Plant Pathology (2014) Doi: 10.1111/ppa.12272

Complex interplay of future climate levels of CO_2, ozone and temperature on susceptibility to fungal diseases in barley

B. L. Mikkelsen[a], R. B. Jørgensen[b] and M. F. Lyngkjær[a]*

[a]*Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg C; and*
[b]*Department of Chemical and Biochemical Engineering, Technical University of Denmark, Frederiksborgvej 399, 4000 Roskilde, Denmark*

Barley (*Hordeum vulgare*) was grown in different climatic environments with elevated [CO_2] (700 vs 385 ppm), [O_3] (60/90 vs 20 ppb) and temperature (24/19 vs 19/12°C day/night) as single factors and in combinations, to evaluate the impact of these climatic factors on photosynthesis and susceptibility to powdery mildew and spot blotch disease. No significant increase in net CO_2 assimilation rate was observed in barley grown under elevated [CO_2] at ambient temperature. However, this rate was positively stimulated under elevated temperature together with a slightly higher potential quantum efficiency of PSII, both at ambient and elevated [CO_2], suggesting that photosynthesis was not limited by [CO_2] at ambient temperature. When growing under elevated temperature or [O_3], infection by the biotrophic powdery mildew fungus decreased, whereas disease symptoms and growth of the toxin-secreting hemibiotrophic spot blotch fungus increased compared to ambient conditions, implying that climate-induced changes in disease severity could be linked to the trophic lifestyle of the pathogens. Elevated [CO_2] decreased powdery mildew infection but had no effect on spot blotch disease compared to ambient condition. However, the effect of elevated [CO_2], [O_3] and temperature did not act in an additive manner when combined. This led to a surprising disease development in the combination treatments, where powdery mildew infection increased despite the individual reducing effect of the climatic factors, and spot blotch disease decreased despite the individual promoting effect of temperature and ozone, emphasizing the importance of conducting multifactorial experiments when evaluating the potential effects of climate change.

Keywords: climate change, CO_2, ozone, photosynthesis, powdery mildew, spot blotch

Introduction

Development and severity of a plant disease depends on the combination of a susceptible host plant, a virulent pathogen and suitable environmental conditions for infection. The future environmental increase in CO_2 and ozone concentrations, and the derived changes in temperature and precipitation patterns, will affect plant physiology, which may have significant effects on the sensitivity of plants to environmental stresses such as drought, waterlogging or heat, and to plant diseases caused by pathogenic microorganisms. These stresses, known as abiotic and biotic stresses, are a major cause of yield and quality losses and are significant constraints for plant production globally.

The concentration of CO_2 in the atmosphere has continuously increased from pre-industrial levels at 280 ppm to about 395 ppm at present, and is expected to reach above 700 ppm by the end of the century (IPCC, 2013). Tropospheric ozone (O_3) is a greenhouse gas and an air pollutant increasing in concentration due to the burning of hydrocarbons. The global mean concentration of ozone is now about 40 ppb, but the concentration fluctuates according to geographical location and weather conditions, and does occasionally reach values well above the threshold for damage in many crop plants. Ozone mean concentration is expected to increase up to 18% by 2100 (IPCC, 2013). Followed by the increase in greenhouse gases, the global temperature may rise up to 4·5°C in the same period, accompanied by more extreme weather events (IPCC, 2013).

Generally, it is believed that climate change-mediated effects on host plant physiology will be a key factor determining susceptibility to plant diseases in the future (Garrett *et al.*, 2006). Elevated [CO_2], [O_3] or temperature as a single factor has quite different effects on plant physiology. Elevated [CO_2] may enhance the photosynthetic rate, which would increase sugar content in leaves, decrease photorespiration, provide a protective role against oxidative stress and increase biomass and yield (Gillespie *et al.*, 2011). However, elevated [CO_2] may also result in premature leaf senescence and lower nitrogen and chlorophyll content in plants (Fangmeier *et al.*, 2000). An increase in average temperature of more than 3°C during the growth season may lead to reduced photosynthetic capacity, increased oxidative stress, accelerated leaf senescence and reduced biomass and yield (Sharkey, 2005; Clausen *et al.*, 2011). The same negative effects can be induced by elevated [O_3]. Inside the plant, ozone rapidly reacts to form reactive oxygen species (ROS) that may suppress photosynthesis and accelerate

*E-mail: mlyn@plen.ku.dk

leaf senescence (reviewed by Fiscus *et al.*, 2005). Altered plant physiology could affect plant–pathogen interaction in relation to availability and quality of nutrients for pathogen feeding or change contents of stress compounds such as ROS, which may promote defence-like responses including oxidative burst, cell wall strengthening and induction of pathogen-associated defence genes (Sandermann *et al.*, 1998; Fiscus *et al.*, 2005).

Several reviews have extensively covered possible effects of elevated [CO_2], [O_3] and temperature on future severity of plant diseases (e.g. Manning & von Tiedemann, 1995; Garrett *et al.*, 2006; Eastburn *et al.*, 2011), but experimental studies have so far focused mainly on single climatic factors such as CO_2, ozone and temperature. The general findings and expectations are that increased levels of CO_2, ozone and temperature tend to stress plants and may change susceptibility to some, but not to other plant diseases. However, only a few experimental studies have investigated effects of combined climatic factors on plant disease severity, and mainly CO_2 and ozone in combination (von Tiedemann & Firsching, 2000; Plessl *et al.*, 2005; Eastburn *et al.*, 2010). Because combinations of climatic factors have been shown to act in a non-additive and unpredictable manner on plant physiology and growth (Plessl *et al.*, 2005; Clausen *et al.*, 2011; Gillespie *et al.*, 2011), and that the outcome of a specific plant–pathogen interaction is highly dependent on the environment, it is important to include combinations of multiple climatic factors in experimental studies addressing questions concerning the outcome of plant–pathogen interactions in a future climate.

