Plant, Cell & Environment

Plant, Cell and Environment (2010) **33**, 2056–2064 doi: 10.1111/j.1365-3040.2010.02205.x

Elevated CO_2 shifts the focus of tobacco plant defences from cucumber mosaic virus to the green peach aphid

XUE FU[1,2], LEFU YE[1,3], LE KANG[1] & FENG GE[1]

[1]State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China, [2]College of Agricultural Resource and Environment, HeiLongjiang University, Haerbin 150086, China and [3]Graduate University of Chinese Academy of Sciences, Beijing 100080, China

ABSTRACT

Elevation in CO_2 concentration broadly impacts plant physiological characteristics, which influences herbivores and biotrophic pathogens, which in turn regulate the plant defensive response. In this study, responses of tobacco plants to stress in the form of the green peach aphid, *Myzus persicae* (Sulzer), or cucumber mosaic virus (CMV), or both aphid and CMV combined were investigated in open-top chambers under ambient and elevated CO_2 concentrations. We measured aboveground biomass and foliar chlorophyll, nitrogen, non-structural carbohydrates, soluble protein, total amino acid and nicotine content in tobacco plants and also measured aphid population dynamics, body weight, honeydew production and anti-oxidative enzyme activities in individual aphids. Plants produced more secondary metabolites for defence in both CO_2 treatments when treated with aphid and CMV combined than with either alone. Aphid density significantly increased on CMV-infected tobacco plants (relative to uninfected plants) under ambient CO_2 but not under elevated CO_2. This suggests that plant defences against virus and aphid would be more efficient under elevated CO_2. Plant defence appears to shift from plant virus to aphid under increasing CO_2 levels, which highlights the potential influences of multiple biotic stressors on plants under elevated CO_2.

Key-words: cucumber mosaic virus; *Myzus persicae* (Sulzer); defence response; elevated CO_2; tobacco.

INTRODUCTION

Atmospheric CO_2 concentration has risen from 280 mg g^{-1} to 379 mg g^{-1} since the industrial revolution, and now exceeds any level in the past 65 000 years (Anderson 2006; IPCC 2007). Atmospheric CO_2 is predicted to double by 2100 (IPCC 2007). Increases in atmospheric CO_2 alter a variety of plant physiological systems and can reduce water requirements, increase biomass accumulation and increase photosynthesis rates (Ainsworth & Long 2005; Ainsworth *et al*. 2008). These effects can then lead to changes in the physiology and behaviour of the insect herbivores feeding on these plants (Anderson 2006), and also affect the incidence and severity of plant diseases caused by pathogens (Manning & Tiedemann 1995; Chakraborty & Datta 2002). Given the complexity of the interactions between plant, insect herbivore/plant pathogen and environment, it is not surprising that our understanding of how elevated CO_2 influences plant pests and disease agents is still incomplete.

Plants have evolved many responses to biotic stressors including herbivorous insects and pathogens (Coaker, Falick & Staskawicz 2005). Defence responses, such as the direct production of secondary metabolites or proteinase inhibitors (Kliebenstein 2004; Lawrence *et al*. 2006; Metlen, Aschehoug & Callaway 2009) and indirect defences (those involving the creation of volatile compounds that can direct herbivore predators to the site of the prey infestation) can inhibit the insect (Paré & Tumlinson 1999; Howe & Jander 2008; Unsicker, Kunert & Gershenzon 2009). Furthermore, simultaneous occurrence of several stresses is more damaging to crops than single stresses (Mittler 2006). Therefore, how a plant allocates energy to different defensive responses is critical for plant survival. The optimal defence hypothesis predicts that plants will show trade-offs in biomass allocation among maintenance, growth, storage, reproduction and defence (Coley, Bryant & Chapin 1985). Studies showed that responses of plants to combinations of stresses cannot be directly extrapolated from knowledge about plant responses to individual stresses (Mittler 2006; Post & Pedersen 2008). Little is known about the acclimation of plants to combinations of different biotic stressors, although plant responses to simultaneous abiotic stresses (Rizhsky *et al*. 2004; Sih, Bell & Kerby 2004) and to the interactions of biotic and abiotic environments (Bilgin *et al*. 2008) have been studied; for example, prolonged exposure to abiotic stress caused by drought or nutrient deprivation weakens plant defences and enhances susceptibility to pests or pathogens (Grodzki *et al*. 2004; Matros *et al*. 2006).

This study concerns the responses of tobacco (*Nicotiana tabacum*), cucumber mosaic virus (CMV) and the green peach aphid *Myzus persicae* (Sulzer) to elevated CO_2. The

Correspondence: F. Ge. Fax: +86 10 64807099; e-mail: gef@ioz.ac.cn

Beichen West Road, Chaoyang District, Beijing 100101, PR China. Lefu Ye and Xue Fu contributed equally to the manuscript.

model organism and C_3 plant, *N. tabacum*, appears to be especially responsive to CO_2 (Sage, Sharkey & Seeman 1989). The green peach aphid is a major pest and principal vector of CMV on tobacco, and CMV is a non-persistently transmitted virus that does not affect the aphid directly but does affect the aphid via the plant host.

In this study, responses of the 'tobacco–CMV–green peach aphid' system to elevated CO_2 were explored in open-top chambers (OTC). Our objectives were: (1) To determine whether tobacco plants have different defensive responses to individual stresses (virus or aphid) under elevated CO_2 versus ambient CO_2; (2) To quantify individual and density responses of aphids on tobacco plants to elevated CO_2; and (3) To explore the potential specific 'tradeoff' between quality and biomass of plants when exposed to the herbivore and the virus.

