in chamber psychrometers (Merrill Instruments, Logan, UT, USA). Psychrometers were measured with a microvoltmeter (Wescor Instruments, Logan, UT, USA) using the psychrometric method described in Pallardy *et al.* (1991). A 2-h equilibration was used for initial temperature and water vapor equilibration within the cuvette. After determination of total leaf water potential, the psychrometers were disassembled, and the chamber with the leaf tissue was wrapped in aluminum foil and frozen in a −20°C freezer (predawn tissue) or liquid nitrogen (noon tissue), and allowed to warm to room temperature. Psychrometers were then reassembled, and leaf osmotic plus matric potential (Ψ_{S+M}) was determined after a 75-min equilibration in a water bath. Turgor pressure was calculated from the difference between the two measurements (Ψ_T and Ψ_{S+M}).

Statistical analyses

A one-way analysis of variance was used to test for differences in attributes between sensitive and insensitive plants using S-Plus (MATHSOFT 2000), and to test for differences among stomatal behavior types (none, muted, and normal). A spline regression (S-Plus, MATHSOFT 2000) fit was applied to describe the stomatal response to changes in VPD for both sensitive and insensitive cutleaf coneflowers. Unless otherwise specified, the statistical significance is reported at the $P = 0.05$ level.

Results

Stomatal responses to dynamic light conditions

There were three stomatal responses observed to simulated sunflecks (changes in light from low to high, then back again): no response (Fig. 2a), muted response (< 100 mmol m^{-2} s^{-1} change in g_s with a 1400 μmol m^{-2} s^{-1} change in light; Fig. 2b), and normal response (g_s and A increased concurrently, with greater rates of change; Fig. 2c). Although there appeared to be perturbations to g_s in cutleaf coneflower with 'no response' to light (Fig. 2a), the g_s returned to similar values (in all cases within 40 mmol m^{-2} s^{-1}) in high and low light. The perturbations, i.e. increased g_s when light was reduced and decreased g_s when light was increased, were artifacts caused by transient changes in air temperature and pressure within the cuvette.

Two A responses to sunflecks were observed: muted (< 4 μmol CO_2 m^{-2} s^{-1} change in A with a 1400 μmol m^{-2} s^{-1} change in light), and normal. Of the six O_3-sensitive plants tested, three had no g_s response to changes in light, and three had a muted g_s response. All of the sensitive plants had muted A responses to changes in light. Of the four insensitive cutleaf coneflowers tested, one had a flat stomatal response with a muted A response, one had muted g_s and A responses, and two had normal g_s and A responses.

The magnitudes of the g_s and A responses differed significantly among the three groups (for summary statistics, see Table 1). The stomatal opening and closure rates in response to increased and decreased light intensities differed significantly among the three groups of stomatal responses. The up- and down-regulation of A in response to increased and decreased light intensities differed significantly among the three groups of stomatal responses. Ambient light intensities (light external to the cuvette) were similar throughout the experiment (~300 μmol m^{-2} s^{-1} ranging ± 50 μmol m^{-2} s^{-1}), and did not explain the response types.

