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Elevated CO_2 reduces disease incidence and severity of a red maple fungal pathogen via changes in host physiology and leaf chemistry

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Abstract

Atmospheric CO_2 concentrations are predicted to double within the next century. Despite this trend, the extent and mechanisms through which elevated CO_2 affects plant diseases remain uncertain. In this study, we assessed how elevated CO_2 affects a foliar fungal pathogen, *Phyllosticta minima*, of *Acer rubrum* growing in the understory at the Duke Forest free-air CO_2 enrichment experiment in Durham, North Carolina. Surveys of *A. rubrum* saplings in the 6th, 7th, and 8th years of the CO_2 exposure revealed that elevated CO_2 significantly reduced disease incidence, with 22%, 27%, and 8% fewer saplings and 14%, 4%, and 5% fewer leaves infected per plant in the three consecutive years, respectively. Elevated CO_2 also significantly reduced disease severity in infected plants in all years (e.g. mean lesion area reduced 35%, 50%, and 10% in 2002, 2003, and 2004, respectively). To assess the mechanisms underlying these changes, we combined leaf structural, physiological and chemical analyses with growth chamber studies of *P. minima* growth and host infection. *In vitro* exponential growth rates of *P. minima* were enhanced by 17% under elevated CO_2, discounting the possibility that disease reductions were because of direct negative effects of elevated CO_2 on fungal performance. Scanning electron micrographs (SEM) verified that conidia germ tubes of *P. minima* infect *A. rubrum* leaves by entering through the stomata. While stomatal size and density were unchanged, stomatal conductance was reduced by 21–36% under elevated CO_2, providing smaller openings for infecting germ tubes. Reduced disease severity under elevated CO_2 was likely due to altered leaf chemistry and reduced nutritive quality; elevated CO_2 reduced leaf N by 20% and increased the C : N ratio by 20%, total phenolics by 15%, and tannins by 14% ($P < 0.05$ for each factor). The potential dual mechanism we describe here of reduced stomatal opening and altered leaf chemistry that results in reduced disease incidence and severity under elevated CO_2 may be prevalent in many plant pathosystems where the pathogen targets the stomata.

Keywords: climate change, Duke FACE, elevated CO_2, fungal plant pathogen, free-air CO_2 enrichment, plant disease, plant–pathogen interactions, red maple

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Introduction

Atmospheric CO_2 concentrations are rapidly increasing and are expected to double in the next century (IPCC, 2001). The growth and physiological responses of numerous plant species to elevated CO_2 are well documented (reviewed in Drake *et al*., 1997; Saxe *et al*., 1998). Despite the vast number of studies documenting plant responses to elevated CO_2, few studies have assessed how plant interactions with pathogens will change under future climatic conditions (Coakley *et al*., 1999; Chakraborty *et al*., 2000a; Percy *et al*., 2002; Runion, 2003).

Pathogens drastically reduce plant growth in agricultural and natural ecosystems worldwide. Current

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estimates of crop losses to all plant pathogens in the US alone are ~ $33 billion annually (Pimentel *et al*., 2000). The makeup and functioning of natural ecosystems can also change dramatically because of pathogens. In extreme cases, entire species may disappear, such as the American Chestnut *Castanea dontata* decimated by the fungal pathogen, *Cryphonectria parasitica* (Agrios, 1997). Despite the paramount importance of plant disease to agricultural and natural ecosystems, little is known of how disease will be affected by global climate change.

The expression of disease symptoms is influenced by three main components: (1) host, (2) pathogen, and (3) environmental conditions. A change in any single component can greatly alter the magnitude of symptoms. For example, many plants exhibit increased susceptibility to disease during drought (Boyer, 1995). Because plant predisposition to disease is altered by abiotic factors, changes in environmental variables such as elevated CO_2 will also likely affect the severity and range of pathogens. Understanding such relationships is vital to making predictions about overall plant health and for managing agricultural and natural ecosystems in the future.

Structural, physiological, and chemical changes are common to many plants grown under elevated CO_2 conditions and could also alter interactions with microbial pathogens (Coakley *et al*., 1999; Chakraborty *et al*., 2000a; Gill *et al*., 2002; Karnosky *et al*., 2002). Such changes include reduced stomatal density (SD), size and/or conductance, reduced leaf nutritional quality, increased defensive compounds, and increased leaf wax (Jackson *et al*., 1994; Field *et al*., 1995; Drake *et al*., 1997; Saxe *et al*., 1998; Urban, 2003). Because many plant pathogens infect leaves through the stomata (Agrios, 1997), any changes in stomatal structure and function induced by elevated CO_2 may affect the infection process (Coakley *et al*., 1999; Chakraborty *et al*., 2000a). For example, reductions in SD and aperture could provide pathogens with fewer and smaller points of entry, respectively. In addition, once infection occurs, changes in leaf chemistry induced by elevated CO_2 may alter the severity of infection.

In the current study, we assessed how elevated CO_2 affects the susceptibility of *Acer rubrum* (red maple) to foliar leaf spot disease. This common disease is caused by the fungus *Phyllosticta minima*. Species of the genus *Phyllosticta* affect hundreds of economically important plant species globally, including corn, soybean, orchids, and numerous tree species. We examined the mechanisms governing changes in infection for both fungal and plant characteristics, including changes in *P. minima* growth and mode of infection as well as *A. rubrum* leaf physiology, structure, and chemical composition with elevated CO_2. We examined these interactions in the 6th, 7th, and 8th years of the Duke Forest free-air CO_2 enrichment (FACE) experiment and in accompanying growth chamber and pathogen-culture studies.