MATERIALS AND METHODS

Open-top chambers

The experiment was carried out in eight 4.2-m-diameter octagonal OTCs at the Observation Station of the Global Change Biology of the Institute of Zoology, Chinese Academy of Sciences (CAS) in Xiaotangshan County, Beijing, China (40°11′N, 116°24′E). Atmospheric CO_2 treatments were the ambient level (375 μL L^{-1}) and double the ambient level (750 μL L^{-1}) and were applied continuously. CO_2 concentrations were monitored continuously and were adjusted using an infrared CO_2 analyser (Ventostat 8102, Telaire Company, Goleta, CA, USA) to maintain the assigned CO_2 concentrations. The automatic-control systems for adjusting the levels of CO_2 concentration, as well as specifications for the OTCs, are described in detail in Chen, Ge & Su (2005a,b). CO_2 concentrations were measured hourly, and actual CO_2 concentrations (mean ± SD per day) were 383 ± 26 μL L^{-1} in the ambient CO_2 chambers and 769 ± 23 μL L^{-1} in the elevated CO_2 chambers. Tops of chambers were covered with nylon nets to exclude other insects. Air temperature was measured three times per day and did not differ significantly between the two sets of chambers (24.8 ± 3.40 °C in the ambient CO_2 chambers versus 25.5 ± 4.55 °C in the elevated CO_2 chambers) throughout the experiment.

Plant growth conditions

Tobacco (*N. tabacum*) seeds were sown in trays on 1 June 2007. Seedlings germinated on 10 June, and 20 d later (1 July), tobacco seedlings were transplanted into plastic pots (diameter: height = 10 cm: 12 cm) filled with 8:1 peat soil: vermiculite. Sixteen pots with tobacco plants (one plant per pot) were randomly placed in each chamber on 5 July 2007. Plants were watered daily (200 mL $plant^{-1}$, excluding cloudy or rainy day) and fertilized once per week with 100 mL of a 0.5% solution of an NPK fertilizer (15-15-15). Pots were randomly rotated within the chamber once weekly to minimize chamber effects.

Green peach aphids and CMV

Green peach aphids, *M. persicae* (Sulzer), were collected from tobacco fields in the Observation Station of the Global Change Biology of the Institute of Zoology, CAS in Xiaotangshan County, Beijing, China (40°11′N, 116°24′E). Before experiments were conducted, *M. persicae* (Sulzer) was reared in the laboratory with a L14: D10 photoperiod on tobacco for at least three successive generations.

CMV was isolated from tomato plants in Yanqing County, Beijing, China. The isolate was identified with RT-PCR and maintained in tobacco plants at the Beijing Agriculture and Forestry Academy of Sciences, where infected tobacco foliage was kept at –20 °C until used.

Experimental treatments

Eight OTCs were used in four blocks, each block comprising one ambient (375 μL L^{-1}) and one double ambient (750 μL L^{-1}) CO_2 concentration treatment. Each CO_2 concentration OTC contained four combinations of treatments: two levels of virus (infected versus uninfected) and two levels of aphid (infested versus uninfested).

Tobacco leaves of CMV-infected plants stored in at –20 °C were homogenized in 100 mM K-phosphate buffer, pH 7.0 (1 g of leaf material in 20 mL of buffer), to obtain viral extract. The experiment was begun at the five- to seven-leaf stage of tobacco (30–35 d after planting), and the treated plants were mechanically infected with viral extracts by rubbing the adaxial surface of the fifth leaf with virus inoculum and carborundum powder on 5 August 2007. After 5 min, the treated leaves were washed with water. Ten plants were selected randomly for inoculation with CMV, and another six plants were not inoculated with virus (they were inoculated with normal saline) in each OTC. Half of the inoculated (five) and half of the non-inoculated (three) plants were then infested with aphids; the remaining eight plants were kept as controls. Five apterous adult aphids were transferred to the abaxial side of the third leaf of the tobacco plants. Five newborn nymphs were kept as a cohort on the infested leaf, and the other aphids were removed after the adults produced offspring. Aphids were confined with nylon nets during the experiment. Over all OTCs, treatments with plants that were inoculated with virus and uninfested with aphids or non-inoculated with virus and infested with aphids were represented by 12 replicate pots for each CO_2 concentration. Treatments with plants that were inoculated with virus and uninfested with aphids or inoculated with virus and infested with aphids were represented by 20 replicate pots for each CO_2 concentration.

Plants inoculated with virus exhibited typical symptoms of CMV infection 5–7 d after inoculation, i.e. the infected leaves became mottled or had a light-green dark-green mosaic pattern. Non-inoculated plants did not have these symptoms. We hereafter use the terms 'infected' for the inoculated plants and 'uninfected' for the non-inoculated plants.

Aphid abundance, aphid weight and honeydew production

Myzus persicae were counted every 3 d during the experiment between 7 August and 5 September 2007. Twenty adult aphids from each tobacco plant (five replicates) were weighed using an electronic balance (HANGPING, FA1004N, 1/100000) to measure average biomass of a single adult aphid at the end of the experiment. Five fourth-instar aphids were singled out randomly and starved for 1 h and transferred to the uninfected or infected leaves of tobacco plants from which the original aphids had been removed, which were not used for other determinations 14 d after virus inoculation. Weighed sealing film was placed 10 cm below the leaf with aphids to collect honeydew, and each film was reweighed every day.

Chemical determination

Four weeks after aphid infestation (10 September 2007), tobacco plants from each OTC were cut at ground level, weighed and dried at 80 °C for 72 h, except that the 5th upper expanded fresh leaf of each plant was removed to measure the variables described in the next paragraph. Dried leaves from each treatment were ground in a mill. Foliar nitrogen content (with eight replicates per treatment) was analysed using a CNH analyser (Coviella, Stipanovic & Trumble 2002), and total sugar content was determined using the DNS (3,5-dinitrosalicylic acid) method (Suh, Noh & Choi 2002). Foliar nicotine content was quantified by an HPLC System (Agilent 1100 Series LC System). The mobile phase consisted of 40% (v/v) methanol containing 0.2% (v/v) phosphoric acid buffered to pH 7.25 with triethylamine (Saunders & Blume 1981).

Fresh leaves were used to examine foliar soluble protein content, total amino acid content and chlorophyll content. About 0.2 g of fresh leaf tissue was used for soluble protein content determination. Fresh leaves were homogenized in 1:10 (fresh weight/buffer volume ratio) 100 mM phosphate buffer, pH 7.4, containing 100 mM KCl and 1 mM ethylenediaminetetraacetic acid (EDTA) for 1.5 min at 4 °C. The homogenate was centrifuged at 10 000 *g* at 4 °C for 15 min, and the supernatants were used to analyse soluble protein content by the Bradford (1976) assay. Approximately 0.5 g of leave tissue was used to determine total amino acid content using the absorbance spectrophotometry method by ninhydrin (Yang & Miller 1963). Foliar chlorophyll content was quantified using the absorbance spectrophotometry method (Porra, Thompson & Kriedemann 1989) using about 0.3 g of fresh leaf tissue.