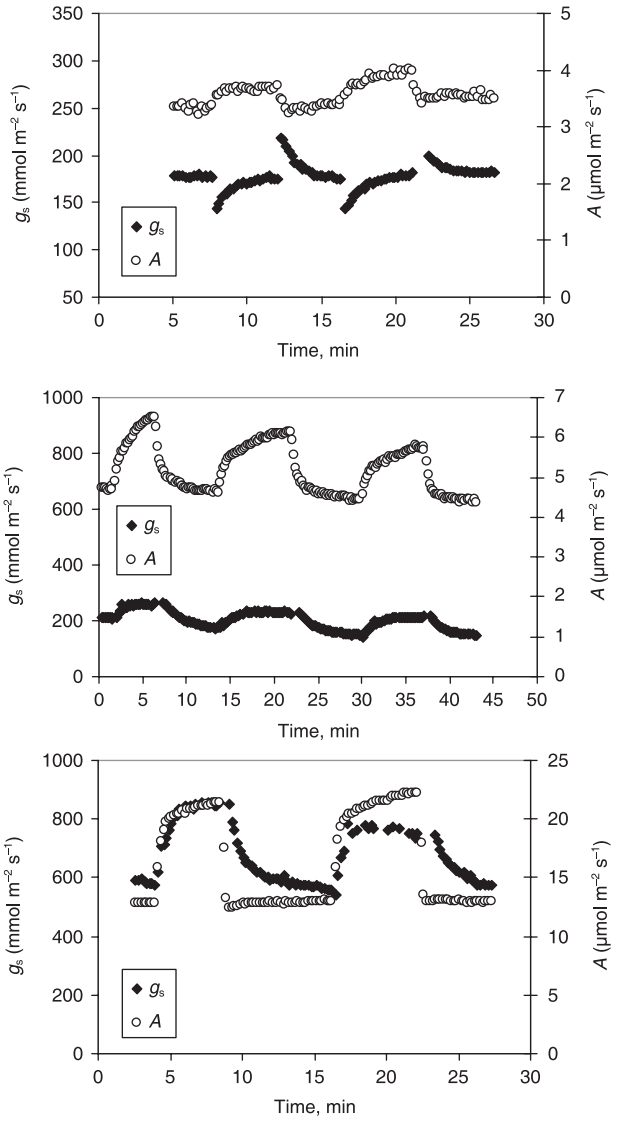

Fig. 2 Example of three types of stomatal behavior [no response (top), muted response (middle), and normal response (bottom)] and A response [muted (top) and normal (middle, bottom)] to changes from low (375 μmol m^{-2} s^{-1}) to high (1400 μmol m^{-2} s^{-1}) light. Several cycles of high and low light are presented. O_3-sensitive cutleaf coneflowers (*Rudbeckia laciniata* var. *digitata*) had no or muted g_s response; O_3-insensitive plants were represented in all response types, but the majority of plants had normal stomatal responses.

Table 1 Summary statistics for stomatal response from low (375 μmol m^{-2} s^{-1}) to high (1800 μmol m^{-2} s^{-1}) photosynthetic flux density (Q); cutleaf coneflower (*Rudbeckia laciniata* var. *digitata*) exhibited three stomatal responses: flat, muted, and normal (see Fig. 2)

	Flat	Muted	Normal	*P*
g_s closure rate	0.53 ± 1.9	9.1 ± 14.5	36.9 ± 49.6	0.002
g_s in low light	194 ± 53	207 ± 54.3	491 ± 188	0.007
g_s opening rate	0.3 ± 1.1	11.4 ± 12.8	61.5 ± 40.9	0.003
g_s in high light	193 ± 53	261 ± 67	641 ± 41	0.001
A decr in decr light	0.84 ± 0.31	0.52 ± 0.33	6.66 ± 1.04	0.017
A in low light	5.83 ± 1.68	3.97 ± 1.07	12.00 ± 2.38	0.093
A incr in incr light	0.72 ± 0.20	0.29 ± 0.27	3.18 ± 1.62	0.126
A in high light	6.81 ± 2.05	6.51 ± 1.97	18.25 ± 6.27	0.032
Ratio of sensitivity types	3 S : 1 I	3 S : 1 I	0 S : 2 I	

g_s, stomatal conductance; *A*, assimilation; decr, decrease; incr, increase; S, sensitive plants; I, insensitive plants.
The rate of stomatal closure and opening (g_s min^{-1}), the rate of up- and down-regulation of *A* (*A* min^{-1}), the value of g_s (mmol m^{-2} s^{-1}), *A* (μmol m^{-2} s^{-1}), and instantaneous transpiration (T) efficiency (ITE; *A*/*T* in mmol m^{-2} s^{-1}) at equilibrium in both high and low *Q* are given (mean, ± 1 standard error). The probability of significance of a one-way analysis of variance is also given (*P*).