Materials and methods

Field site description

The Duke FACE experiment is located in the Blackwood Division of the Duke Forest, Orange County, NC, USA (35°97′N 79°09′W). The site contains a loblolly pine (*Pinus taeda*) plantation established in 1983. Since then, the plantation has not been managed to prevent other plant species from establishing. The most abundant understory tree species include red maple (*A. rubrum*), sweetgum (*Liquidambar styraciflua*), eastern red bud (*Cercis canadensis*), and winged elm (*Ulmus alata*). This forest grows on nutrient-poor, clay-rich loam soil that is typical of many upland areas of southeastern USA. Additional site details are available in DeLucia *et al*. (1999).

Within the Duke FACE site, six 30-m diameter circular plots (rings) were established in 1996. Each ring is equipped with 32 vertical pipes that extend from the forest floor through the canopy and deliver either elevated CO_2 or ambient air. Three experimental rings are fumigated with CO_2 to raise the atmospheric CO_2 concentration $200\,\mu L\,L^{-1}$ above ambient (elevated). Three additional rings are fumigated with ambient air only and serve as controls to accommodate any effects of air movement on the vegetation (ambient). Further details about the use of FACE technology at this site are available in Hendrey *et al*. (1999).

Pathosystem description: host plant, fungal pathogen, and disease verification

A. rubrum is one of the most widely distributed and locally abundant tree species in North America. Its range extends from southeastern Manitoba to southern Florida and as far west as eastern Texas (Walters & Yawney, 1990). *A. rubrum* is deciduous and occurs in a broad range of habitats, from wetlands to drier upland forests, and across an extreme range of soil types, textures, and pH. It occurs as both an understory and canopy species and as a dominant or co-dominant species in many plant communities of Southeast USA (Gleason & Cronquist, 1991).

The fungus *P. minima* (Berk. & M. A. Curtis) Underw. & Earle causes an eye spot or a purple-bordered, tan-centered, leaf spot of ~ 5 mm diameter on *A. rubrum*. The disease occurs nearly everywhere red maples grow, and can occasionally become severe and cause partial

defoliation (Sinclair *et al*., 1987). The symptomatic leaf spots are irregularly round and with pycnidia containing conidial spores generally open to the upper leaf surface and usually arranged in a circle in the tan central portion of the lesion. If infection is severe, lesions may coalesce to form large, irregular dead areas. Species of *Phyllosticta* that infect deciduous plants are generally assumed to overwinter in fallen leaves, in buds, or on twigs, and to produce spores in spring that disperse to new foliage and that start another cycle of disease (Sinclair *et al*., 1987).

P. minima occurs abundantly on *A. rubrum* throughout the Duke Forest, and a preliminary survey confirmed the presence of *P. minina* in all Duke FACE rings. Dr Gloria Abad at the Plant Disease Clinic at North Carolina State University verified our identification of *P. minima* as the causal agent of the leaf spot on *A. rubrum*. For this verification, *P. minima* was isolated from *A. rubrum* tissue collected at Duke FACE and initially cultured in water agar media at 22 °C. The culture was then transferred to 30% potato dextrose agar (PDA) medium where it produced abundant pycnidia and spores after growing under continuous fluorescent light for 7–10 days. Anatomical characteristics of these structures matched descriptions for *P. minima* and reconfirmed it as the causal organism.

Disease incidence and severity assessment (digital analysis of images)

In all years of the study, we surveyed disease incidence and severity on even-aged *A. rubrum* saplings planted in herbivore exclosures within the Duke FACE rings. The saplings were originally established in eight 1.44 m^2 exclosures inside the canopy of each experimental ring as part of herbivory study in the summer of 1998 (see Mohan, 2002 for details). At the time of the initial planting, *A. rubrum* represented $\sim 10\%$ of the saplings in these plots. We used the saplings in the exclosure plots to preclude damage from herbivory and to obtain quantitative assessments of disease.

We surveyed all *A. rubrum* saplings in each of the 48 exclosures ($n \geq 76$ for both ambient and elevated CO_2 in each year of the study) for disease incidence (% saplings infected and % leaves of each sapling with infection). We also sampled one randomly selected *A. rubrum* in each exclosure for disease severity (mean lesion area and lesion area per leaf area; 24 plants in elevated and 24 plants in ambient CO_2). We used a Nikon CoolPix 995 digital camera (Nikon Corporation, Tokyo, Japan) set at fine quality and medium range optical zoom to capture images of all leaves on the study plants. Each intact *A. rubrum* leaf was photographed *in situ* over a background of graph paper (one block $\simeq 40.1\ mm^2$) for scale during image capture. In the lab, images were converted to tif format using Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA, USA; version 5.5) and then transferred to Scion Image software (Scion Corporation, Frederick, MD, USA) to measure total leaf area (mm^2) and *P. minima* lesion area (mm^2). Each lesion on diseased leaves was analyzed and recorded separately. To assess the changes across years, we monitored disease severity on these 48 individuals for 3 years at the Duke FACE site.