Aphids on each tobacco plant were collected and kept at –20 °C for enzyme determination before the tobacco seedlings were sampled. About 0.02 g of aphids were homogenized in 1:10 (aphid fresh weight : buffer volume) 100 mM phosphate buffer, pH 7.4, containing 100 mM KCl and 1 mM EDTA for 1.5 min at 4 °C. The homogenate was centrifuged at 3500 rpm at 4 °C for 10 min, and the supernatants were used to examine the enzymes. Three enzymes, including glutathione S-transferase (GST), superoxide dismutase (SOD) and acetylcholinesterase (AchE) in the aphid (four replicates per treatment) were also quantified separately as indicated by the kit protocol (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu Province, China). GST activity was determined spectrophotometrically at 340 nm by use of the 1-chloro-2,4-dinitrobenzene (CDNB) and GSH system. The specific activity of GST is expressed in μmol min^{-1} mg^{-1} of protein. SOD activity was assayed spectrophotometrically at 550 nm by use of the xanthine and xanthine oxidase system. One unit (U) of SOD activity was defined as the amount of GST required for 50% inhibition of xanthine and xanthine oxidase system reaction per minute and per milligram of total protein in the homogenate. AChE assays are developed with Ellman's Reagent, which contains acetylthiocholine as the substrate. The final product of the enzymatic reaction, 5-thio-2-nitrobenzoic acid, is bright yellow, and can be read at 405–420 nm. One unit is the amount of enzyme catalysing an increase of 1 absorbance unit in a 1 cm light path of 1 mL of Ellman's reagent.

Statistical analyses

Where necessary, proportional data were arcsine transformed and aphid densities data were log-transformed to ensure that data were normally distributed and variances were homogenous before analysis. In the blocked split-plot design, CO_2 and block (a pair of ambient and elevated OTCs) were the main effects, the virus (plus or minus infection) was the subplot effect and the aphid (plus or minus infestation) was the sub-subplot effect. Analysis of variance (ANOVA; PROC MIXED, Littell *et al*. 1996) was used for analysis of tobacco biomass and nutrient content data by the following ANOVA model:

$$y_{jklm} = \mu + \beta_j + C_k + \varphi_{jk} + V_l + (CV)_{kl} + \delta_{jkl} + A_m + (CA)_{km} + (VA)_{lm} + (CVA)_{klm} + \varepsilon_{jklm}$$

where X_{jklm} represents the average response of block j, CO_2 level i, virus treatment k and aphid treatment l. CO_2 level (C_k), virus treatment (V_l), aphid treatment (A_m) and each of the interaction terms [$(CV)_{kl}$ $(CA)_{km}$ $(VA)_{lm}$ and $(CVA)_{klm}$] represent fixed effects. Random effects include block (β_j), whole plot error (φ_{jk}), subplot error (δ_{jkl}) and sub-subplot error (ε_{jklm}). We computed F-tests for C_k with φ_{jk} as the error term ($F_{1,6}$), for V_l and $(CV)_{kl}$ with δ_{jkl} as the error term ($F_{1,6}$), and for A_m $(CA)_{km}$ $(VA)_{lm,}$ and $(CVA)_{klm}$ with ε_{jklm} as the error term ($F_{1,12}$). The effects of block and its interaction with other factors were not significant ($P > 0.512$) and are not presented to simplify data presentation in tables and text. Differences between means were determined using Tukey's HSD test at $P < 0.05$ (SAS Institute Inc, Cary, NC, USA, 1996).

Two-way ANOVAs were used to analyse the effects of the CO_2 level (ambient versus elevated), virus (without virus and with virus) and their interactions on the aphid abundance, mass, honeydew excretion and enzyme

Table 1. *P* values from ANOVA for the effect of CO_2 level, CMV and green peach aphid on the aboveground biomass and foliar chemical constituents of tobacco

Main effects and interactions	Dependent variable						
	Biomass	Nitrogen	TNCs	Protein	[d]TAA	[e]Chl	Nicotine
[a]CO_2	<0.001***	0.538	0.355	0.709	0.004**	0.011*	0.782
[b]Virus (V)	<0.001***	<0.001***	0.146	<0.001***	0.005**	0.011*	<0.001***
$CO_2 \times V$	0.912	<0.001***	0.018*	0.765	0.109	0.080	<0.001***
[c]Aphid (A)	<0.001***	<0.001***	<0.001***	0.063	0.002**	0.070	<0.001***
$CO_2 \times A$	0.001**	<0.001***	<0.001***	0.383	0.001**	0.232	0.019*
$V \times A$	0.860	<0.001***	<0.001***	0.121	0.001**	0.035*	0.117
$CO_2 \times V \times A$	0.001**	0.123	0.012*	0.025*	<0.001***	0.284	0.543

[a]CO_2 levels (ambient and elevated), [b]virus (with and without), [c]aphid (with and without), [d]total amino acids, [e]chlorophyll content. Significance levels are indicated by $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

activities using a split-plot design with CO_2 and block as the main effects and virus as the subplot effect. A paired-samples *t*-test was conducted to examine any change between observations for the same treatment made at two different times. CO_2 level (C_k), virus treatment (V_l) and the interaction terms $(CV)_{kl}$ represent fixed effects. Random effects include block (β_j), whole plot error (φ_{jk}) and subplot error (δ_{jkl}). The effects of block and its interaction with the factors were not significant, and are not presented to simplify data presentation in tables and text. Correlation analysis (Pearson's correlation coefficient) was used to examine the relationship between aphid variables and tobacco variables (SPSS Inc, Chicago, IL, USA).

RESULTS

Plant biomass

Elevated CO_2 concentration increased the aboveground biomass of tobacco in the treatments of uninfected and uninfested tobacco, CMV-infected tobacco and aphid infested + virus infected tobacco by 36.2, 20.8 and 25.1%, respectively (Table 1, Fig. 1). Aphid infestation decreased tobacco aboveground biomass under elevated CO_2 (Table 1, Fig. 1).