Plant diseases are caused by a large number of different pathogenic microorganisms, often very specialized to attack a specific plant species, or even specific genotypes. Plant pathogens can be divided into three main categories based on their lifestyle: either biotrophic, i.e. the pathogen is feeding on living plant tissue and does not kill the infected host tissue; necrotrophic, i.e. the pathogen kills the infected tissue and feeds on the released nutrients; or hemibiotrophic, i.e. has a biotrophic lifestyle in the initial part of the infection and later switches to a necrotrophic lifestyle. Even though it is generally hypothesized that biotrophic pathogens favour healthy and non-stressed plants and hemibiotrophic/necrotrophic pathogens favour stressed plants that are weakened or damaged (Manning & von Tiedemann, 1995), it is difficult to predict effects of climatic conditions on disease susceptibility (Eastburn *et al.*, 2011).

The objective of this study was to examine how predicted future levels of temperature, CO_2 and ozone, individually and in combination, would affect severity of plant diseases in the crop plant barley (*Hordeum vulgare*) caused by fungal pathogens with opposite lifestyles, and how this relates to changes in host plant photosynthesis. The diseases studied were powdery mildew (caused by the biotrophic pathogen *Blumeria graminis* f. sp. *hordei*) and spot blotch (caused by the hemibiotrophic pathogen *Bipolaris sorokiniana*). These diseases are already or may become serious problems in most cereal growing areas.

Powdery mildew causes considerable yield losses in wheat (*Triticum aestivum*) and barley every year. This disease is most prominent in temperate regions and it is predicted that increase in canopy size, density and humidity due to climate change will increase powdery mildew disease severity (Manning & von Tiedemann, 1995). Powdery mildew infection is established when a fungal spore germinates to form an appressorium from which a penetration peg attempts to directly penetrate the attacked plant epidermal cell wall. If penetration succeeds, the peg differentiates into a nutrient-absorbing haustorium within the living epidermal cell, which supports further colony growth. However, plant epidermal cells can and do actively resist penetration by local production of fungitoxic components such as phenolic compounds and ROS, and by deposition of a papilla that reinforces the plant cell wall at the site of attempted penetration (Zeyen *et al.*, 2002). It is the speed and degree of host cell response that appears to determine the efficiency of this initial basal defence and this process is dependent on plant genotype, age and environmental growth conditions.

Spot blotch disease is particularly important under conditions of high relative humidity and high temperature and has become a major production constraint of wheat in South Asia and Latin America. This increase has been linked to climate change and increased temperature (Sharma *et al.*, 2007). Spot blotch disease is characterized by causing necrotic lesions on the infected leaf. The mechanism of infection is similar to powdery mildew and includes germination of the fungal spore on the leaf surface, followed by appressorium formation that supports direct penetration through the cell wall of the attacked host plant's epidermal cell (Kumar *et al.*, 2002). The fungus enters its necrotrophic stage after invasion of the underlying mesophyll cells, and pathogenicity is associated with the production of toxins (reviewed by Kumar *et al.*, 2002). The phytotoxins probably affect plant photosynthesis by inhibiting electron transport from photosystem II to photosystem I (Adeishvili *et al.*, 1989).

Materials and methods

Climate treatments

Experiments were conducted in the Risø Environmental Risk Assessment Facility (RERAF) (Clausen *et al.*, 2011). The phytotron consists of six 24 m^2 gastight chambers each able to run specific climatic conditions. Plants were grown in different combinations of ambient or elevated levels of [CO_2], temperature (T) and [O_3]. The climatic conditions were designed to resemble an average day in the growth season in Denmark at present (ambient, [CO_2]: 385 ppm, temperature: 19/12°C (day/night) and [O_3]: 20–30 ppb) and around year 2075 (elevated, [CO_2]: 700 ppm, temperature: 24/17°C (day/night) and [O_3]: 60–90 ppb), as predicted by IPCC (2013). Six conditions were tested: ambient, increased single climatic factors (CO_2, T or O_3) and the combination of increased climatic factors (CO_2 + T or CO_2 + T + O_3). The growth conditions included a 16/8 h day/night light cycle

with a light intensity of 400–700 μmol photons m^{-2} s^{-1}. During the first and last hour of the day, sunrise and sunset was simulated by a gradual change in light intensity. The relative humidity (RH) was kept at 55/70% (day/night) in all treatments. Gas concentrations, temperature and RH in the chambers were monitored and adjusted continuously. Examples of intended and measured climatic conditions are shown in Table S1.

Plants and growth conditions

Five seeds of spring barley (*H. vulgare*) cv. Pallas were sown in *c.* 1 kg of a mixture of peat with granulated clay (Pindstrup substrate no. 6; Pindstrup Mosebrug A/S). Plants were grown until full expansion of the third leaf, which was used for photosynthetic measurements and pathogen treatment. To make measurements and pathogen inoculation possible in all treatments at the same day, plant development was synchronized by delayed sowing of plants grown under treatments with elevated temperature. The pots were sufficiently watered to avoid drought stress, using an automatic surface dripping system that delivered the same amount of water to all treatments. To avoid chamber-specific biases, treatments and plants were shifted to new chambers once a week in the growing phase before experimentation.

Photosynthetic gas exchange

Measurements were done on uninfected leaves on 15- and 18-day-old plants when grown in elevated temperature, and on 18- and 21-day-old plants when grown at ambient temperature, corresponding to 3 days before and on the day of pathogen inoculation. Measurements of net CO_2 assimilation rate (A) and stomatal conductance (g_s) were performed using a portable open-path infrared gas analyser (LI-6400; LI-COR Biosciences). The in-built red–blue LED light source provided a photosynthetic photon flux density (PPFD) of 2000 μmol photons m^{-2} s^{-1}. Temperature of the internal heating block and reference [CO_2] was regulated according to daytime growth conditions for every treatment. Relative air humidity in the leaf chamber was set to 50 $\pm$ 2·5%. Plants remained in the growth chambers during the measurements. The measurements were done on four or five individual plants, and three technical replicates per leaf were performed after stabilization of A. The average of the technical replicates was used for further data analysis.

Chlorophyll *a* fluorescence

Possible effects of the climatic conditions on photosystem II (PS II) were recorded by measurements of chlorophyll fluorescence using a Handy PEA (Plant Efficiency Analyzer; Hansatech Instruments). A flash of saturating light (3000 μmol photons m^{-2} s^{-1}) lasting 2 s was applied to leaves, which had been dark adapted for 30 min. The fluorescence data were analysed and the F_v/F_m ratio calculated using the Handy PEA software (v. 1.30). Measurements were done on five leaves of individual uninfected plants. The plants were 15 days old when grown at elevated temperature and 18 days old when grown at ambient temperature, and the same leaf stage as applied for the infection studies was used.