Stomatal impressions and leaf gas exchange

To measure SD and size, casts were made of one leaf each from 12 to 15 *A. rubrum* in each Duke FACE ring on 18 October 2002. To preserve leaf material of the saplings in the herbivore exclosures, these casts were made on other *A. rubrum* in the treatment rings. Each cast consisted of a 2–3 cm leaf section made by pressing the abaxial leaf surface onto a plastic slide covered with polyvinylsiloxane dental impression material ('Extrude' Medium; Kerr Manufacturing Co., Orange, CA, USA) until the polymer hardened (Williams & Green, 1988; Maherali *et al*., 2002). Each cast was analyzed using scanning electron microscopy (see ESEM description below) under fossil mode at 400 × magnification. For each impression, five field-of-view images were taken using ESEM and analyzed using NIH Image 1–58 (US National Institutes of Health; http://rsb.info.nih.gov/nih-image/). SD (the number of stomata per mm^2) was counted and averaged across each ring and CO_2 treatment to avoid pseudoreplication. Length of the stomatal aperture (AP) was calculated by measuring the length between the junctions of the guard cells at each end of the stomata (Malone *et al*., 1993).

Stomatal conductance (g_s) was measured on *A. rubrum* saplings in the herbivore exclosures using a Li-Cor 6400 photosynthetic system (Li-Cor Inc., Lincoln, NE, USA). Measurements of g_s were made on the most recent fully expanded leaf between 1045 and 1245 h on 27 June and 16 August 2003 (during the second summer of our experiment).

Growth chamber experiments

To study the mechanism of *P. minima* infection in a controlled environment and independent of CO_2 effects, *A. rubrum* saplings were grown at ambient CO_2 in growth chambers at the National Phytotron at Duke. Dormant two-year-old saplings from a nursery were transplanted individually in pots, watered twice daily, and fertilized three times a week with half-strength Hoagland's solution. To simulate late spring conditions, the saplings were grown in a 14-h photoperiod

and a temperature regime of 24 °C : 12 °C day : night. Five fully expanded leaves on each sapling were inoculated with approximately 1.0 mL of *P. minima* spore suspension (concentration = 10^6 spores per mL) by misting each leaf (Kuo & Hoch, 1996). All leaves were then bagged with clear plastic for 18 h to insure a high relative humidity and spore germination. At 12, 24, and 48 h after inoculation, leaf material was collected to document the infection process, sputter coated (Anatech Hummer 6.2 sputter coater; Anatech Ltd, Demer, NC, USA), and viewed using a Philips XL 30 Environmental Scanning Electron Microscope (ESEM; FEI Company, Eindhoven, The Netherlands) in the Biological Sciences SEM Facility at Duke University for signs of spore germination.

Axenic culture growth of P. mimina

To test for direct CO_2 effects on fungal growth, we axenically cultured *P. minima* on agar plates in growth chambers at the National Phytotron. *P. minima* used for inoculations and cultures were collected from *A. rubrum* trees outside Duke FACE rings to prevent bias of the previous growing condition (i.e. ambient or elevated CO_2) on fungal performance. We inoculated thirty 30% PDA plates ($n = 15$ randomly assigned to each CO_2 treatment) with a small section ($\simeq 8.0\,mm^2$) of *P. minima* cultured on PDA. Two growth chambers were used, one at ambient CO_2 (360 ppm) and the other at elevated CO_2 (560 ppm). CO_2 settings of the two chambers were reversed three times during the experiment and the samples were switched between chambers to control for any chamber effect. Except for CO_2 levels, growth chamber conditions were the same as described above. Digital images of each fungal plate were captured in the first 24 h and then every 2 days starting 1 day after initial plate preparation. These images were processed with the digital imaging software SCION to measure the radial growth of the fungus over time.

Leaf wax and chemistry analysis

Leaf C and N content was determined for 24 fully expanded *A. rubrum* leaves on saplings from a growth chamber experiment in which both CO_2 levels and chamber conditions were the same as described above. Leaf disks ($\sim 3.0\,cm^2$) were harvested from green leaf material and dried at 65 °C for 14 days. Within each CO_2 treatment, four disks were randomly selected and pooled to form one sample ($n = 6$ for each CO_2 treatment). The pooled sample was ground with a ball grinder and combusted in a CE Elantech NC 2100 Elemental Analyzer (Thermoquest Italia, Milan, Italy) for leaf C and N analysis. Leaf C and N measurements on *A. rubrum* leaves from Duke FACE showed results similar to ours from growth chambers (Finzi & Schlesinger, 2002).

Total phenolic and tannin content was determined using the Folin–Ciocalteu method (Makkar, 2003) on leaves from 24 replicate *A. rubrum* saplings for each CO_2 treatment of the growth chamber experiment. A disk ($\sim 3.0\,cm^2$) was punched from each leaf, placed in an aluminum foil envelope, flash frozen in liquid N_2, and stored at −80 °C until analysis. Within both elevated and ambient CO_2 treatments, four disks were randomly selected and pooled to form one sample ($n = 6$ for each CO_2 treatment). Frozen samples were vacuum freeze dried (Labconco Freezone 12 Freeze Dry System; Labconco Corporation, Kansas City, MO, USA), ground with a ball grinder, prepared according to Makkar (2003), and measured for sample absorbance at 725 nm on a Perkin-Elmer Lambda 3B UV/VIS spectrophotometer (Perkin–Elmer Corporation, Norwalk, CT, USA. Total phenolics as tannic-acid equivalent was calculated from a tannic acid calibration curve (Makkar, 2003). For tannin determination, an aliquot of the supernatant was treated with insoluble polyvinyl pyrrolidone (PVPP) (Sigma Chemical Company, St Louis, MO, USA), which binds tannins and removes them from the solution. Tannin content was calculated by subtracting the total phenolic content of the PVPP-treated sub-sample from the total phenolic content of the first solution.