Foliar chemicals

CMV decreased foliar protein content of aphid-infested tobacco leaves by 37.9% under ambient CO_2 (Table 1, Fig. 2a). Elevated CO_2 increased the protein content in tobacco leaves infected/infested with both virus and aphid by 27.5% (Table 1, Fig. 2a). Those plants infested with aphids or infected with CMV and those with both aphids and virus had increased foliar amino acid content under elevated CO_2 (Table 1, Fig. 2b). Under ambient CO_2, the response of foliar amino acid content to aphid feeding was negative; under elevated CO_2, however, the response was positive (Table 1, Fig. 2b). Elevated CO_2 enhanced amino acid content of plants infested with aphids ($CO_2 \times$ Aphid interaction, Table 1, Fig. 2b).

Under ambient CO_2, foliar nitrogen content was decreased by 26.0% in the CMV infection treatment compared with the uninfected and uninfested tobacco treatment (Table 1, Fig. 2c). Foliar nitrogen, however, decreased by 18.1% if plants were infested with aphids and decreased by 16.5% if plants were infected with CMV under elevated CO_2 (Table 1, Fig. 2c). Under ambient CO_2, foliar sugar content decreased markedly for plants infested with aphids but increased for plants infected with CMV (Table 1, Fig. 2d). Under elevated CO_2, foliar sugar content was greater with CMV alone than with combined stressors or with no stressors (Table 1, Fig. 2d). Elevated CO_2 enhanced the sugar content of plants infested with aphids ($CO_2 \times$ Aphid interaction). Elevated CO_2 markedly increased the foliar chlorophyll content of uninfested and uninfected plants (Table 1, Fig 2e).

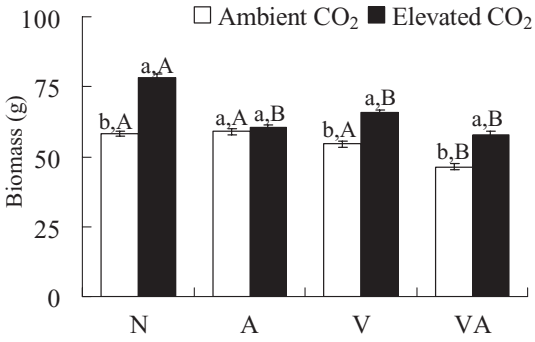

Figure 1. Aboveground biomass of tobacco (*Nicotiana tabacum*) grown under ambient or elevated CO_2 and as affected by CMV infection and aphid infestation. N = uninfested with aphids and uninfected with virus, A = aphid infested, V = CMV infected, VA = aphid infested and virus infected. Values are the means (±1 SE) of 8 replicates. For each pair of bars, different lowercase letters indicate significant differences between CO_2 levels (Tukey's HSD test: d.f. = 1,14; $P < 0.05$). Within each CO_2 level, different uppercase letters indicate significant differences among N, A, V and AV treatments (Tukey's HSD test: d.f. = 3,28; $P < 0.05$).

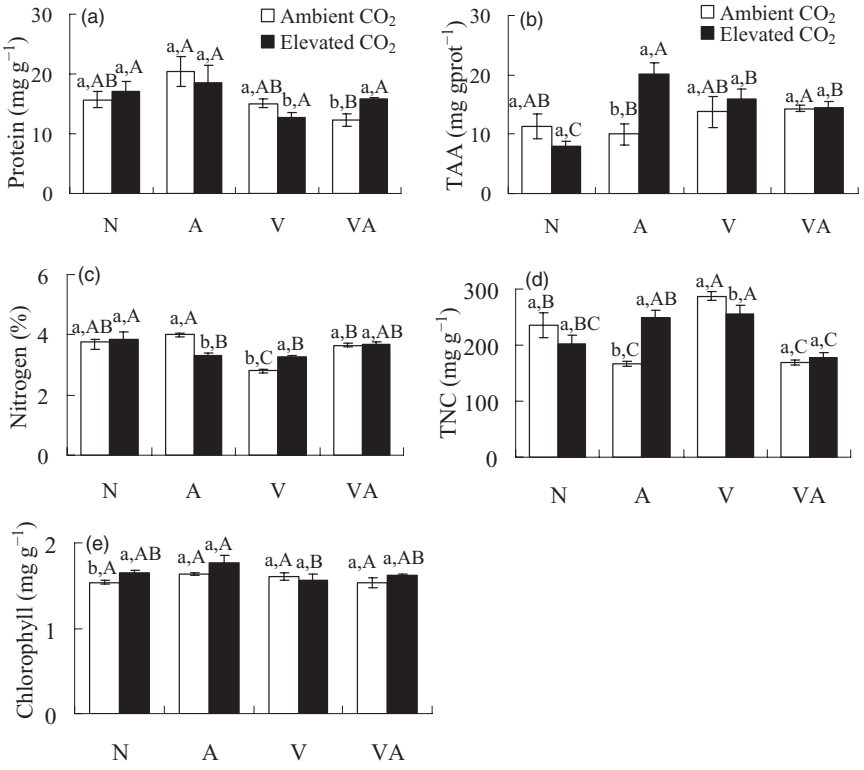

Figure 2. Chemical content of tobacco (*Nicotiana tabacum*) grown under ambient or elevated CO_2 and as affected by CMV (Cucumber mosaic virus) infection and green peach aphid infestation. (a) protein content; (b) total amino acid (TAA); (c) nitrogen content; (d) total non-structural sugar (TNC); and (e) total chlorophyll content. N = uninfested with aphids and uninfected with virus, A = aphid infested, V = CMV infected, VA = aphid infested and virus infected. Values are the means (±1 SE) of six replicates for TAA and eight replicates for the others. For each pair of bars, different lowercase letters indicate significant differences between CO_2 levels (Tukey's HSD test: $d.f._{(a),(c),(d),(e)} = 1,14$; $d.f._{(b)} = 1,6$; $P < 0.05$). Within each CO_2 level, different uppercase letters indicate significant differences among N, A, V and AV treatments (Tukey's HSD test: d.f. = 3,28; $P < 0.05$).