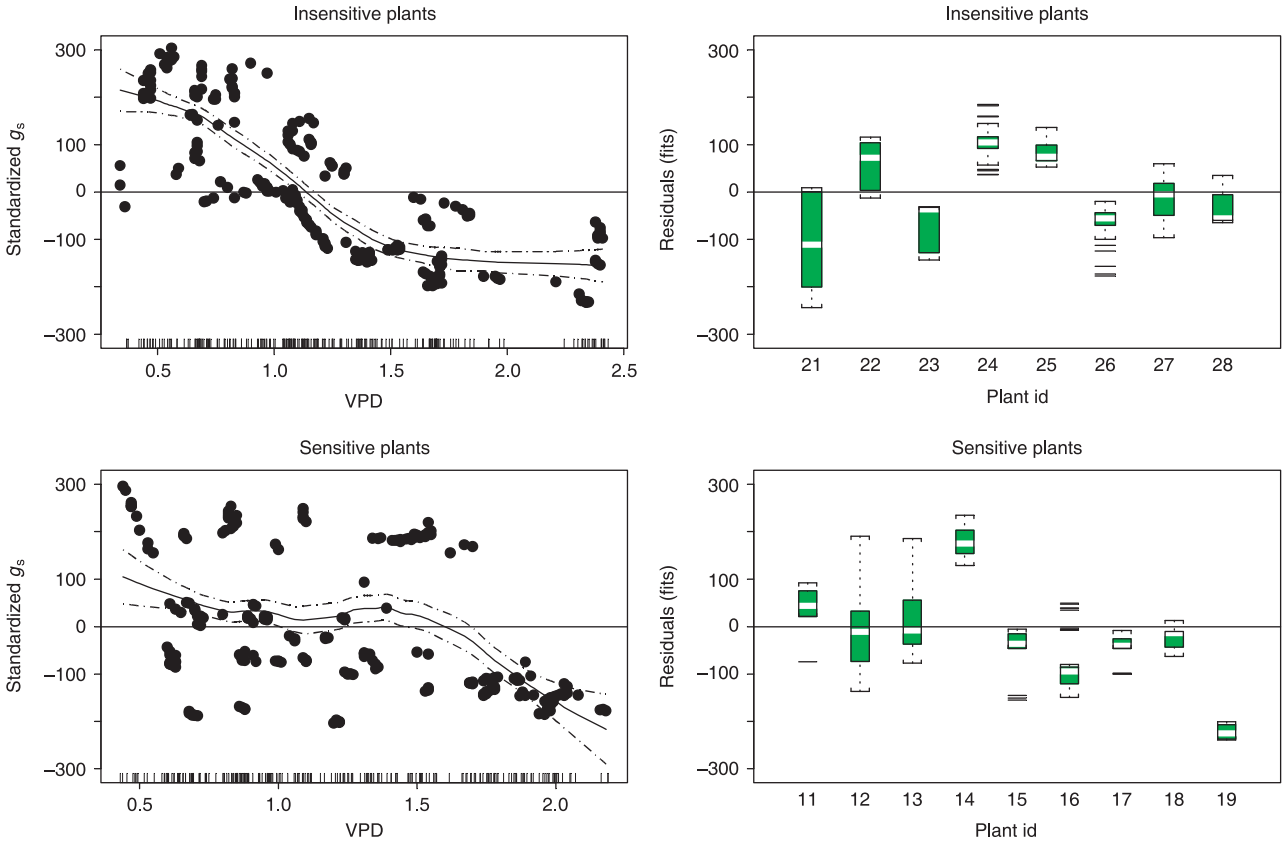

Fig. 3 Spline fit to stomatal conductance (g_s, mmol m^{-2} s^{-1}, *y*-axis) response to leaf-to-air vapor pressure deficit (VPD, kPa, *x*-axis) under 1400 μmol m^{-2} s^{-1} external photosynthetic photon flux density (Q). The zero-line value of the standardized g_s (G) is unique to each graph, and corresponds to the mean subpopulation g_s; the dotted lines indicate the 95% confidence intervals. Hatch marks on the *x*-axis indicate sampling frequency. The box plot on the right indicates mean 5% (white bar in middle), 67% of the observations (outlined box containing the shaded bar) and the maximum and minimum observed values (brackets) for each cutleaf coneflower (*Rudbeckia laciniata* var. *digitata*).

Dynamic responses to VPD

A spline function was fit to determine the response of g_s to incremental decreases in VPD for the O_3-sensitive and -insensitive cutleaf coneflowers (Fig. 3). The regression lines of the two O_3 sensitivity types differed significantly ($P < 0.001$). O_3-sensitive plants had no stomatal response, or a sluggish response, to declining VPD in the range from 0.5 to 1.5 kPa.

