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
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 dentata 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 CO2 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 CO2 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 CO2 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 CO2 may alter the severity of infection.

In the current study, we assessed how elevated CO2 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 CO2. We examined these interactions in the 6th, 7th, and 8th years of the Duke Forest free-air CO2 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° 0’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 CO2 or ambient air. Three experimental rings are fumigated with CO2 to raise the atmospheric CO2 concentration 200 μL 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. minima 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 m² 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 ~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 76 for both ambient and elevated CO₂ 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 CO₂). 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 40.1 mm²) 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 (mm²) and P. minima lesion area (mm²). 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 polyvinylsloxane dental impression material (Extrude’ Medium; Kerr Manufacturing Co., Orange, CA, USA) until the polymer hardened (Williams & Green, 1988; Maherati et al., 2002). Each cast was analyzed using scanning electron microscopy (see ESEM description below) under fossil mode at 400 x 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 mm²) was counted and averaged across each ring and CO₂ 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 (gₛ) 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 gₛ 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 CO₂ effects, A. rubrum saplings were grown at ambient CO₂ 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. minima

To test for direct CO2 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 CO2) on fungal performance. We inoculated thirty 30% PDA plates (n = 15 randomly assigned to each CO2 treatment) with a small section (~ 8.0 mm²) of P. minima cultured on PDA. Two growth chambers were used, one at ambient CO2 (360 ppm) and the other at elevated CO2 (560 ppm). CO2 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 CO2 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 CO2 levels and chamber conditions were the same as described above. Leaf disks (~ 3.0 cm²) were harvested from green leaf material and dried at 65 °C for 14 days. Within each CO2 treatment, four disks were randomly selected and pooled to form one sample (n = 6 for each CO2 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 CO2 treatment of the growth chamber experiment. A disk (~ 3.0 cm²) was punched from each leaf, placed in an aluminum foil envelope, flash frozen in liquid N2, and stored at ~80 °C until analysis. Within both elevated and ambient CO2 treatments, four disks were randomly selected and placed to form one sample (n = 6 for each CO2 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 (~ 3.0 cm² 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 desiccator under N2-saturated air. The remaining epicuticular wax was weighed and expressed as μg 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, gsc, C:N ratio, %N, total phenols, tannins, and epicuticular wax were pooled within rings and evaluated using analysis of variance (ANOVA) with CO2 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 CO₂ 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 CO₂ 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 CO₂ in the field (Fig. 1a–d). Fewer saplings from elevated CO₂ rings were infected in all years (Fig. 1a, \( P < 0.003, 22\%, \ 27\%, \ \text{and} \ 8\%, \ 2002, \ 2003, \ \text{and} \ 2004, \ \text{respectively} \)) and significantly fewer leaves were infected per plant (Fig. 1b, 2002 only \( P = 0.074; 14\%, \ 4\%, \ \text{and} \ 5\% \) less each year, respectively). Similarly, disease severity was reduced under elevated CO₂; elevated CO₂ 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) \( (P = 0.045) \).

*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

![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.](image)

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 CO₂ 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 \( F = 4.744, \ P = 0.045. \)

show the germinating *Phyllosticta minima* conidia directly targeting and entering *A. rubrum* stomata (Fig. 2a–c). We found no germinating spores forming penetration pegs or appressoria 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 CO2 (Fig. 3a–c). Abaxial SD (Fig. 3a) and stomatal aperture length (Fig. 3b) did not differ significantly between CO2 treatments (*P* = 0.296). However, elevated CO2 reduced *g*ₙ 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 CO2 treatments, but the functional size of the stomatal openings (i.e. conductance) was reduced during the day under elevated CO2.

Despite reduced disease incidence and severity in the field (Fig. 1), *P. minima* cultures exhibited enhanced radial growth under elevated CO2 in growth chamber experiments (Fig. 4, *P* < 0.0001). Exponential growth rates of *P. minima* were 17% greater under elevated CO2 (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 CO2 is important because it suggests that the reduced infection and severity observed for the fungus in the field were not caused directly by increased CO2. These reductions must have instead resulted from interactions with other variables affected by CO2, such as plant chemistry or morphology.

*A. rubrum* leaves grown at elevated CO2 were less hospitable and of poorer nutrient quality for *P. minima* once the fungus entered the leaf through the stomata (Table 1). Elevated CO2 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 CO2 (*P* = 0.113, Table 1). Stomata, leaf surface topography, and wax structure analyzed from the SEM images appeared similar between the two CO2 treatments.

**Discussion**

Although direct effects of elevated CO2 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...
ELEVATED CO₂ REDUCES DISEASE INCIDENCE AND SEVERITY OF A RED MAPLE FUNGAL PATHOGEN

Table 1 Acer rubrum leaf chemistry and surface wax characteristics for plants grown under elevated and ambient CO₂ at the Duke FACE site (wax) or Duke Phytotron (leaf N and C; total phenolics; tannins) in Durham, North Carolina

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Leaf nitrogen (% dry wt.)</th>
<th>Carbon : nitrogen</th>
<th>Total phenolics (% dry wt.)</th>
<th>Tannins (% dry wt.)</th>
<th>Epicuticular wax (µg cm⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ambient CO₂</td>
<td>2.21 (0.08)a*</td>
<td>21.35 (0.64)a*</td>
<td>3.99 (0.12)a*</td>
<td>3.63 (0.11)a*</td>
<td>103.0 (30.0)a</td>
</tr>
<tr>
<td>Elevated CO₂</td>
<td>1.77 (0.06)b</td>
<td>26.76 (1.00)b</td>
<td>4.70 (0.11)b</td>
<td>4.23 (0.12)b</td>
<td>127.0 (42.0)a</td>
</tr>
</tbody>
</table>

Data are means (SE). FACE, free-air CO₂ enrichment. Values in each parameter column with different superscripts indicate a significant CO₂ 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 CO₂ 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 CO₂. Our results are consistent with the only other study documenting reduced foliar disease incidence under elevated CO₂. 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 CO₂. 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 CO₂. Severity is the more commonly measured disease parameter in the handful of studies examining fungal disease under elevated CO₂. 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 CO₂ 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 CO₂. 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 CO₂-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 CO₂ 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 x₀ in A. rubrum under elevated CO₂, a result also documented in a previous growth chamber study on A. rubrum (Groninger et al., 1996). Reduced x₀ 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 CO₂ 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 CO₂ 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 CO₂ 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 CO₂ 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 CO₂ (Agrell et al., 2000). Thompson et al. (1993) and Thompson & Drake (1994) found similar results with reduced disease severity under elevated CO₂ 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 CO₂. Altered leaf nutritional quality caused by reduced N and increased defensive chemistry under elevated CO₂ 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 CO₂ on *P. minima*, particularly on conidia germination prior to infection, could have altered disease incidence in our pathosystem. We found that elevated CO₂ enhanced *P. minima* growth in vitro (Fig. 4), but we did not document whether CO₂ affected spore viability. Recent studies have shown that elevated CO₂ 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 CO₂.

Conclusions

Here we propose a dual mechanism underlying reductions in *P. minima* leaf spot disease incidence and severity under elevated CO₂. Our results verify predictions made nearly a decade ago (Manning & Tiedemann, 1995) that elevated CO₂-induced changes in gₑ 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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