Nicotine content

Under ambient CO_2, CMV infection increased the nicotine level in tobacco leaves by 83.5% and CMV infection + aphid infestation increased the nicotine level by 135.5% (Table 1, Fig. 3). Under elevated CO_2, tobacco nicotine content in treatments of aphid infestation and CMV infection + aphid infestation increased by 56.8 and 103.6% (Table 1, Fig. 3).

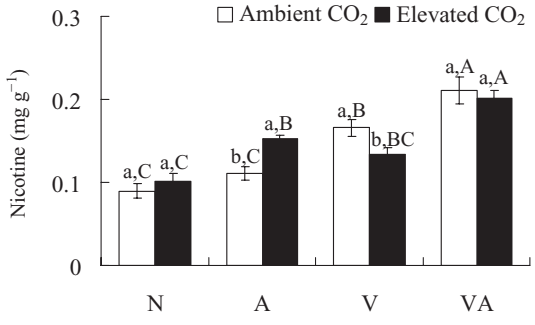

Figure 3. Nicotine content of tobacco (*N. tabacum*) grown under ambient or elevated CO_2 and as affected by CMV infection and green peach aphid infestation. N = uninfested with aphids and uninfected with virus, A = aphid infested, V = CMV infected, VA = aphid infested and virus infected. Values are the means (±1 SE) of eight replicates. For each pair of bars, different lowercase letters indicate significant differences between CO_2 levels (Tukey's HSD test: d.f. = 1,14; $P < 0.05$). Within each CO_2 level, different uppercase letters indicate significant differences among N, A, V and AV treatments (Tukey's HSD test: d.f. = 3,28; $P < 0.05$).

Aphid abundance

CO_2 concentration increased aphid abundance on the 12th, 15th, 18th, 24th and 30th day of the experiment. CMV decreased aphid abundance on the 6th day. The interaction between CO_2 levels and CMV infection affected aphid abundance on each day (Table 2, Fig. 4).

In all four treatments, two plateaus in aphid density occurred over the whole observation period; the first plateau appeared later (21 August) on uninfected plants under ambient CO_2 than on plants in the other three treatments (18 August). The second plateau, which lasted to the end of the study, appeared earlier on uninfected plants under elevated CO_2 (24 August) and later on infected plants under elevated CO_2 (August 30) than on plants from the ambient CO_2 treatments (27 August). Under ambient CO_2, aphid density was twofold greater on CMV-infected than on uninfected tobacco (Fig. 4). Under elevated CO_2, however, aphid density was high on both CMV-infected and uninfected plants but was nearly two times higher on uninfected than on infected plants during the investigation period (Fig. 4). Generally speaking, no significant response of aphid density to elevated CO_2 was found on infected plants (Fig. 4).

Table 2. ANOVA results (*P* values) for the effects of CO_2 level, CMV and their interactions on the abundance of green peach aphids

	Main effects and interactions and interactions		
Day	[a]CO_2	[b]Virus (V)	$CO_2 \times V$
6	0.930	0.007**	0.031*
9	0.063	0.050	0.031*
12	0.012*	0.131	0.022*
15	0.005**	0.260	0.006**
18	0.037*	0.763	0.008**
21	0.057	0.879	0.040*
24	0.035*	0.642	0.033*
27	0.055	0.666	0.026*
30	0.044*	0.541	0.025*

[a]CO_2 levels (ambient and elevated); [b]virus (with and without).
Significance levels are indicated by $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

Aphid body and honeydew weight

Elevated CO_2 decreased aphid weight by 27.8% and decreased honeydew excretion by 49.3% on plants uninfected with virus (Table 3, Fig. 5a,b). Elevated CO_2 did not affect aphid weight but increased honeydew excretion by 114.7% in plants infected with CMV (Table 3, Fig. 5a,b). Virus infection and elevated CO_2 had similar negative effects on aphid per capita body weight. Under ambient CO_2, CMV infection reduced aphid weight by 29.3% and reduced honeydew excretion by 75.3% (Table 3, Fig. 5a,b).

Aphid chemicals

Elevated CO_2 increased GST activity by 79.2%, SOD activity by 17.0% and AChE activity by 78.2% in aphids feeding on uninfected tobacco (Table 3, Fig. 5c–e). Under elevated CO_2, GST activity was reduced by 23.9% in aphids feeding on virus-infected rather than on uninfected tobacco plants (Fig. 5c). In aphids feeding on virus-infected tobacco plants, GST, SOD and AChE enzyme activities were increased by 28.5, 25.0 and 35.5%, respectively, under elevated CO_2 rather than ambient CO_2 (Fig. 5c–e).

Tobacco nitrogen content and aphid body weight were positively correlated (correlation coefficient = 0.402, $P = 0.020$, Weight = $0.22 + 0.08 \times$ nitrogen content $R^2 = 0.16$). Aphid abundance and aphid body weight were negatively correlated (correlation coefficient = –0.528, $P = 0.002$, Weight = $0.58 \pm 0.0006 \times$ population size $R^2 = 0.28$). Additionally, aphid abundance and tobacco nitrogen content were negatively correlated (correlation coefficient = –0.242, $P = 0.036$).

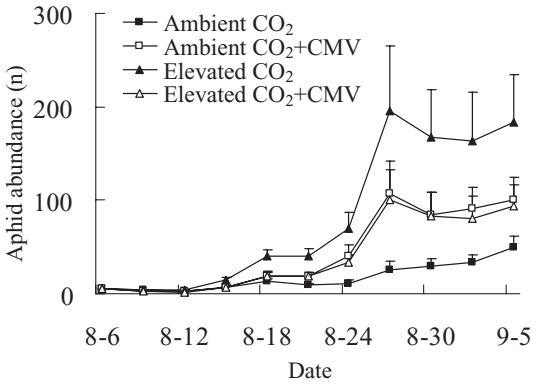

Figure 4. Aphid abundance per plant over time as affected by ambient CO_2, ambient CO_2 + CMV, elevated CO_2 and elevated CO_2 + CMV. Values are the means (±1 SE) of eight replicates.