Epicuticular wax concentration was determined for six fully expanded leaves from each ring at the Duke FACE site. Five leaf disks ($\sim 3.0\,cm^2$ each) were punched from each of the six leaves to exclude primary veins from material analyzed and to strictly control the leaf area sampled. Wax was stripped from the leaf material using two successive chloroform (HPLC grade) rinses (5.0 mL) in separate vials. The solvent/wax solution was filtered and the solvent was subsequently evaporated in a vacuum dessicator under N_2-saturated air. The remaining epicuticular wax was weighed and expressed as $\mu g\,cm^{-2}$ leaf area (Karnosky *et al.*, 2002).

Statistical analysis

We used a nested analysis of data values within a ring to avoid pseudoreplication. Treatment effects on disease incidence, severity, SD, aperture length, g_s, C : N ratio, %N, total phenols, tannins, and epicuticular wax were pooled within rings and evaluated using analysis of variance (ANOVA) with CO_2 as the main effect (SAS Institute, Cary, NC). A repeated measures ANOVA was used to compare radial growth rate of *P. minima*

cultures exposed to ambient or elevated CO_2 conditions over time. Radial growth data were also natural log transformed and the slopes from linear regressions of the exponential growth portion of the curves (i.e. growth rates) were analyzed for a CO_2 effect with ANOVA.

Results

Throughout the study, we found that the incidence and severity of leaf spot disease were significantly reduced on *A. rubrum* saplings growing at elevated CO_2 in the field (Fig. 1a–d). Fewer saplings from elevated CO_2 rings were infected in all years (Fig. 1a, $P = 0.003$, 22%, 27%, and 8%, 2002, 2003, and 2004, respectively) and significantly fewer leaves were infected per plant (Fig. 1b, 2002 only $P = 0.074$; 14%, 4%, and 5% less each year, respectively). Similarly, disease severity was reduced under elevated CO_2; elevated CO_2 reduced lesion area per leaf area by 26%, 45%, and 6% (Fig. 1c) ($P = 0.005$) and reduced mean lesion area by 35%, 50%, and 10% in the three consecutive years, respectively (Fig. 1d) ($P = 0.045$).

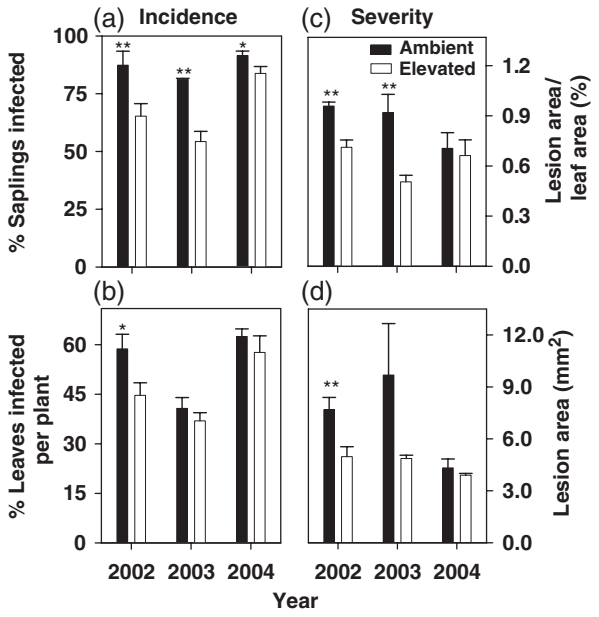

Fig. 1 *Phyllosticta minima* leaf spot disease incidence (a and b) and severity (c and d) measured on understory *Acer rubrum* saplings in August 2002 and 2003 and July 2004. The plants were growing in ambient and elevated CO_2 at the Duke FACE site, Durham, NC. Measures of disease incidence are percentage of saplings infected (a) and percentage of leaves infected per infected plant (b), and for disease severity they are lesion area/ leaf area (c) and mean lesion area (d). Paired treatment bars within a year are significantly different at $P \leq 0.05$ (**) or $P \leq 0.10$ (*). Statistical analysis results: (a) overall: $F = 12.315$, $P = 0.003$; (b) overall $F = 2.099$, $P = 0.167$; (c) overall $F = 10.327$, $P = 0.005$; (d) overall $F = 4.744$, $P = 0.045$.

P. minima infected *A. rubrum* leaves by entering through the stomata, a mechanism common to many fungal foliar pathogens (Fig. 2a–c). The SEM images