At greater VPD values, g_s declined with VPD. Insensitive cutleaf coneflowers exhibited a linear decline in g_s with increasing VPD from 0.5 to 1.5 kPa. At greater VPD values, no further change in g_s was observed. From a plot of the residuals, it can be seen that one of the sensitive plants had a high endogenous g_s (~472 mmol H_2O m^{-2} s^{-1}, plant number 14), and one had a low endogenous g_s (~99 mmol H_2O m^{-2} s^{-1}, plant number 19). These two plants had nearly flat responses to changes in VPD, suggesting that their stomata were physiologically inactive.

Steady-state stomatal responses

Under steady-state conditions, the g_s of insensitive plants was higher than that of sensitive plants, which would likely cause an increase, not a decrease, in foliar symptoms (Fig. 4). Stomatal conductance under nonlimiting VPD (0.5 kPa) and high light (1400 μmol m^{-2} s^{-1}) declined with increasing foliar injury.

The apparent quantum efficiency of insensitive plants was significantly greater (1.6×) than that of sensitive plants (Table 2). The light compensation point was significantly lower in insensitive plants: more light was required for positive carbon balance in O_3-sensitive cutleaf coneflowers. For insensitive plants, the carboxylation efficiency was significantly greater (2.6×) than that of sensitive plants. The maximum rate of assimilation under saturating light and CO_2 was also significantly greater (1.5×) for insensitive plants.

The intrinsic transpiration efficiency (ITE, A/T) was calculated at equilibrium to test for differences between O_3-sensitive and -insensitive cutleaf coneflowers (Table 2). Insensitive plants had a significantly greater ITE, and sensitive plants had lower CO_2 uptake for every mole of H_2O lost.

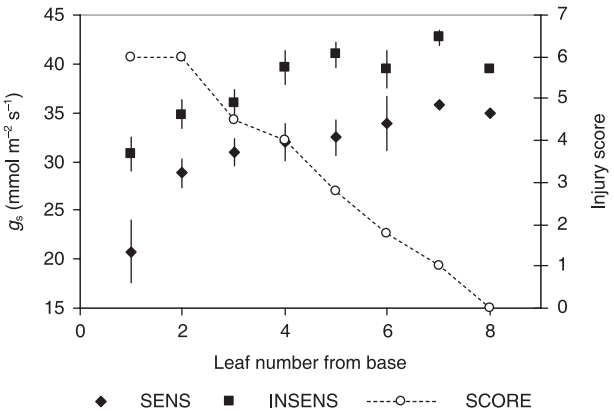

Fig. 4 Relationship between leaf position and maximum stomatal conductance (g_s) for symptomatic, O_3-sensitive (diamonds) and asymptomatic, insensitive (squares) cutleaf coneflowers (*Rudbeckia laciniata* var. *digitata*). The foliar injury scores for leaves on the O_3-sensitive plants were assigned according to our modified injury index (open circles). Symbols represent the mean g_s of five sensitive and four insensitive plants, ± 1 standard error.

Table 2 Statistical summary of steady-state attributes for ozone (O_3)-sensitive and -insensitive cutleaf coneflowers (*Rudbeckia laciniata* var. *digitata*)

Attribute	O_3-sensitive	O_3-insensitive	*P*
Quantum efficiency	0.031 ± 0.002	0.048 ± 0.003	0.014
Compensation point	17 ± 5	3 ± 1	0.046
Carboxylation efficiency	0.017 ± 0.002	0.045 ± 0.006	0.001
A (1800)	12.4 ± 1.4	18.4 ± 2.2	0.046
ITE (1800)	3.1 ± 0.5	5.6 ± 0.4	0.003
ITE (375)	4.2 ± 0.4	10.8 ± 0.6	0.000

The average apparent quantum efficiency is given in μmol CO_2 m^{-2} s^{-1} μmol^{-1} m^{-2} s^{-1} of light (*Q*), the light compensation point is given in *Q*, the carboxylation efficiency is given in μmol CO_2 m^{-2} s^{-1} per ppm substomatal CO_2 concentration, and assimilation in saturating light (*Q*) [*A* (1800)] is given in μmol m^{-2} s^{-1}. At equilibrium, the instantaneous transpirational efficiency (ITE; μmol CO_2 $mmol^{-1}$ H_2O) is given in high and low *Q* (1800 and 375, respectively). For all attributes, the mean values ± 1 standard error are given. The probability of significance of a one-way analysis of variance is also given (*P*).