DISCUSSION

Elevated CO_2 increased the biomass of healthy plants, plants infested by green peach aphids, plants infected by CMV and plants affected by both stresses by 36, 3, 21 and 25%, respectively, in our study (Fig. 1). In a previous study, *Barley yellow dwarf virus* reduced the enhancement of plant growth by elevated CO_2 (Malmström & Field 1997). Aphid herbivory alone can also reduce the enhancement of plant growth by elevated CO_2 (Dan Flynn, Sudderth & Bazzaz 2006). The biomass results confirm that aphid infestation and virus infection tend to reduce the CO_2 fertilization effect. In the current study, however, reduction in the CO_2 fertilization effect was much greater with the aphid alone than with the virus alone or with the aphid and virus together. The optimal defence hypothesis predicts that plants will show trade-offs in biomass allocation among various processes, including those related to defence (Coley *et al*. 1985). Resource availability is the primary force

Table 3. ANOVA results (*P* values) for the effects of CO_2 level, CMV and their interactions on aphid weight per capita, the weight of honeydew produced by green peach aphid per capita, and enzyme activities in the body of aphids

Main effects and interactions	Dependent variable				
	Aphid weight	Honeydew weight	GST	SOD	AChE
[a]CO_2	<0.001***	0.003**	<0.001***	<0.001***	<0.001***
[b]Virus (V)	<0.001***	<0.001***	0.002**	0.957	0.258
$CO_2 \times V$	<0.001***	<0.001***	<0.001***	0.351	0.008**

Enzymes were GST (glutathione S-transferase), SOD (superoxide dismutase), and AchE (acetylcholinesterase).
[a]CO_2 levels (ambient and elevated); [b]virus (with and without).
Significance levels are indicated by $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

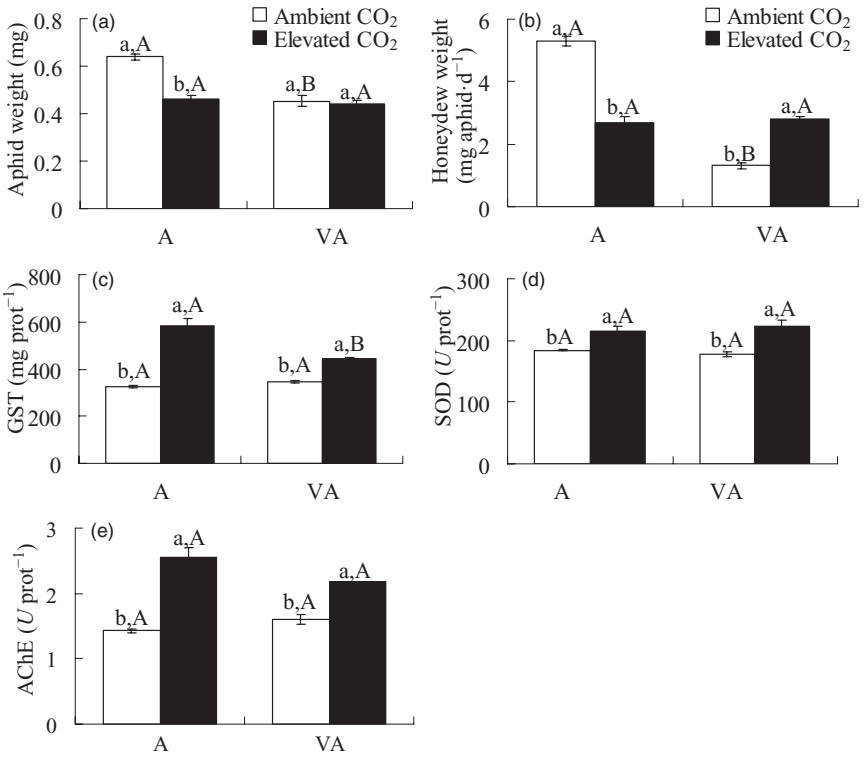

Figure 5. Traits of aphids fed on uninfected and virus-infected plants under ambient or elevated CO_2. (a) aphid weight; (b) honeydew weight excreted by aphids; (c) GST activity of aphid; (d) SOD activity of aphid; and (e) AChE activity of aphid. A = aphids on uninfected plants, VA = aphids on CMV-infected plants. For each pair of bars, different lowercase letters indicate significant differences between CO_2 levels. For each CO_2 level, different uppercase letters indicate significant differences between A and VA treatments.

determining whether there is a surplus of photosynthate available for allocation to the secondary metabolism needed for plant defence (Bryant, Tuomi & Niemela 1988). Under ambient CO_2 conditions, no significant changes occurred in the biomass, nitrogen content or nicotine content of aphid-infested plants, which indicates that no significant energy was allocated to produce increased nicotine to fight aphids under ambient CO_2 (Figs 1–3). This is consistent with the idea that evolution of defence mechanisms against herbivores will result in only minor reductions in growth according to the environmental constraint hypothesis (Bryant *et al*. 1988). CMV infection under ambient CO_2 increased nicotine and TNC content and decreased nitrogen content, which suppressed viral damage; in these cases, response to the virus occurred without any changes in Chl levels, allowing a sufficient photosynthesis rate in the tobacco plants. These results suggest that plants directed more energy to resist CMV than to resist the aphid under ambient CO_2.

CMV resides in aphid mouth parts (James & Keith 2004) without harming the aphid carrier, but CMV infection could indirectly affect aphids by changing the quantity and composition of host-plant secondary metabolites and chemical composition. Aphids weighed less and consumed less on CMV-infected tobacco than on uninfected plants (Fig. 5). Aphid numbers, however, were 106% greater on CMV-infected than on uninfected tobacco under ambient CO_2 but were 49% smaller on CMV-infected than on uninfected tobacco under elevated CO_2 over 3 weeks. A previous study found that the green peach aphid showed a preference for *Potato Leafroll Virus*-infected plants over uninfected plants (Srinivasan, Alvarez & Eigenbrode 2006). The current study also indicated that under elevated CO_2, aphid density increased almost four-fold while aphid consumption per capita (as indicated by honeydew excretion) was half of that under ambient CO_2. This finding is supported by the report that peach aphid abundance was enhanced by CO_2 treatment (Bezemer & Jones 1998). It follows that elevated CO_2 should increase consumption by the entire aphid population by two-fold and that aphids would be more damaging under elevated than under ambient CO_2. Awmack, Harrington & Leather (1997) also reported that numbers of the aphid *Aulacorthum solani* increased when CO_2 was elevated.