Inoculation with pathogens and disease assessment

Powdery mildew

Evaluation of powdery mildew disease was done in three individual experiments that varied in O_3 concentration during plant growth and in the age of the tested leaves when inoculated (Table S2). In experiments 1 and 2, the central 8 cm of the third leaf from five plants grown in each of the six tested climatic conditions was cut off and placed in Petri dishes on 0·4% water agar with the adaxial surface up. The leaves were subsequently inoculated (about 20 spores mm^{-2}) with the compatible isolate A6 of the fungus *B. graminis* f. sp. *hordei* that causes powdery mildew disease in barley. All the boxes were incubated for 48 h under ambient conditions before the leaf segments were fixed and cleared for microscopy. In experiment 3, attached leaves were fixed into a horizontal position to secure uniform inoculation and the infected plants were kept in the appropriate climatic conditions for 48 h before harvesting leaf segments for microscopy. The fungal structures were stained with 0·01% Evans blue and observed by light microscopy, and fungal infection attempts from germinating fungal spores were recorded as successful or unsuccessful in penetrating the cell wall of the attacked barley epidermal cells. A successful penetration was identified by formation of a fungal haustorium inside the attacked cell. At least 100 observations of fungal penetration attempts were made per leaf segment and the results are reported as percentage successful infections.

Spot blotch

Evaluation of spot blotch disease was done in four individual experiments, which varied in O_3 concentration during plant growth and in the age of the tested leaves when inoculated (Table S3). Twelve leaves were fixed into a horizontal position before being spray inoculated with a spore solution (25 000 conidia mL^{-1}) prepared from the Danish single spore field isolate 1623 of the fungus *B. sorokiniana* that causes spot blotch disease on barley. The inoculation was performed with a glass hand sprayer until run-off. To ensure optimal conditions for *B. sorokiniana* infection, the plants were immediately covered with a plastic tent and incubated in darkness to obtain near 100% humidity under ambient condition for 24 h. Thereafter, the plants were returned to the original climatic conditions and the tops of the plastic tents were removed. Disease development was recorded 3 and 7 days after inoculation by digital images and the amount of disease was measured as: number of symptoms per leaf, percentage leaf area covered with symptoms, and the appearance in size and extent of chlorosis around symptoms. In experiment 1, the fungal biomass of *B. sorokiniana* was also estimated 3 and 7 days after inoculation by measuring the amount of fungal DNA relative to plant DNA using quantitative real-time PCR (qPCR).

Estimation of the relative amount of fungal DNA by qPCR

Genomic DNA was extracted from 100 mg leaf material from a pooled sample of five leaves using the DNeasy Plant Mini kit (QIAGEN). Pure fungal DNA was extracted from a *B. sorokiniana* culture grown in liquid potato dextrose agar and pure barley DNA was extracted from uninoculated leaves, to prepare a standard dilution series for quantification. Primers for detection of *B. sorokiniana* were designed to amplify an 81 bp fragment specific to *Bipolaris* species from the internal transcribed spacer (ITS) region from strain R174, GenBank: JN689946.1, using the program PRIMER 3 PLUS (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Primers used were: ITS forward primer 5′-TGGGAGACTCGCCTTAAAAC-3′ and reverse primer 5′-CTCCTGATACAAAGCGCAAA-3′. To normalize the amount of fungal DNA in the leaves to the amount of plant

DNA, the elongation factor (EFO) gene from barley was used, using EFO forward primer 5′-ACCCTGACAAGGTTCCCTTC-3′ and reverse primer 5′-ACCAGTCAAGGTTGGTGGAC-3′. The qPCR was performed using the real-time PCR system Mx3000P (Stratagene). All DNA samples were analysed using both primer sets. The PCR reaction was set up in duplicates for each DNA sample. The lowest detectable amount of pure *B. sorokiniana* DNA was 0·2 pg, and *B. sorokiniana* DNA well above this limit was present in all infected leaves.

Statistical analysis

Data from measurement of gas exchange, chlorophyll fluorescence, number of symptoms and infected leaf areas represent continuous variables and were analysed by analysis of variance (ANOVA). Data from penetration attempts by the powdery mildew fungus had a binomial distribution and data for number of *B. sorokiniana* symptoms a Poisson distribution. These data were analysed by logistic regression (corrected for over-dispersion when present). In all tests the null hypothesis was rejected at $P \leq 0{\cdot}05$. Data were analysed by PC-SAS v. 9.2 (SAS Institute Inc.). Two-sample *t*-tests (two-tailed) were used to compare means between time points, and *F*-test was performed to test for equal or unequal variances. Means are presented with standard errors (SE) unless otherwise stated.

Results

The growth of plants was visibly affected by the different climatic conditions. Especially in the elevated temperature (+5°C) treatments, plants developed faster and reached full maturity of the third leaf about 2 days earlier than plants grown under ambient temperature. Elevated ozone treatments were modest in all experiments, with intended elevated values at either 60 or 90 ppb. Although the plants were exposed to short periods with [O_3] higher than intended, ozone leaf spots were generally not observed on the leaves used for experiments.