Table 3 Summary statistics for morphological attributes of ozone (O_3)-sensitive and -insensitive cutleaf coneflowers (*Rudbeckia laciniata* var. *digitata*)

	O_3-sensitive	O_3-insensitive
Leaf number for measurements	7.1 ± 0.4	7.6 ± 0.2
Number dead below	4.1 ± 0.6	2.1 ± 0.6
Number live below	2.0 ± 0.4	4.5 ± 0.6
Total number of leaves	11.9 ± 0.8	11.9 ± 0.4
Total number of live leaves	7.8 ± 0.7	9.8 ± 0.6
Injury score	4.1 ± 0.2	0 ± 0

Differences in *A* (assimilation) contributed to a greater extent to the changes in ITE than did differences in g_s between the two sensitivity types.

Morphological traits of O_3-sensitive and -insensitive plants

The leaf positions sampled for gas exchange measurements were similar between the O_3-sensitive and -insensitive cutleaf coneflowers (Table 3). Total numbers of leaves produced on the two types of plant were also similar, but, on average, there were two more dead leaves per plant in O_3-sensitive plants. O_3-sensitive plants had an average injury score of 4 (51–75%), but scores ranged from 3 to 5, whereas insensitive plants had average foliar injury ratings of < 2.

Stomata were located mainly on the abaxial surface, and there were no differences in the absolute densities for either surface between O_3-sensitive and -insensitive individuals. The ratio of abaxial to adaxial stomatal density did not differ significantly between O_3-sensitive and -insensitive foliage

(0.94 ± 0.03 and 0.91 ± 0.03 (± 1 S.E.), respectively; $P = 0.097$). A uniform stomatal ratio (0.93) was applied in all gas exchange measurements.

Differences in water relations between O_3-sensitive and -insensitive plants

There were no statistically significant differences between O_3-sensitive and -insensitive cutleaf coneflowers with regard to predawn or noon Ψ_T, Ψ_{S+M}, or Ψ_P (data not presented).

Discussion

Over the last 5 years, we have reported on variation in symptom expression in cutleaf coneflower (Chappelka *et al.*, 2003), the genetic make-up of populations and the effects of environment on symptoms (Davison *et al.*, 2003), the flux of O_3 to the leaves during development (Davison *et al.*, 2003; Finkelstein *et al.*, 2004), and biochemical antioxidant capacity (Burkey *et al.*, 2006). The research reported here is the first study to describe physiological aspects of O_3-insensitive and -sensitive cutleaf coneflowers under field conditions.

O_3-sensitive cutleaf coneflowers exposed to low, ambient O_3 exposures, but with level 4 foliar injury symptoms (51–75%), had little or no stomatal response to large changes in light intensity, and the rate of stomatal opening or closing was significantly lower than that of insensitive plants with a 'normal' stomatal response, whose foliage had less than level 2 injury symptoms (7–25%). Response of A to changes in light in sensitive plants was always muted relative to that of insensitive plants with a 'normal' stomatal response. In insensitive plants with normal stomatal responses to rapid changes in light, A was tightly coupled with g_s. At each light intensity or VPD value, full stabilization was achieved before the next adjustment was made, as suggested by Maier-Maercker & Koch (1992). Interestingly, one insensitive individual did not exhibit stomatal adjustments to changes in light. It is possible that this plant may have been a sensitive genotype, and would have become symptomatic if given a higher ambient O_3 exposure, but the threshold O_3 concentration to induce visible symptoms was higher than that of other sensitive plants. It should be noted that O_3 exposures during the summer when this study was conducted were among the lowest in the past 15 years, as a result of unusually wet and cool weather that year.