Under elevated CO_2, aphids would consume more biomass, leading to a marked decrease of biomass, and stimulate the host plant to allocate more material and energy for synthesis of more secondary metabolites (Metlen *et al*. 2009). Plants reacted less strongly, however, to viral infection than to aphid feeding under elevated CO_2 in the current study, indicating that virus effects would be less severe than aphid effects under elevated CO_2. In other words, we infer that the plant will transfer more energy to defend against aphids than virus under elevated CO_2. Changes in plant responses to herbivorous insects and virus pathogens may be explained by elevated CO_2 changing the nutrition of host plants in ways that are more favourable to herbivores than to virus. How changes in the host plant caused by elevated CO_2 affects disease incidence will depend on host-plant growth stage and type of pathogen (Manning & Tiedemann 1995).

Multiple stresses that induce new defence or acclimation responses should be studied and regarded as a new

state in plants (Rizhsky *et al*. 2004; Mittler 2006). In our study, the combination of aphid and virus caused no marked changes in nitrogen content or Chl content relative to uninfected and uninfested plants under ambient CO_2. The combination, however, increased nicotine content by 136% (Fig. 3), indicating that different defence tactics were used when the plants were exposed to multiple stresses. Moreover, chloroplasts isolated from healthy leaves did not differ from those isolated from *Soybean Mosaic Virus*-infected leaves (Magyarosy, Buchanan & Schurmann 1973); there was, however, a shift in biosynthesis from sugars to amino acids in chloroplasts from infected leaves (Chakraborty *et al*. 2000), and amino acids were previously found to be an important nutrient source for aphids. This explains why aphid density increased when they fed on CMV-infected tobacco plants in which foliar amino acid content was enhanced.

In conclusion, biotic stressors such as pathogens, pests or weeds significantly affect natural or managed ecosystems (Rosenzweig *et al*. 2001). Elevated CO_2 can accelerate the evolution of pathogens and pests, which will threaten host resistance to biotic stresses (Hibberd, Whitbread & Farrar 1996; Chakraborty *et al*. 2000). With some trade-off between resistances to different stresses, tobacco plant defence under elevated CO_2 shifted in that it focused more on resisting aphids than on resisting CMV infection.

Although this study focused on the effects of elevated CO_2, increases in CO_2 will likely be accompanied by increases in temperature (Ainsworth *et al*. 2008). Temperature was not included as a variable in this study because the OTC system limits the number of variables that can be studied simultaneously. If temperature were included as a variable, multiple temperatures would need to be tested because information about how much temperature will increase per increase in CO_2 concentration is very limited. Another concern is that organisms respond differently to sudden changes versus gradually changes in CO_2 concentration (Klironomos *et al*. 2005). Because of the limited number of OTCs available, the current study could only examine a sudden change in CO_2 concentration represented by two quite different concentrations. Future research should consider the interactive effects of temperature and CO_2 level, and should also consider more CO_2 levels and more gradual changes in CO_2 level.

ACKNOWLEDGMENTS

We thank Prof. Marvin Harris of Texas A&M University for reviewing a draft of the manuscript. We are grateful to Prof. Qiu of the Beijing Agriculture and Forestry Academy of Sciences for providing CMV and to Dr Duan CG of the National Gene Research Center of China for advice about planting and growing tobacco. This project was supported by the 'National Basic Research Program of China' (973 Program) (No. 2006CB102002) and the National Nature Science Fund of China (Nos. 30770382, 30621003).

REFERENCES

Ainsworth E.A. & Long S.P. (2005) What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO_2. *The New Phytologist* **165,** 351–372.

Ainsworth E.A., Beier C., Calfapietra C., *et al*. (2008) Next generation of elevated [CO_2] experiments with crops: a critical investment for feeding the future world. *Plant, Cell & Environment* **31,** 1317–1324.

Anderson L.J. (2006) Atmospheric CO_2 changes: past and future. *Ecology* **87,** 262–263.

Awmack C.S., Harrington R. & Leather S.R. (1997) Host plant effects on the performance of the aphid *Aulacorthukm solani* (Kalt.) (Homoptera: Aphididae) at ambient and elevated CO_2. *Global Change Biology* **3,** 545–549.

Bezemer T.M. & Jones T.H. (1998) Plant-insect herbivore interactions in elevated atmospheric CO_2: quantitative analyses and guild effects. *Oikos* **82,** 212–222.

Bilgin D.D., Aldea M., O'Neill B.F., Benitez M., Li M., Clough S.J. & Delucia E.H. (2008) Elevated ozone alters soybean-virus interaction. *Molecular Plant-Microbe Interactions* **21,** 1297–1308.

Bradford M.M. (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. *Analytical Biochemistry* **72,** 248–254.

Bryant J.P., Tuomi J. & Niemela P. (1988) Environmental constraint of constitutive and long-term inducible defences in woody plants. In *Chemical Mediation of Coevolution* (ed. K.C. Spencer), pp. 367–389. Academic Press, San Diego, CA, USA.

Chakraborty S. & Datta S. (2002) How will plant pathogens adapt to host plant resistance at elevated CO_2 under a changing climate? *The New Phytologist* **159,** 733–742.

Chakraborty S., Pangga I.B., Lupton J., Hart L., Room P.M. & Yates D. (2000) Production and dispersal of *Colletotrichum gloeosporioides* spores on *Stylosanthes scabra* under elevated CO_2. *Environmental Pollution* **108,** 381–387.

Chen F.J., Ge F. & Su J.W. (2005a) An improved open-top chamber for research on the effects of elevated CO_2 on agricultural pests in field. *Chinese Journal of Ecology* **24,** 585–590.

Chen F.J., Wu G., Ge F., Parajulee M.N. & Shrestha R.B. (2005b) Effects of elevated CO_2 and transgenic Bt cotton on plant chemistry, performance, and feeding of an insect herbivore, the cotton bollworm. *Entomologia Experimentalis et Applicata* **115,** 341–350.