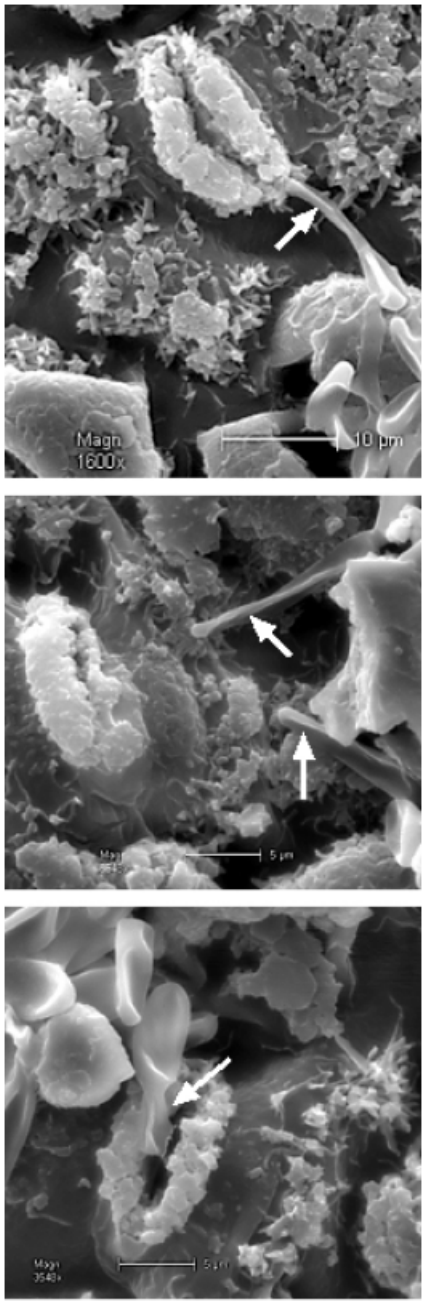

Fig. 2 Environmental scanning electron micrographs of *Phyllosticta minima* spores germinating on *Acer rubrum* leaf surfaces of saplings grown in Duke Phytotron growth chambers. Images were used to verify that germ tubes (white arrows) of *P. minima* conidia target *A. rubrum* stomata in order to gain entry to the leaves. Images were captured in the Biological Sciences Scanning Electron Microscopy lab at Duke University. We found no evidence of appresoria or penetration pegs that would indicate that the fungus enters leaves via forceful entry.

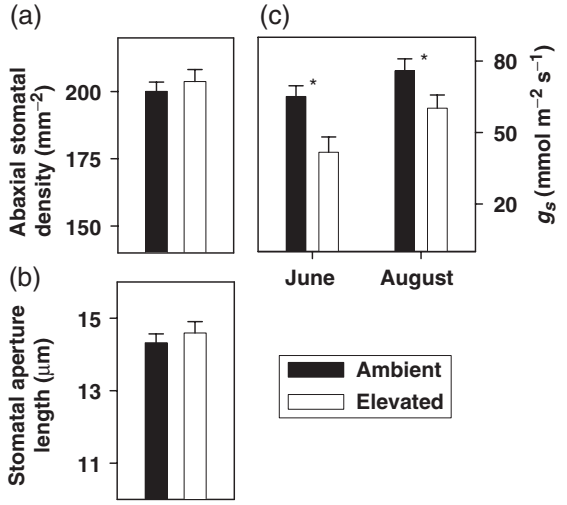

Fig. 3 Structural characteristics (abaxial stomatal density (a) and length of the stomatal aperture (b); mean ± SE, $n = 9$) and functional characteristics (stomatal conductance, g_s (c); mean SE, $n = 18$) characteristics of *Acer rubrum* stomata exposed to ambient or elevated CO_2 at the Duke FACE site. Paired treatment bars designated with a * are significantly different at $P < 0.05$ within a given year. Statistical results: (a) $F = 1.96$, $P = 0.296$; (b) $F = 1.96$, $P = 0.296$; (c) $F = 9.19$, $P = 0.008$; June: $t = 3.032$, $P < 0.01$; August: $t = 2.262$, $P < 0.025$.

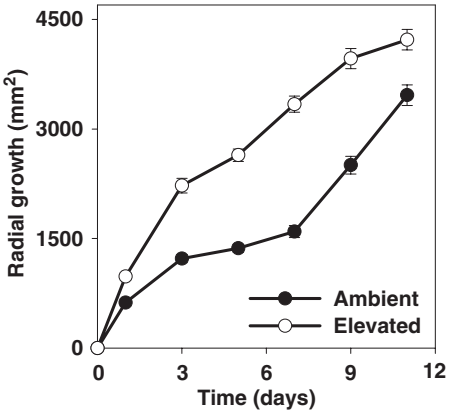

Fig. 4 Radial growth of *Phyllosticta minima* cultured axenically on 30% potato dextrose agar media and exposed to ambient or elevated CO_2 conditions in growth chambers at the Duke Phytotron (mean ± SE, $n = 15$ per treatment). Repeated Measures ANOVA results: $F = 44.48$, $P < 0.0001$. Data were natural log transformed to obtain linear regression slopes (i.e. growth rate) from the exponential growth portion of the curve; mean (SE) ambient and elevated CO_2 slopes from this analysis were 0.344 (.022) and 0.416 (.017), respectively.

show the germinating *P. minima* conidia directly targeting and entering *A. rubrum* stomata (Fig. 2a–c). We found no germinating spores forming penetration pegs or appresoria to bore directly through the leaf epidermis. These data suggest that the stomatal openings are the sole point of entry by *P. minima* into the plant.

Stomata were structurally similar but functionally different between elevated and ambient CO_2 (Fig. 3a–c). Abaxial SD (Fig. 3a) and stomatal aperture length (Fig. 3b) did not differ significantly between CO_2 treatments ($P = 0.296$). However, elevated CO_2 reduced g_s by 36% and 21% on the June and August 2003 sampling dates, respectively ($P < 0.025$) (Fig. 3c). Therefore, the number and structural size of the infection targets (i.e. stomata) for *P. minima* did not change between CO_2 treatments, but the functional size of the stomatal openings (i.e. conductance) was reduced during the day under elevated CO_2.

Despite reduced disease incidence and severity in the field (Fig. 1), *P. minima* cultures exhibited enhanced radial growth under elevated CO_2 in growth chamber experiments (Fig. 4, $P < 0.0001$). Exponential growth rates of *P. minima* were 17% greater under elevated CO_2 (Fig. 4, $P < 0.016$; natural log transformed growth curves). We found similar results when we repeated the *in vitro* growth analysis two additional times in different growth chambers. This result of greater growth by *P. minima* at elevated CO_2 is important because it suggests that the reduced infection and severity observed for the fungus in the field were not caused directly by increased CO_2. These reductions must have instead resulted from interactions with other variables affected by CO_2, such as plant chemistry or morphology.