Sluggish stomatal responses to changes in light during O_3 exposure were first observed in 1984 (*Populus deltoides* × *trichocarpa*, Reich & Lassoie, 1984; *Picea abies* and *Abies alba*, Keller & Häsler, 1984). Stomatal response of *Helianthus annuus* to changing light intensities during O_3 exposure was 13–15 min slower than for control plants (Younglove *et al.*, 1988). Sluggish stomatal responses to changes in light during or after O_3 exposure have continued to be reported by numerous authors across a range of species (*P. abies*, Keller & Häsler, 1987; Wieser & Havranek, 1993; *Pinus ponderosa*, Grulke & Preisler, 2002; *Quercus kellogii*, Grulke *et al.*, 2005; *Arbutus unedo*, Paoletti, 2005). Our research describes physiological aberrations in stomatal functioning for plants of differing O_3 sensitivity type under low ambient O_3 exposures in the field. The comparison of sensitive and insensitive plants at ambient O_3 concentrations was an effective tool for estimating effects of O_3 in natural ecosystems, as has been shown for such model indicator plants as white clover (*Trifolium repens*, Heagle *et al.*, 1994).

O_3-sensitive cutleaf coneflowers had a sluggish stomatal response to changes in VPD relative to insensitive plants. Sluggish stomatal responses to changes in VPD during O_3 exposure were reported for *P. abies* (Maier-Maercker & Koch, 1992 and Maier-Maercker, 1989, as reported in the former). Similar findings with long-term O_3 exposure have also been obtained for *Pinus sylvestris* (Kellomäki & Wang, 1997) and are similar to our observations of sensitive cutleaf coneflowers, i.e. a muted stomatal response to VPD. Although both sensitive and insensitive *Pinus jeffreyi* had a linear response to decreased VPD, the rate of stomatal closure was slower in the sensitive phenotype (Patterson & Rundel, 1989) when exposed to moderate, ambient O_3 concentrations in Sequoia National Park, CA.

Under steady-state conditions, the ITE of sensitive plants was significantly lower than that of insensitive plants at both low (375 Q) and high (1800 Q) light intensities, despite no measurable difference in plant water status between the two sensitivity types. Insensitive plants had much greater ITE than sensitive plants at low light, probably because stomata were more effectively closed. The differences in ITE between sensitivity types were a result of changes in both A and g_s, but were dominated by changes in A. *Betula pendula* (Matyssek *et al.*, 1991) and *Acer saccharum* (Tjoelker *et al.*, 1995) experimentally exposed to high O_3 concentrations had significant reductions in foliar water use efficiency (WUE; Tjoelker *et al.*, 1995). Older leaves had much lower WUE than younger leaves (produced in the same year) in more polluted areas relative to less polluted sites in cloned *Populus deltoides* (Gregg *et al.*, 2003). Although sun flecks in the field can cause significant, transitory drops in Ψ_T (A. W. Davison, unpublished data), the data presented here for cutleaf coneflowers were obtained during periods of overcast sky (~300 Q) or before direct sunlight in the morning.

Stomata control O_3 uptake, and there is a great deal of current interest in the regulation of conductance and estimation of O_3 flux (Emberson *et al.*, 2000). In general (and in most physiological modeling), plant physiologists assume that stomatal aperture is tightly coupled to A and governed by C_i. Under chronic low, or short moderate levels of O_3 exposure, a change in A is accompanied by a proportional change in g_s for many plant species (pines, hardwoods, and crops, Reich, 1987; *Pinus taeda*, Sasek & Richardson, 1989; *Solanum tuberosum*, Dann & Pell, 1989; *P. abies*, Schweizer & Arndt, 1990; *P. ponderosa*, Weber *et al.*, 1993; *P. sylvestris*, Kellomäki

& Wang, 1997; *Populus tremuloides*, Noormets *et al.*, 2001). In the above examples, a reduction in A is the primary response to O_3 exposure, and a reduction in g_s is a secondary response to guard cell sensing of increased C_i from O_3-induced reductions in A.

From comparisons of stomatal responses described above, it appears that O_3-sensitive plants respond similarly to plants exposed to experimentally elevated O_3. O_3-sensitive plants may simply have a lower injury threshold to the same O_3 exposure experienced by insensitive plants. With higher, longer term exposures (as described in other studies above), and as shown here with sensitive cutleaf coneflowers in low ambient O_3, sluggish stomatal responses to changes in environmental conditions (e.g. light and VPD) were most likely primary responses to O_3, not secondary responses to changes in A. For example, in some O_3-sensitive cutleaf coneflowers, g_s was not responsive to changes in light and was not coupled to A. This has been previously observed in other herbaceous species, such as *Plantago major*, where O_3 reduced g_s, but did not always reduce A (Reiling & Davison, 1995; Zheng *et al.*, 2000).