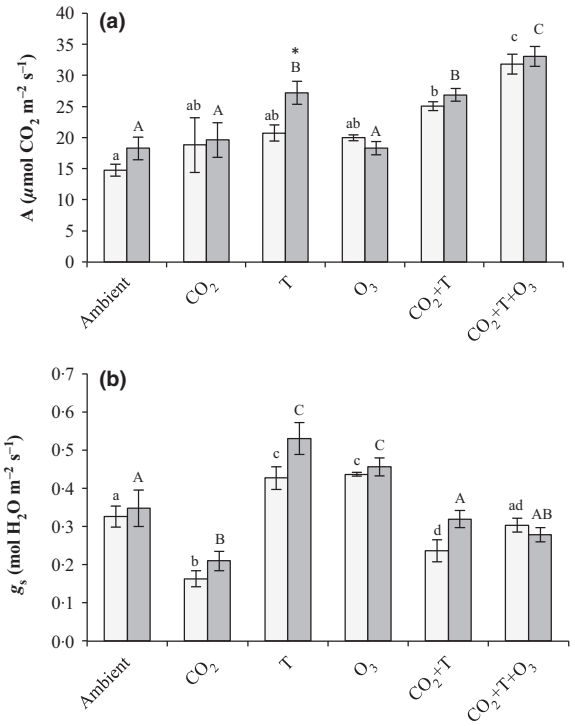

Figure 1 Measurements of gas exchange on uninfected leaves, from barley plants grown under ambient, elevated CO_2 (CO_2), elevated temperature (T), elevated ozone (O_3), combined elevated CO_2 and temperature (CO_2 + T) or combined CO_2, temperature and ozone (CO_2 + T + O_3) conditions. (a) Net CO_2 assimilation rate (A); (b) stomatal conductance (g_s). Light grey columns: 3 days before inoculation (15/18-days-old in elevated/ambient temperature); dark grey columns: day of inoculation (18/21-days-old in elevated/ambient temperature). Values are means of four/five leaves. Error bars ± SE of the mean. Identical letters within a time point are not significantly different at $P = 0{\cdot}05$. *indicates significant difference between the two time points within the treatment.

The effect of climate factors on photosynthesis

Gas exchange

Except from the elevated temperature treatment T, the net CO_2 assimilation rate in the leaves did not change significantly between the early and late time point in the different treatments (Fig. 1a). In general, elevated temperature had a strong positive effect on the net CO_2 assimilation rate. In the single and multiple treatments with elevated temperature (T, CO_2 + T and CO_2 + T + O_3), the net CO_2 assimilation rate was substantially higher than under ambient growth conditions, and except for the early time point in the elevated temperature treatment, this difference was significant (Fig. 1a). Elevated ozone did not affect net CO_2 assimilation rate and surprisingly, this was also the case under elevated [CO_2] growth conditions (Fig. 1a).

Stomatal conductance did not change significantly between the two time points in either of the treatments (Fig. 1b). Elevated temperature and ozone increased opening of stomata and these treatments had significantly increased stomatal conductance compared to ambient growth conditions. In contrast, elevated [CO_2] induced stomatal closure and significantly reduced stomatal conductance compared to ambient growth conditions. In the multifactor CO_2 + T and CO_2 + T + O_3 treatments, an antagonistic interaction of CO_2 and temperature/ozone was observed and plants had stomatal conductance similar to ambient growth conditions (Fig. 1b).

Chlorophyll a fluorescence

The maximum quantum efficiency of PSII was estimated as the F_v/F_m ratio. In plants grown under ambient conditions, the dark-adapted F_v/F_m ratio was 0·78 and there were only slight differences between the treatments (Table 1). Interestingly however, the CO_2 and O_3 treatments resulted in a lower F_v/F_m, ratio compared to ambient, indicating less optimal photosynthesis under these treatments. In contrast, elevated temperature seemed to have a positive effect on PSII, as the F_v/F_m ratio was

Table 1 Maximum quantum efficiency of photosystem II (F_v/F_m) in dark adapted leaves

Treatment	F_v/F_m	SE
Ambient	0·784 ab	0·010
CO_2	0·770 b	0·014
T	0·795 ab	0·007
O_3	0·775 b	0·009
CO_2 + T	0·811 a	0·012
CO_2 + T + O_3	0·800 ab	0·016

Leaves were 15–18 days old at the time of measurements in elevated/ambient temperature. F_v/F_m values with the same letter are not significantly different at $P = 0·05$.

slightly higher than ambient in all treatments with elevated temperature (T, CO_2 + T and CO_2 + T + O_3: Table 1).

The effect of climate factors on powdery mildew disease

Evaluation of powdery mildew disease was measured as percentage successful penetration attempts observed by microscopy 48 h after fungal inoculation. At this time point the fungus had developed into microcolonies that were easily recognized. Disease measurement was done in three individual experiments that varied in ozone concentration during plant growth and in the age of the tested leaves when inoculated (Table S2). The total level of disease varied between the experiments, but the relative differences in disease susceptibility between the treatments were consistent. Single CO_2, T and O_3 treatments led to a decrease in *B. graminis* infection, whereas plants became substantially more susceptible when grown in the multifactor treatments CO_2 + T or CO_2 + T + O_3 (Fig. 2). Cell death responses were observed in <5% of the attacked epidermal cells and this proportion was not affected by the growth conditions. No direct effects of the climatic condition on fungal infection were observed. The relative infection rate in the single and multifactor treatments was identical both when the fungus was introduced directly to attached barley leaves in the growth chambers, and when leaves were detached and challenged with mildew disease outside the growth chambers (Table S2). Autofluorescence below papillae, which may be used as an estimate of phenolic defence compounds (Zeyen *et al.*, 2002), was also evaluated. However, no conclusive differences between treatments were observed (data not presented).

The effect of climate factors on spot blotch disease

Evaluation of spot blotch disease caused by the hemibiotrophic fungus *B. sorokiniana* was measured as lesion appearance, percentage leaf area covered with symptoms and amount of fungal biomass, at 3 and 7 days after inoculation. The spot blotch disease tests were repeated four times in experiments that varied in ozone concentration during plant growth and in the age of the tested leaves when inoculated (Table S3). The relative disease levels between treatments were consistent in the individual experiments. Three days after inoculation, the first spot blotch symptoms (small necrotic lesions) were detected on plants from all treatments (Fig. 3). At this time point, there were no major deviations in size and appearance of lesions among treatments, but significant differences were found in the leaf area covered with symptoms despite a low coverage (0·9–4·4%). Leaves from the single CO_2, T and O_3 treatments, and the multifactor CO_2 + T + O_3 treatment, showed significantly more symptoms compared to ambient growth conditions (Fig. 3).

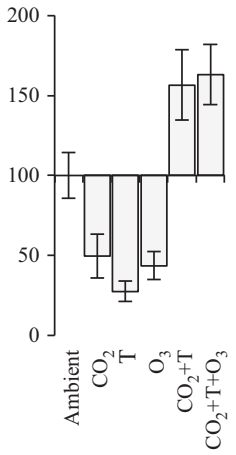

Figure 2 Powdery mildew infection. Successful fungal penetration in the climate treatments, elevated CO_2 (CO_2), elevated temperature (T), elevated ozone (O_3), combined elevated CO_2 and temperature (CO_2 + T) or combined CO_2, temperature and ozone (CO_2 + T + O_3), relative to ambient conditions (100%). Average of the three experiments listed in Table S2. Error bars ± SE of the mean.