A. rubrum leaves grown at elevated CO_2 were less hospitable and of poorer nutrient quality for *P. minima* once the fungus entered the leaf through the stomata (Table 1). Elevated CO_2 reduced leaf N by 20% on average ($P = 0.001$; Table 1), increased C : N ratios in the leaves by 20% ($P = 0.001$), total phenolics by 15% ($P = 0.002$), and tannins by 14% ($P = 0.004$; Table 1). Although not significant, leaf surface wax concentration tended to increase at elevated CO_2 ($P = 0.113$, Table 1). Stomata, leaf surface topography, and wax structure analyzed from the SEM images appeared similar between the two CO_2 treatments.

Discussion

Although direct effects of elevated CO_2 on plant physiology and growth are well documented (e.g. Drake *et al*., 1997; Saxe *et al*., 1998), predicted changes in plant disease under future climatic conditions are based primarily on host plant responses and rarely on direct assessment of plant disease under future environmental conditions (Runion, 2003). Here we document a reduction in disease incidence and severity under

Table 1 *Acer rubrum* leaf chemistry and surface wax characteristics for plants grown under elevated and ambient CO_2 at the Duke FACE site (wax) or Duke Phytotron (leaf N and C; total phenolics; tannins) in Durham, North Carolina

Treatment	Leaf nitrogen (% dry wt.)	Carbon : nitrogen	Total phenolics (% dry wt.)	Tannins (% dry wt.)	Epicuticular wax ($\mu g\,cm^{-2}$)
Ambient CO_2	2.21 (0.08)^{a*}	21.35 (0.64)^{a*}	3.99 (0.12)^{a*}	3.63 (0.11)^{a*}	103.0 (30.0)^{a}
Elevated CO_2	1.77 (0.06)^{b}	26.76 (1.00)^{b}	4.70 (0.11)^{b}	4.23 (0.12)^{b}	127.0 (42.0)^{a}

Data are means (SE). FACE, free-air CO_2 enrichment.
Values in each parameter column with different superscripts indicate a significant CO_2 effect at $*P<0.01$.
Statistical results: Leaf N : F = 18.99, $P = 0.001$; C : N: $F = 20.66$, $P = 0.001$; total phenolics: $F = 18.10$, $P = 0.002$; tannins: $F = 13.91$, $P = 0.004$; wax: $F = 2.70$, $P = 0.113$.

elevated CO_2, and provide support for a potential mechanism driven by changes in *A. rubrum* sapling physiology and leaf chemistry that underlie the disease reductions. We found that field-grown *A. rubrum* saplings exhibited lower leaf spot disease incidence under elevated CO_2. Our results are consistent with the only other study documenting reduced foliar disease incidence under elevated CO_2. In their study, Pangga *et al*. (2004) found that a foliar Anthracnose disease of *Stylosanthes scabra* exhibited reduced disease incidence (i.e. lesions per leaf) when exposed to elevated CO_2. Their study, however, did not document physiological changes in the host plant possibly responsible for reduced disease incidence.

We also found that *P. minima* disease severity was reduced under elevated CO_2. Severity is the more commonly measured disease parameter in the handful of studies examining fungal disease under elevated CO_2. Among these recent studies, severity was decreased in three (Thompson *et al*., 1993; Thompson & Drake, 1994; Chakraborty *et al*., 2000b), unchanged in three (Hibberd *et al*., 1996; Tiedemann & Firsching, 2000; Percy *et al*., 2002), and increased in two pathosystems (Thompson & Drake, 1994; Mitchell *et al*., 2003). Below we discuss the changes in *A. rubrum* under elevated CO_2 that we believe drive the reduced disease incidence and severity of *P. minima* leaf spot. We relate these changes to other pathosystems in order to make predictions based on changes in host plants under elevated CO_2. Such mechanistically and physiologically based predictions are needed to test generalized responses across other pathosystems.

The *P. minima* disease reduction that we observed is potentially driven by a dual mechanism of elevated CO_2-induced changes in *A. rubrum*. The first part of our proposed mechanism deals with reduced disease incidence because of changes in *A. rubrum* stomatal functioning. Recent reviews (Manning & Tiedemann, 1995; Coakley *et al*., 1999; Chakraborty *et al*., 2000a) suggested that changes in stomatal structure and function induced by elevated CO_2 may alter plant disease in the future because many foliar pathogens infect plants via the stomata (Agrios, 1997). However, to date no experimental studies to our knowledge had linked changes in stomatal function with reductions in disease incidence. We have shown that *P. minima* targets stomata and initiates infection by gaining entry into *A. rubrum* leaves via the stomata. Reduced disease incidence coincided with reduced g_s in *A. rubrum* under elevated CO_2, a result also documented in a previous growth chamber study on *A. rubrum* (Groninger *et al*., 1996). Reduced g_s to water vapor in our study is driven solely by stomatal closure because we found no significant changes in SD and size.

Not only does stomatal closure reduce the pore size for stomatal-infecting pathogens to enter the plants, but it could also alter the microclimatic conditions on the leaf surface via localized humidity reductions around stomatal pores. Kiefer *et al*. (2002) showed that *Plasmopara viticola*, which causes downy mildew in grapes, utilizes leaf surface microclimate (i.e. localized humidity around open stomata) to track stomatal pores for infection; abscissic acid-induced stomatal closure in grape leaves caused reductions in stomatal targeting by *P. viticola* in the same study. They suggested that reduced stomatal targeting was caused by reduced diffusion of volatile compounds necessary for chemotaxis or other mechanical or electrical cues. Similarly, germ tubes of *Colletotrichum gloeosporioides* that penetrate and infect mulberry leaves via the stomata were attracted to open stomata and away from closed stomata (Kumar *et al*., 2001).