Coaker G., Falick A. & Staskawicz B. (2005) Activation of a phytopathogenic bacterial effector protein by a eukaryotic cyclophilin. *Science* **308,** 548–550.

Coley P.D., Bryant J.P. & Chapin F.S. (1985) Resource availability and plant antiherbivore defense. *Science* **230,** 895–899.

Coviella C.E., Stipanovic R.D. & Trumble J.T. (2002) Plant allocation to defensive compounds: interactions between elevated CO_2 and nitrogen in transgenic cotton plants. *Journal of Experimental Botany* **53,** 323–331.

Dan Flynn F.B., Sudderth E.A. & Bazzaz F.A. (2006) Effects of aphid herbivory on biomass and leaf-level physiology of *Solanum dulcamara* under elevated temperature and CO_2. *Environmental and Experimental Botany* **56,** 10–18.

Grodzki W., McManus M., Kníek M., Meshkova V., Mihalciuc V., Novotny J., Turani M. & Slobodyan Y. (2004) Occurrence of spruce bark beetles in forest stands at different levels of air pollution stress. *Environmental Pollution* **130,** 73–83.

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

Howe G.A. & Jander G. (2008) Plant immunity to insect herbivores. *Annual Review of Plant Biology* **59,** 41–66.

Intergovernmental Panel on Climate Change (IPCC) (2007) *Climate Change 2007; the physical science basis. Summary for policy makers. Report of Working Group I of the Intergovernmental Panel on Climate Change*. (eds S. Solomon, D. Qin, M. Manning, Z. Chen, M. Marquis, K.B. Averyt, M. Tignor & H.L. Miller). Cambridge University Press, Cambridge, UK.

James C.K.N. & Keith L.P. (2004) Transmission of plant viruses by aphid vectors. *Molecular Plant Pathology* **5,** 505–511.

Kliebenstein D.J. (2004) Secondary metabolites and plant/environment interactions: a view through *Arabidopsis thaliana* tinged glasses. *Plant, Cell & Environment* **27,** 675–684.

Klironomos J.N., Allen M.F., Rillig M.C., Piotrowski J.S., Makvandi-Nejad S., Wolfe B.E. & Powell J.R. (2005) Abrupt rise in atmospheric CO_2 overestimates community response in a model plant-soil system. *Nature* **433,** 621–624.

Lawrence S.D., Dervinis C., Novak N. & Davis J.M. (2006) Wound and insect herbivory responsive genes in poplar. *Biotechnology Letter* **28,** 1493–1501.

Littell R.C., Milliken G.A., Stroup W.W. & Wolfinger R.D. (1996) *SAS System for Mixed Models*. SAS Institute, Cary, NC, USA.

Magyarosy A.C., Buchanan B.B. & Schurmann P. (1973) Effect of a systemic virus infection on chloroplast function and structure. *Virology* **55,** 426–438.

Malmström C.M. & Field C.B. (1997) Virus-induced differences in the response of oat plants to elevated carbon dioxide. *Plant, Cell & Environment* **20,** 178–188.

Manning W.J. & Tiedemann A.v. (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.

Matros A., Amme S., Kettig B., Buck-Sorlin G.H., Sonnewald U. & Mock H.P. (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 & Environment* **29,** 126–137.

Metlen K.L., Aschehoug E.T. & Callaway R.M. (2009) Plant behavioural ecology: dynamic plasticity in secondary metabolites. *Plant, Cell & Environment* **32,** 641–653.

Mittler R. (2006) Abiotic stress, the field environment and stress combination. *Trends in Plant Science* **11,** 15–19.

Paré P.W. & Tumlinson J.H. (1999) Plant volatiles as a defense against insect herbivores. *Plant Physiology* **121,** 325–331.

Porra R.J., Thompson W.A. & Kriedemann P.E. (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochimica et Biophysica Acta.* **975,** 384–394.

Post E. & Pedersen C. (2008) Opposing plant community responses to warming with and without herbivores. *Proceedings of the National Academy of Sciences of the United States of America* **105,** 12353–12358.

Rizhsky L., Liang H.J., Shuman J., Shulaev V., Davletova S. & Mittler R. (2004) When defense pathways collide: the response of Arabidopsis to a combination of drought and heat stress. *Plant Physiology* **134,** 1683–1696.

Rosenzweig C., Iglesias A., Yang X.B., Epstein P.R. & Chivian E. (2001) Climate change and extreme weather events; implications for food production, plant diseases, and pests. *Global Change & Human Health* **2,** 90–104.

Sage R.F., Sharkey T.D. & Seeman J.R. (1989) Acclimation of photosynthesis to elevated CO_2 in five C_3 species. *Plant Physiology* **89,** 590–596.

Saunders J.A. & Blume D.E. (1981) Quantitaton of major tobacco alkaloids by high-performance liquid chromatography. *Journal of Chromatography* **205,** 147–154.

Sih A., Bell A.M. & Kerby J.L. (2004) Two stressors are far deadlier than one. *Trends in Ecology and Evolution* **19,** 274–276.

Srinivasan R., Alvarez J.M. & Eigenbrode S.D. (2006) Influence of Hairy Nightshade *Solanum sarrachoides* (Sendtner) and Potato leafroll virus (Luteoviridae: Polerovirus) on the host preference of *Myzus persicae* (Sulzer) (Homoptera: Aphididae). *Environmental Entomology* **35,** 546–553.

Suh H.J., Noh D.O. & Choi Y.M. (2002) Solubilization of onion with polysaccharide-degrading enzymes. *International Journal of Food Science and Technology* **37,** 65–71.

Unsicker S.B., Kunert G. & Gershenzon J. (2009) Protective perfumes: the role of vegetative volatiles in plant defense against herbivores. *Current Opinion in Plant Biology* **12,** 479–485.

Yang S.F. & Miller G.W. (1963) Biochemical studies on the effect of fluoride on higer plants. *Biochemical Journal* **88,** 505–509.

Received 5 March 2010; received in revised form 12 June 2010; accepted for publication 28 June 2010