Seven days after inoculation, the infected leaves in all treatments had started to become yellow (chlorotic). Leaves from the CO_2 and O_3 treatments were most chlorotic, followed by leaves from the T treatment which showed more patchy areas of chlorotic tissue. Development of lesions (lesion size and appearance) was highest in the elevated temperature treatments (T, CO_2 + T and CO_2 + T + O_3), and here the leaves were covered with large necrotic spots (Fig. 4). In the treatments with ambient temperature condition (ambient, CO_2 and O_3) the single lesions were smaller and less developed. The leaf area covered with symptoms was significantly increased in all treatments from 3 to 7 days (Fig. 3). Leaves from plants grown in the single treatments T and O_3 were strongly affected by spot blotch disease, and had the largest area covered with symptoms. However, when elevated temperature and ozone was combined with elevated [CO_2] in the multiple CO_2 + T and CO_2 + T + O_3 treatments, the plants were less affected and the leaf area covered with spot blotch disease was close to that of plants grown under ambient conditions (Fig. 3).

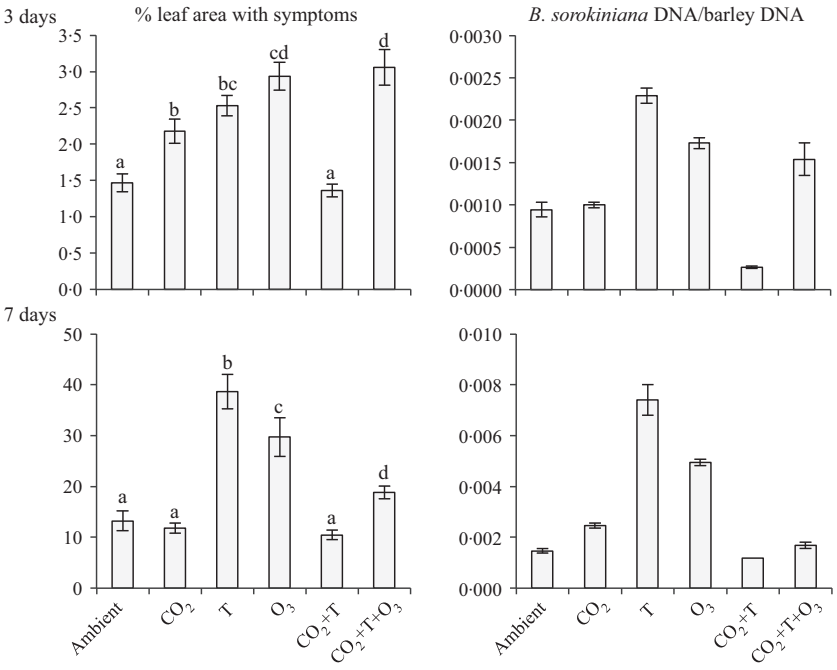

Figure 3 Spot blotch infection. Percentage leaf area covered with symptoms and amount of fungal biomass measured as ng *Bipolaris sorokiniana* DNA/ng barley DNA, at 3 and 7 days after inoculation on barley plants grown under ambient, elevated CO_2 (CO_2), elevated temperature (T), elevated ozone (O_3), combined elevated CO_2 and temperature (CO_2 + T) or combined CO_2, temperature and ozone (CO_2 + T + O_3) conditions. Error bars ± SE of the mean. Identical letters within a time point are not significantly different at P = 0·05.

To compare the visual assessment of spot blot symptoms with the actual level of fungal growth in the leaves, the fungal biomass was estimated by measuring the ratio of fungal versus plant genomic DNA using qPCR (Fig. 3). The T treatment strongly promoted fungal growth in the plants. B 3 days after inoculation there was already two times more fungal biomass than in leaves from plants grown under ambient conditions. The O_3 and CO_2 + T + O_3 treatments also promoted fungal growth in the leaves but less than in the T treatment. However, in the multifactor CO_2 + T treatment fungal growth seemed to be suppressed, although elevated [CO_2] alone did not affect fungal growth. Seven days after inoculation, the stimulating effect of elevated temperature on fungal growth was even more apparent (Fig. 3). The amount of fungal biomass had increased dramatically in the T treatment, and more than in any of the other treatments. The O_3 treatment also strongly promoted fungal growth 7 days after inoculation, but less than in the T treatment. Remarkably, when elevated temperature was combined with elevated [CO_2] in both the multifactor treatments CO_2 + T and CO_2 + T + O_3, the strong promoting effect of temperature on fungal growth in the leaves was clearly absent, and the amount of fungal biomass was at the same level as in plants grown under ambient conditions (Fig. 3).

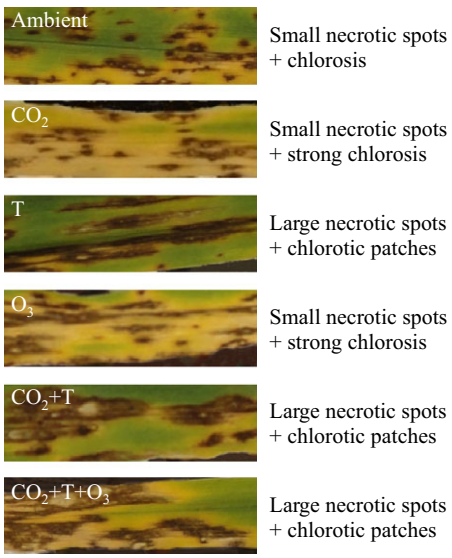

Figure 4 Typical spot blotch symptom observed 7 days after inoculation on barley plants grown under ambient, elevated CO_2 (CO_2), elevated temperature (T), elevated ozone (O_3), combined elevated CO_2 and temperature (CO_2 + T) or combined CO_2, temperature and ozone (CO_2 + T + O_3) conditions.

Discussion

Despite the large attention on possible effects of climate change on plant diseases during recent decades, there is still a lack of experimental studies evaluating the effect of combined climatic factors on development of plant diseases and the underlying physiological mechanisms in the host plant. In this study, elevated [CO_2], [O_3] and temperature, individually and in combination, affected severity of powdery mildew and spot blotch disease in barley.