In the second part of the mechanism, reductions in disease severity under elevated CO_2 increase defensive chemistry and reduce nutritional quality of *A. rubrum* tissue. Disease severity was reduced despite the increased growth potential of *P. minima* at elevated CO_2 observed in our growth chamber study, so changes in host tissue quality are likely responsible for reduced pathogen success once inside *A. rubrum*. We found that

elevated CO_2 reduced leaf N while increasing C : N ratios, total phenolics, and tannins in *A. rubrum*. Previous work has demonstrated that *A. rubrum* leaves have lower N concentrations under elevated CO_2 at Duke FACE (Finzi & Schlesinger, 2002), and other members of the *Acer* genus have exhibited reduced leaf N and increased tannins and phenolics under elevated CO_2 (Agrell *et al*., 2000). Thompson *et al*. (1993) and Thompson & Drake (1994) found similar results with reduced disease severity under elevated CO_2 that were related to reduced leaf N. To our knowledge, ours is the first study to associate increased defensive chemistry (i.e. total phenolics and tannins) to reductions in foliar disease severity under elevated CO_2. Altered leaf nutritional quality caused by reduced N and increased defensive chemistry under elevated CO_2 is a likely mechanism functioning in many pathosystems, given the frequent occurrence of these responses across numerous plant species (Hartley *et al*., 2000).

Direct negative effects of elevated CO_2 on *P. minima*, particularly on conidia germination prior to infection, could have altered disease incidence in our pathosystem. We found that elevated CO_2 enhanced *P. minima* growth *in vitro* (Fig. 4), but we did not document whether CO_2 affected spore viability. Recent studies have shown that elevated CO_2 has no effect on conidia germination (Manning & Tiedemann, 1995; Hibberd *et al*., 1996) and stimulatory effects on pathogen fecundity (Coakley *et al*., 1999). Therefore, it is unlikely that disease reductions were because of lower initial inoculum available for infection or altered spore viability under elevated CO_2.

Conclusions

Here we propose a dual mechanism underlying reductions in *P. minima* leaf spot disease incidence and severity under elevated CO_2. Our results verify predictions made nearly a decade ago (Manning & Tiedemann, 1995) that elevated CO_2-induced changes in g_s and leaf chemistry would alter plant disease in the future. These results provide concrete evidence for a potentially generalizable mechanism to predict disease outcomes in other pathosystems under future climatic conditions. More data are needed to strengthen these predictions, especially considering the importance of plant disease to agronomic and natural ecosystems and because microbial pathogens are expected to respond faster to northward species range shifts than plants (Coakley, 1995).

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References

Agrell J, McDonald EP, Lindroth RL (2000) Effects of CO_2 and light on tree phytochemistry and insect performance. *Oikos*, **88**, 259–272.

Agrios GN (1997) Plant Pathology, 4th edn. New Academic Press, New York.

Boyer JS (1995) Biochemical and biophysical aspects of water deficits and the predisposition to disease. *Annual Review of Phytopathology*, **33**, 251–274.

Chakraborty S, Tiedemann AV, Teng PS (2000a) Climate change: potential impact on plant diseases. *Environmental Pollution*, **108**, 317–326.

Chakraborty S, Pangga IB, Lupton J *et al*. (2000b) Production and dispersal of *Colletotrichum gloeosporioides* spores on *Stylosanthes scabra* under elevated CO_2. *Environmental Pollution*, **108**, 381–387.

Coakley SM (1995) Biospheric change-will it matter in plant pathology. *Canadian Journal of Plant Pathology*, **17**, 147–153.

Coakley SM, Scherm H, Chakraborty S (1999) Climate change and plant disease management. *Annual Review of Phytopathology*, **37**, 399–426.

DeLucia EH, Hamilton JG, Naidu SL *et al*. (1999) Net primary production of a forest ecosystem with experimental CO_2 enrichment. *Science*, **284**, 1177–1179.

Drake BG, Gonzalez-Meler MA, Long SP (1997) More efficient plants: a consequence of rising atmospheric CO_2. *Annual Review of Plant Physiology and Plant Molecular Biology*, **48**, 609–639.

Field CB, Jackson RB, Mooney HA (1995) Stomatal responses to increased CO_2: implications from the plant to the global scale. *Plant Cell Environment*, **18**, 1214–1225.

Finzi AC, Schlesinger WH (2002) Species control variation and litter decomposition in a pine forest exposed to elevated CO_2. *Global Change Biology*, **8**, 1217–1229.

Gill RA, Polley HW, Johnson HB *et al*. (2002) Nonlinear grassland responses to past and future atmospheric CO_2. *Nature*, **417**, 279–282.

Gleason HA, Cronquist A (1991) *Manual of Vascular Plants of Northeastern United States and Adjacent Canada*. The New York Botanical Garden, Bronx, NY.

Groninger JW, Seiler JR, Zedaker SM *et al*. (1996) Effects of CO_2 concentration and water availability on growth and gas exchange in greenhouse-grown miniature stands of Loblolly Pine and Red Maple. *Functional Ecology*, **10**, 708–716.

Hartley SE, Jones CG, Couper GC *et al*. (2000) Biosynthesis of plant phenolic compounds in elevated atmospheric CO_2. *Global Change Biology*, **6**, 497–506.