The mechanisms responsible for sluggish stomatal responses during O_3 exposure are not wholly understood. O_3 exposure mediates increases in apoplastic H_2O_2, which alters membrane permeability, specifically to cation influx (Castillo & Heath, 1990; Torsethaugen *et al.*, 1999; Pei *et al.*, 2000; McAinsh *et al.*, 2002). Although we found no statistically significant differences in the degree of drought stress experienced by the two O_3 sensitivity types, if poor stomatal control resulted in a net increase in transpiration, abscisic acid could further modify the H_2O_2-mediated membrane hyperpolarization and cation influx (Pei *et al.*, 2000). Furthermore, guard cell zeaxanthin modulates CO_2-dependent changes in stomatal aperture (Zhu *et al.*, 1998), and its oxidation state and activity are directly modified by O_3 exposure. In Norway spruce, sluggish stomatal response to O_3 exposure was attributable to reduced cell wall lignification. Reduced cell wall lignification resulted in greater g_s, but also in slower stomatal movement because cellulose has a higher affinity for water than lignin (Maier-Maercker & Koch, 1992). These mechanisms suggest potential primary effects of O_3 exposure on the guard or subsidiary cells, or on the surrounding epidermal cells.

O_3-insensitive cutleaf coneflowers had greater g_s than sensitive plants, which would lead to more, not less, O_3 uptake and foliar injury. O_3-insensitive tobacco (*Nicotiana tabacum*) also had greater g_s than O_3-sensitive plants (Pasqualini *et al.*, 2002), also suggesting that sensitivity was not attributable to greater O_3 uptake. There were no physical or morphological attributes identified that could have led to increased O_3 sensitivity. In cutleaf coneflowers, the foliar injury (purple-brownish stippling) was confined to the adaxial surface; the abaxial surface was green, with no visible cellular injury. However, leaves with level 4 injury also had lower chlorophyll concentrations (Neufeld *et al.*, 2006). The sluggish stomatal responses observed in sensitive coneflower plants suggest that there may also be internal cues governing stomatal behavior once foliar injury is observed. Some possible candidates might include enhanced ethylene production and premature leaf senescence, induced by hyper-sensitivity to O_3 exposure in sensitive plants.

Carbon acquisition attributes differed significantly between O_3 sensitivity types of cutleaf coneflower. O_3-insensitive plants had greater maximum assimilation rates under saturating light, and greater quantum and carboxylation efficiencies, all contributing to a more positive plant carbon balance. Although these plants grow in a highly exposed sun environment on the margin of the forest and meadow, the very low light compensation point of insensitive plants suggests adaptation to, and persistence in, small forest gaps. O_3-sensitive plants had higher light requirements for positive carbon balance, similar to that of symptomatic giant sequoia seedlings (*Sequoiadendron giganteum* (Lindl.) J. Buchholz; Grulke *et al.*, 1989), another species adapted to establishing in forest gaps. Attributes contributing to greater positive carbon balance in insensitive coneflower could improve the antioxidant defenses or provide more energy for reparation activities. An earlier study found that starch concentrations were slightly higher in leaves and rhizomes of insensitive coneflowers (Peoples, 2005).

Sensitive plants appear to have collections of physiological attributes that predispose them to be susceptible to lower levels of O_3 exposure than insensitive plants (Heath & Taylor, 1997). In addition, both sensitive and insensitive cutleaf coneflowers had high variability within a trait, but not necessarily in all traits. For example, sensitive plants had significant variability in response to changes in VPD while insensitive plants had a uniform response. Sensitive plants had either no stomatal response or a muted response to changes in light intensity. Insensitive plants were represented in each of the stomatal responses observed: no response (one plant), muted response (one plant), and normal response (two plants). A high degree of variability of multiple traits related to O_3-sensitivity has also been found in wild populations of brown knapweed (*Centaurea jacea*) (Bassin *et al.*, 2004). Randomly varying collections of physiological attributes, some of which lead to sensitivity and others of which permit insensitivity, do not lend themselves to incorporating biochemical 'thresholds' of response in modeling efforts (Martin *et al.*, 2001). The whole-plant response to O_3 is a complex of independently varying physiological attributes that result in varying degrees of sensitivity among individual plants.

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