After *c.* 3 weeks of growth, photosynthesis was significantly altered, as seen from the net CO_2 assimilation rate, stomatal conductance and to a lesser extent, from the potential quantum efficiency of PSII, i.e. the F_v/F_m

ratio. Elevated [CO_2] generally increases photosynthesis of C_3 plants, due to elevated supply of CO_2 and reduced carbon loss through decreased photorespiration, but in this study, no significant increase in net CO_2 assimilation rate was observed at ambient temperature (19/12°C, day/night). This suggests that photosynthesis is not limited by the CO_2 concentration at ambient temperature. However, net CO_2 assimilation rate was positively stimulated by the 5°C increase in temperature, both at ambient and elevated [CO_2], which was also reflected by a slightly higher potential quantum efficiency of PSII compared to the ambient temperature treatments. Increased temperature is known to positively stimulate net CO_2 assimilation rate in C3 plants until they reach their optimum (Sage & Kubien, 2007), and the 24°C in the elevated temperature treatments is probably closer to the optimum for barley than the 19°C in the ambient treatment.

The lower quantum efficiency of PSII in the ambient temperature treatments indicates that the conditions here are less optimal for photosynthesis, as less light energy is used for photochemistry, and more is emitted as heat or fluorescence. Ozone is a known inhibitor of photosynthesis (Fiscus *et al.*, 2005) and exposure of barley to high chronic ozone concentrations (180 ppb) resulted in reduced net CO_2 assimilation rate and decreased stomatal conductance (Plazek *et al.*, 2000). However, in the present study, the moderately elevated ozone concentration (60 ppb) did not affect these factors, although a slight reduction of the potential quantum efficiency was observed, indicating that PSII may be affected by the ozone treatment. Short-term exposure of barley seedlings to 100 ppb ozone affected the F_v/F_m ratio at normal light conditions (200 μmol m^{-2} s^{-1}), but did result in damage to the thylakoid membrane (Guéra *et al.*, 2005) and the plants consequently became more sensitive to photo-oxidative stress conditions. Therefore, undetected damages of the photosynthetic apparatus are likely to be present in plants grown at elevated ozone levels in the present study.

Powdery mildew and spot blotch disease levels were strongly affected by the climatic growth conditions in a treatment-specific way. For powdery mildew disease, the main reason was climatically induced changes in the efficiency of basal defence responses in the plant. Direct effect on *B. graminis* fitness only had a minor effect, because the disease levels were similar independently if infected leaves were incubated in ambient conditions or in treatment-specific conditions. When evaluating the infection of spot blotch disease in the climatic treatments, it was not possible to separate any direct climatic effect on *B. sorokiniana* fitness from growth condition effects on plant defence responses, as both plant and fungus were always exposed to the climatic treatments during infection.

Barley growing in elevated [CO_2] as a single factor became more resistant to the biotrophic powdery mildew fungus. Likewise, exposure of wheat and barley to elevated [CO_2] (700 ppm) resulted in reduced infection and higher penetration resistance to powdery mildew compared to plants grown in ambient [CO_2] (Thompson *et al.*, 1993; Hibberd *et al.*, 1996). It was suggested that the increased penetration resistance was related to photosynthesis, because the effect of elevated [CO_2] was not present at low light intensities restricting photosynthesis (Hibberd *et al.*, 1996). However, photosynthesis was not positively stimulated by elevated [CO_2] as a single factor in the present study, and despite a higher net CO_2 assimilation rate at elevated [CO_2] in zucchini (*Cucurbita pepo*), no effect on development of powdery mildew (*Podosphaera xanthii*) was observed (Pugliese *et al.*, 2012). Because powdery mildew infection was highest in the treatments with simultaneously elevated temperature and [CO_2], having the highest photosynthetic rate, the defence response must be controlled by other changes in leaf biochemistry not related to a higher photosynthetic rate. Elevated [CO_2] has shown to increase activity of the defence-related enzymes β-1,3-glucanase (potato and *Phytophthora infestans* (hemibiotrophic)) and PAL (tobacco and *Potato virus Y* (biotrophic)), which both resulted in increased host plant resistance (Matros *et al.*, 2006; Plessl *et al.*, 2007) and are important components for basal resistance. However, elevated [CO_2] had little or no effect on *B. sorokiniana* infection, which was also the finding for another hemibiotrophic fungal barley pathogen, *Drechslera teres*, where exposure to 700 ppm CO_2 did not affect the susceptibility of barley (Plessl *et al.*, 2005).

Elevated temperature is known to modulate plant resistance against pathogens, and higher growth temperature can either increase or decrease disease resistance to pathogens in terms of both basal and race-specific resistance (Cheng *et al.*, 2013). Here, it was found that elevated temperature consistently increased basal resistance against *B. graminis* infection. The mechanisms might involve reinforcement of the cell wall via oxidative cross-linking of the cell wall (Vallélian-Bindschedler *et al.*, 1998). Increased basal resistance against powdery mildew disease when exposed to moderately elevated temperatures is also known in wheat and tomato (Ge *et al.*, 1998; Guzman-Plazola *et al.*, 2003). However, increased powdery mildew disease has been observed in zucchini when growing at elevated temperature (Pugliese *et al.*, 2012).

A substantial increase in spot blotch symptoms and *B. sorokiniana* biomass was observed when barley was exposed to elevated temperature as a single factor. This was expected, as increased severity of spot blotch disease is observed in wheat and barley crops when grown in warm climates, e.g. in South Asia (Sharma *et al.*, 2007). Instead of being the result of a direct temperature-promoting effect on *B. sorokiniana* growth, the increased spot blotch disease probably involves temperature-induced changes in plant physiology and metabolism that affect defence responses, because this effect was not observed in the multifactor treatments. Increased symptom size and severity (symptom development) in the high temperature treatments (T, CO_2 + T and CO_2 + T + O_3) correlated positively with photosynthetic activity. Excess

excitation energy derived from increased photosynthesis in the high temperature treatments could explain the amplified symptom development here, as *B. sorokiniana* toxins have been shown to inhibit electron transport from PSII to PSI and symptom development increases in high light intensities and is related to production of H_2O_2 in mesophyll cells (Adeishvili *et al.*, 1989; Kumar *et al.*, 2001). However, symptom development was not correlated to the amount of fungal biomass or percentage leaf area with symptoms in all treatments, and symptomless growth of the fungus has been observed in dark-grown and white mutant barley leaves, stressing that growth of the fungus in the leaf and symptom development is not necessarily correlated (Schäfer *et al.*, 2004).