Hendrey GR, Ellsworth DS, Lewin KF *et al*. (1999) A free-air enrichment system for exposing tall forest vegetation to elevated atmospheric CO_2. *Global Change Biology*, **5**, 293–309.

Hibberd JM, Whitbread R, Farrar JF (1996) Effect of elevated concentrations of CO_2 on infection of barley by *Erysiphe graminis*. *Physiological and Molecular Plant Pathology*, **48**, 37–53.

Intergovernmental Panel on Climate Change (2001) Technical summary. In: *Climate Change 2001: The Scientific Basis* (eds Houghton JT, Ding Y, Griggs DJ, Noguer M, van den Linden PJ, Dai X, Maskell K, Johnson CA), pp. 21–83. Cambridge University Press, Cambridge.

Jackson RB, Sala OE, Field CB *et al*. (1994) CO_2 alters water use, carbon gain, and yield for the dominant species in a natural grassland. *Oecologia*, **98**, 257–262.

Karnosky DF, Percy KE, Xiang BX *et al*. (2002) Interacting elevated CO_2 and tropospheric O_3 predisposes aspen (*Populus tremuloides* Michx.) to infection by rust (*Melampsora medusae* f. sp *tremuloidae*). *Global Change Biology*, **8**, 329–338.

Kiefer B, Riemann M, Buche C *et al*. (2002) The host guides morphogenesis and stomatal targeting in the grapevine pathogen. *Plasmopara viticola*. *Planta*, **215**, 387–393.

Kumar V, Gupta VP, Babu AM *et al*. (2001) Surface ultrastructural studies on the penetration and infection process of *Colletotrichum gloeosporioides* on mulberry leaf causing black spot disease. *Journal of Phytopathology*, **149**, 629–633.

Kuo K, Hoch HC (1996) The parasitic relationship between *Phyllosticta ampelicida* and *Vitis vinifera*. *Mycologia*, **88**, 626–634.

Maherali H, Reid CD, Polley HW *et al*. (2002) Stomatal acclimation over a subambient to elevated CO_2 gradient in a C3/C4 grassland. *Plant, Cell and Environment*, **25**, 557–566.

Makkar HPS (2003) Treatment of plant material, extraction of tannins and an overview of tannin assays presented in the manual. *Quantification of Tannins in Tree and Shrub Foliage – A Laboratory Manual*. Kluwer Academic Press, Dordrecht.

Malone SR, Mayeux HS, Johnson HB *et al*. (1993) Stomatal density and aperture length in four plant species grown across a subambient CO_2 gradient. *American Journal of Botany*, **49**, 443–452.

Manning WJ, Tiedemann AV (1995) Climate change: potential effects of increased atmospheric carbon dioxide (CO_2), ozone (O_3), and ultraviolet-B (UV-B) radiation on plant diseases. *Environmental Pollution*, **88**, 219–245.

Mitchell CE, Reich PB, Tilman D *et al*. (2003) Effects of elevated CO_2, nitrogen deposition, and decreased species diversity on foliar fungal plant disease. *Global Change Biology*, **9**, 438–451.

Mohan JE (2002) *Atmospheric carbon dioxide effects on temperate forests*. PhD dissertation. Department of Biology, Duke University.

Pangga IB, Chakraborty S, Yates D (2004) Canopy size and induced resistance in *Stylosanthes scabra* determine anthracnose severity at high CO_2. *Phytopathology*, **94**, 221–227.

Percy KE, Awmack CS, Lindroth RL *et al*. (2002) Altered performance of forest pests under atmospheres enriched by CO_2 and O_3. *Nature*, **420**, 403–407.

Pimentel D, Lach L, Zuniga R *et al*. (2000) Environmental and economic costs of nonindigenous species in the United States. *Bioscience*, **50**, 53–65.

Runion GB (2003) Climate change and plant pathosystems – future disease prevention starts here. *New Phytologist*, **159**, 531–533.

Saxe H, Ellsworth DS, Heath J (1998) Tree and forest functioning in an enriched CO_2 atmosphere. *New Phytologist*, **139**, 395–436.

Sinclair WA, Lyon HH, Johnson WT (1987) Diseases of Trees and Shrubs. Cornell University Press, Ithaca, NY.

Thompson GB, Brown JKM, Woodward FI (1993) The effects of host carbon dioxide, nitrogen and water supply on the infection of wheat by powdery mildew and aphids. *Plant, Cell and Environment*, **16**, 687–694.

Thompson GB, Drake BG (1994) Insects and fungi on a C3 sedge and a C4 grass exposed to elevated atmospheric CO_2 concentrations in open-top chambers in the field. *Plant, Cell and Environment*, **17**, 1161–1167.

Tiedemann AV, Firsching KH (2000) Interactive effects of elevated ozone and carbon dioxide on growth and yield of leaf rust-infected vs. non-infected wheat. *Environmental Pollution*, **108**, 357–363.

Urban O (2003) Physiological impacts of elevated CO_2 concentration ranging from molecular to whole plant responses. *Photosynthetica*, **41**, 9–20.

Walters RS, Yawney HW (1990) *Acer rubrum* L. In *Silvics of North America: Volume 2, Hardwoods* (eds Burns RM, Honkala BH), Agricultural Handbook 654, Forest Service, pp. 60–69. United States Department of Agriculture, Washington, DC.

Williams MH, Green PB (1988) Sequential scanning electron microscopy of a growing plant meristem. *Protoplasma*, **147**, 77–79.