Similar to elevated [CO_2] and temperature, elevated [O_3] as a single factor led to increased basal defence against *B. graminis* in barley. Exposure to elevated ozone, both as acute stress and chronic exposure, has been shown to induce responses in the plant similar to defence responses induced by pathogens, including *B. graminis*. Increased transcription of genes from the phenylpropanoid pathway, PR genes and callose formation is known to be induced by elevated ozone (Sandermann *et al.*, 1998; Eastburn *et al.*, 2011). It is therefore tempting to suggest that ozone exposure prior to infection primed a defence response in the plant, leading to increased basal resistance against *B. graminis*. However, exposure to ozone concentrations above 100 ppb in wheat reduced powdery mildew disease as observed in this study, whereas concentrations similar to the ones applied here increased disease (von Tiedemann, 1992b). The effect of ozone, therefore, seems to depend on the specific plant–pathogen interaction and the level of ozone exposure, even within closely related biotrophic pathogens.

Spot blotch disease increased in barley exposed to elevated ozone, indicating a suppressive effect on defence responses which contributed to the increased *B. sorokiniana* growth. This suggests that basal resistance against the biotrophic *B. graminis* and hemibiotrophic *B. sorokiniana* must be different despite the apparent similarities in the early infection process. Exposure to [O_3] comparable to the concentrations used here also increased infection with *B. sorokiniana* in wheat (von Tiedemann, 1992a). However, higher concentrations (above 120 ppb) either had no or a negative effect on infection in *B. sorokiniana* and *D. teres* (von Tiedemann, 1992a; Plessl *et al.*, 2005). The mechanisms for decreased infection by *D. teres* were investigated and correlated with an induced activity of the PR proteins β-1,3-glucanase and chitinase, which is also involved in defence against *B. sorokiniana* (Plessl *et al.*, 2005). However, the molecular background behind the altered infection phenotypes in the climatic treatments in this study is not known.

A few experimental studies have so far examined how combinations of the climatic factors studied here, elevated [CO_2], [O_3] or temperature, affect plant diseases (von Tiedemann & Firsching, 2000; Plessl *et al.*, 2005, 2007; Eastburn *et al.*, 2010; Pugliese *et al.*, 2011, 2012), but none have, to the authors' knowledge, studied the combination of all three factors. Here it is shown that the effect of elevated [CO_2], [O_3] and temperature on development of powdery mildew and spot blotch disease act in an unexpected manner when combined. The elevated single climatic treatments reduced *B. graminis* infection but when combined, the infection was higher than under ambient conditions. In contrast, the effect of single climatic treatments with elevated temperature and [O_3] strongly promoted *B. sorokiniana* growth, but spot blotch disease development was suppressed in the multifactor treatments. For spot blotch disease, this could indicate that elevated [CO_2] has a reducing effect on growth of *B. sorokiniana* in the leaf, but the effect was first apparent when combined with elevated temperature and [O_3]. Recent studies have shown that when treating plants simultaneously with several abiotic and biotic stresses, gene expression, including transcripts involved in defence responses, was altered in a way that could not be predicted from the single stress factors applied alone (Rasmussen *et al.*, 2013). However, the reason for the surprising change in susceptibility of barley to powdery mildew and spot blotch disease in the combined treatments is unknown.

Evidence from the present study and previous multifactor studies emphasizes the need for experiments combining several climatic factors to predict the consequences of climate change on development of plant diseases. The disease phenotypes observed in the present study are probably an outcome of altered host plant physiology and metabolism caused by the climatic growth conditions leading to changed defence responses. The contrasting disease development of powdery mildew and spot blotch disease in relation to the climatic conditions suggests that despite commonalities in the early infection response, the resistance mechanisms against the two pathogens are different and this could be related to their different trophic lifestyles. It would be interesting to learn more about the molecular basis of the early defence responses to *B. graminis* and *B. sorokiniana* under different climatic conditions, to gain an insight into the regulation mechanisms of plant responses to multiple environmental and biotic stresses.

Acknowledgements

Research was financed by the Danish Council for Independent Research: Technology and Production Sciences (FTP) via the project 'Climate Change Effects on Plant Health' (project 0602-00589B).

References

Adeishvili TS, Simonyan GG, Tarabrin GA, Fadeev YN, 1989. Effect of toxins of the fungus *Bipolaris-sorokiniana* (Sacc) on photochemical activity of wheat chloroplasts. *Soviet Plant Physiology* **36**, 116–21.

Cheng C, Gao X, Feng B, Sheen J, Shan L, He P, 2013. Plant immune response to pathogens differs with changing temperatures. *Nature Communications* **4**, 2530.

Clausen SK, Frenck G, Linden LG, Mikkelsen TN, Lunde C, Jørgensen RB, 2011. Effects of single and multifactor treatments with elevated

temperature, CO_2 and ozone on oilseed rape and barley. *Journal of Agronomy and Crop Science* **197**, 442–53.
Eastburn DM, Degennaro MM, Delucia EH, Dermody O, McElrone AJ, 2010. Elevated atmospheric carbon dioxide and ozone alter soybean diseases at SoyFACE. *Global Change Biology* **16**, 320–30.
Eastburn DM, McElrone AJ, Bilgin DD, 2011. Influence of atmospheric and climatic change on plant–pathogen interactions. *Plant Pathology* **60**, 54–69.
Fangmeier A, Chrost B, Högy P, Krupinska K, 2000. CO_2 enrichment enhances flag leaf senescence in barley due to greater grain nitrogen sink capacity. *Environmental and Experimental Botany* **44**, 151–64.
Fiscus EL, Booker FL, Burkey KO, 2005. Crop responses to ozone: uptake, modes of action, carbon assimilation and partitioning. *Plant, Cell & Environment* **28**, 997–1011.
Garrett KA, Dendy SP, Frank EE, Rouse MN, Travers SE, 2006. Climate change effects on plant disease: genomes to ecosystems. *Annual Review of Phytopathology* **44**, 489–509.
Ge Y, Johnson W, Roberts J, Rajaram S, 1998. Temperature and resistance gene interactions in the expression of resistance to *Blumeria graminis* f. sp. *tritici*. *Euphytica* **99**, 103–9.
Gillespie KM, Rogers A, Ainsworth EA, 2011. Growth at elevated ozone or elevated carbon dioxide concentration alters antioxidant capacity and response to acute oxidative stress in soybean (*Glycine max*). *Journal of Experimental Botany* **62**, 2667–78.
Guéra A, Calatayud A, Sabater B, Barreno E, 2005. Involvement of the thylakoidal NADH-plastoquinone-oxidoreductase complex in the early responses to ozone exposure of barley (*Hordeum vulgare* L.) seedlings. *Journal of Experimental Botany* **56**, 205–18.
Guzman-Plazola RA, Davis RM, Marois JJ, 2003. Effects of relative humidity and high temperature on spore germination and development of tomato powdery mildew (*Leveillula taurica*). *Crop Protection* **22**, 1157–68.
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.
IPCC, 2013. *Climate Change 2013: The Physical Science Basis. Working Group I Contribution to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge, UK: Cambridge University Press.
Kumar J, Huckelhoven R, Beckhove U, Nagarajan S, Kogel KH, 2001. A compromised Mlo pathway affects the response of barley to the necrotrophic fungus *Bipolaris sorokiniana* (teleomorph: *Cochliobolus sativus*) and its toxins. *Phytopathology* **91**, 127–33.
Kumar J, Schafer P, Huckelhoven R *et al.*, 2002. *Bipolaris sorokiniana*, a cereal pathogen of global concern: cytological and molecular approaches towards better control. *Molecular Plant Pathology* **3**, 185–95.
Manning WJ, von Tiedemann A, 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**, 219–45.
Matros A, Amme S, Kettig B, Buck-Sorlin GH, Sonnewald U, Mock HP, 2006. Growth at elevated CO_2 concentrations leads to modified profiles of secondary metabolites in tobacco cv. Samsun NN and to increased resistance against infection with potato virus Y. *Plant, Cell and Environment* **29**, 126–37.
Plazek A, Rapacz M, Skoczowski A, 2000. Effects of ozone fumigation on photosynthesis and membrane permeability in leaves of spring barley, meadow fescue, and winter rape. *Photosynthetica* **38**, 409–13.
Plessl M, Heller W, Payer HD, Elstner EF, Habermeyer J, Heiser I, 2005. Growth parameters and resistance against *Drechslera teres* of spring barley (*Hordeum vulgare* L. cv. Scarlett) grown at elevated ozone and carbon dioxide concentrations. *Plant Biology* **7**, 694–705.
Plessl M, Elstner EF, Rennenberg H, Habermeyer J, Heiser I, 2007. Influence of elevated CO_2 and ozone concentrations on late blight resistance and growth of potato plants. *Environmental and Experimental Botany* **60**, 447–57.
Pugliese M, Gullino ML, Garibaldi A, 2011. Effect of climate change on infection of grapevine by downy and powdery mildew under controlled environment. *Communications in Agricultural and Applied Biological Sciences* **76**, 579–82.
Pugliese M, Liu J, Titone P, Garibaldi A, Gullino ML, 2012. Effects of elevated CO_2 and temperature on interactions of zucchini and powdery mildew. *Phytopathologia Mediterranea* **51**, 480–7.
Rasmussen S, Barah P, Suarez-Rodriguez MC *et al.*, 2013. Transcriptome responses to combinations of stresses in *Arabidopsis*. *Plant Physiology* **161**, 1783–94.
Sage RF, Kubien DS, 2007. The temperature response of C3 and C4 photosynthesis. *Plant, Cell & Environment* **30**, 1086–106.
Sandermann H Jr, Ernst D, Heller W, Langebartels C, 1998. Ozone: an abiotic elicitor of plant defence reactions. *Trends in Plant Science* **3**, 47–50.
Schäfer P, Huckelhoven R, Kogel KH, 2004. The white barley mutant Albostrians shows a supersusceptible but symptomless interaction phenotype with the hemibiotrophic fungus *Bipolaris sorokiniana*. *Molecular Plant–Microbe Interactions* **17**, 366–73.
Sharkey TD, 2005. Effects of moderate heat stress on photosynthesis: importance of thylakoid reactions, rubisco deactivation, reactive oxygen species, and thermotolerance provided by isoprene. *Plant, Cell & Environment* **28**, 269–77.
Sharma RC, Duveiller E, Ortiz-Ferrara G, 2007. Progress and challenge towards reducing wheat spot blotch threat in the Eastern Gangetic Plains of South Asia: is climate change already taking its toll? *Field Crops Research* **103**, 109–18.
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 & Environment* **16**, 687–94.
von Tiedemann A, 1992a. Ozone effects on fungal leaf diseases of wheat in relation to epidemiology I. Necrotropic pathogens. *Journal of Phytopathology* **134**, 177–86.
von Tiedemann A, 1992b. Ozone effects on fungal leaf diseases of wheat in relation to epidemiology II. Biotrophic pathogens. *Journal of Phytopathology* **134**, 187–97.
von Tiedemann A, Firsching KH, 2000. Interactive effects of elevated ozone and carbon dioxide on growth and yield of leaf rust-infected versus non-infected wheat. *Environmental Pollution* **108**, 357–63.
Vallélian-Bindschedler L, Schweizer P, Mösinger E, Métraux JP, 1998. Heat-induced resistance in barley to powdery mildew (*Blumeria graminis* f. sp. *hordei*) is associated with a burst of active oxygen species. *Physiological and Molecular Plant Pathology* **52**, 185–99.
Zeyen RJ, Carver TLW, Lyngkjaer MF, 2002. Epidermal cell papillae. In: Bélanger RR, Bushnell WR, Dik AJ, Carver TLW, eds. *The Powdery Mildews: A Comprehensive Treatise*. St Paul, MN, USA: American Phytopathological Society, 107–25.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1. Intended and measured values of the experimental climate conditions.

Table S2. Powdery mildew infection in three independent experiments.

Table S3. Spot blotch symptom in four independent experiments.